The Regulation of Nuclear Factor kappa B (NFκB) Activation Mediated by Proteinase-Activated Receptor-2 (PAR-2)

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Fui Goon Goh

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Department of Physiology and Pharmacology

University of Strathclyde, Glasgow, UK.

ABSTRACT

Proteinase-activated receptor-2 (PAR-2) is the second member of the new subfamily of G-protein coupled receptors with a novel mode of mechanism of activation. As with other family members, PAR-2 is activated through the proteolytic cleavage of the amino terminal of the receptor, thus unmasking a tethered ligand which then binds to the receptor causing intramolecular activation. Serine proteases such as trypsin and tryptase serve as the predominant endogenous activators for PAR-2, whilst synthetic activating peptides derived from the tethered ligand sequence are also able to stimulate receptor activation without proteolysis.

The expression of PAR-2 has been detected in a variety of human tissues and receptor activation has been shown to mediate diverse biological functions including haemostasis and inflammation. However, the signalling mechanisms underlying PAR-2-induced cellular effects remain largely undefined. Thus this study sought to determine the signalling events following PAR-2 stimulation in NCTC-2544 skin cell line stably expressing PAR-2 and normal human epidermal keratinocytes, in particular the activation of NFκB. Initially PAR-2 was demonstrated to activate NFκB at the levels of IκBα loss, p65 NFκB phosphorylation, DNA binding and transcriptional activation. PAR-2 was also found to stimulate the three major MAP kinases, namely ERK, p38 MAPK and JNK. In addition, this study has included the work to examine the effects of putative PAR-2 inhibitors; K-14585 and ENMD-1068. K-14585 was a weak antagonist in some assay systems but not others, whilst ENMD-1068 was ineffective. The intermediates upstream in the NFκB pathway stimulated by PAR-2 were assessed. Phosphorylation of p65 NFκB was found to be dependent upon $G\alpha_{q/11}$, PKC isoforms, calcium mobilisation and IKKβ. Interestingly, the inhibition of $G\alpha_{q/11}$ activity did not affect NFκB-DNA binding but partially suppressed NFκB-driven transcription. These findings point to the differential regulations of these intermediary components on different levels of NFκB activation mediated by PAR-2.

PUBLICATIONS

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Papers

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ABBREVIATIONS

Adv	adenovirus
ANOVA	analysis of variance
AP-1	activating protein-1
AP	activating peptide
APS	ammonium persulphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5'-monophosphate
CHO	Chinese hamster ovary
COX-2	cyclooxgenase-2
CaMK	calmodulin-dependent kinase
cDNA	complementary DNA
CK-2	casein kinase-2
CRE	cyclic-AMP responsive element
CREB	CRE binding protein
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagles medium
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECL-2	extracellular loop-2
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether) N,N,N,N'-tetraacetic acid
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
ET	endothelin
FCS	foetal calf serum
FVIIa	activated factor-VIIa
FXa	activated factor-X

GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GM-CSF	granulocyte macrophage-colony stimulating factor
GPCR	G protein-coupled receptor
G-protein	GTP-binding protein
GRK	G protein-coupled receptor kinase
GTP	Guanosine triphoshate
HEK	human embryonic kidney
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
HRP	horseradish peroxidase
HIV	human immunodeficiency virus
HLH	helix-loop-helix
HSF	heat shock factor
HSP	heat shock protein
HUVEC	human umbilical vein endothelial cells
ICAM-1	intracellular adhesion molecule-1
IKAP	IKK complex-associated protein
IκB	inhibitory kappa-B
IKK	inhibitory kappa-B kinase
IL	interleukin
IL-1R	interleukin-1 receptor
IL-1RacP	inerleukin-1 receptor accessory protein
IRAK	interleukin receptor associated kinase
IP	inositol phosphate
IP_3	inositol 1,4,5-triphosphate
JAK-2	Janus-activated kinase-2
JNK	c-jun amino-terminal kinase
kDa	kilo-Dalton
KNRK	Kirten virus transformed rat kidney
LPA	lysophosphatidic acid

LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEK	MAP kinase kinase
MEKK	MAP kinase kinase kinase
MMP	matrix metalloproteinase
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MSK	mitogen and stress kinases
mSos	mammalian Son of Sevenless
MyD88	Myeloid differentiation primary response protein-88
NEMO	NFκB essential modulator
NFATc	nuclear factor of activated T cell
NFκB	nuclear factor kappa-B
NHEK	normal human epidermal keratinocytes
NIK	nuclear factor kappa-B inducing kinase
NLS	nuclear localisation sequence
NO	nitric oxide
NOS	nitric oxide synthase
P	phosphor
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PAR	Proteinase-activated receptor
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PFU	plaque forming unit
PGE_2	prostaglandin E-2
PI3K	phosphatidyl inositol-3 kinase
PIP_3	phosphatidyl inositol 3,4,5-triphosphate

PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLA_2	phospholipase A-2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonylfluoride
PTX	pertussis toxin
PYK-2	proline-rich tyrosine kinase 2
RIP	receptor-interacting protein
RASMC	rat aortic smooth muscle cell
RGS	regulator of G protein signalling
RHD	rel homology domain
RIP	receptor-interacting protein
RNA	ribonucleic acid
ROI	reactive oxygen intermediate
ROS	reactive oxygen species
RSK	ribosomal S6 kinase
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase-polymerase chain reaction
S1P	spingosine-1-phosphate
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulphate
SHC	Src homology collagen
SHP2	Src homology phosphatase-2
siRNA	small interfering RNA
SP	Substance P
STAT	signal transducer and activator of transcription
TAD	transactivation domain

TEMED	N,N,N',N'-tetramethylenediamine
TNFα	Tumour necrosis factor α
TXA_2	thromboxane A-2
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial cell growth factor

AMINO ACID ABBREVIATIONS

Symbol	3-letter code	Full name
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic
E	Glu	Glutamic
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
X	Xxx	Unknown/Any
Y	Tyr	Tyrosine

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

In order to survive, an organism must be able to distinguish environmental changes and react accordingly. To detect environmental factors, the organism must intercept signals which might exist in diverse forms such as chemicals, light or sound. Therefore a receptor serves as the communication channel between the cell and its surroundings, enabling necessary internal adjustments.

1.1.1 The superfamily of G protein-coupled receptors (GPCRs)

Several families of membrane-bound receptor have been identified to date, including ligand-gated ion channels (Colquhoun, 2006), kinase-linked receptors (Johnson & Ingram, 2005) and cytokine receptors (Grotzinger, 2002). Amongst these, the most common receptor family is the one comprising the guanosine-trisphosphate (GTP)-binding protein (G-protein)-coupled receptors (GPCRs). Since the cloning of bovine rhodopsin, more than 12000 GPCR sequences have been reported with ~600 GPCRs in mammalian and accounting for ~2% of the human genome (Nathans & Hogness, 1983; Horn *et al.*, 2003).

All GPCRs have the common structure of 7 hydrophobic transmembrane helices connected by 3 extracellular loops and 3 cytosolic loops (Baldwin, 1993). The GPCRs bind a wide range of ligands such as bioorganic amines, neuropeptides and large glycoprotein hormones to exert diverse physiological functions (Strader *et al.*, 1994). Thus it is not surprising that GPCRs serve as a primary target for pharmaceutical development and therapeutic approaches.

Several classification systems have been employed to organize this superfamily according to the ligand structure, sequence homology, and receptor function. One of the most frequently used systems categorises both vertebrates and inverterbrates GPCRs into classes A-F (Kolakowski, 1994). Human GPCRs have been grouped into classes A-C, further divided into more than 50 subclasses. Generally, there is a substantial degree of amino acid homology amongst members of the same subfamily, but little or no

sequence similarity is observed between subfamilies. Class A constitutes rhodopsin-like GPCRs, represents the largest family and includes amine, peptide, prostanoid and olfactory receptors. Class B, the secretin-like family, consists of calcitonin, glucagon, and vasointestinal peptide receptors. Metabotropic glutamate, GABA and taste receptors are amongst the members of Class C GPCRs. Class D and Class E which represent fungal pheromone receptors and cAMP receptors respectively, and Class Z that comprise bacteriorhodopsins, are non-mammalian receptors. In addition, the screening of cDNA libraries has identified multiple sequences that contain 7 membrane-spanning domains typical of a GPCR. These are termed orphan receptors as their respective endogenous ligands have yet to be identified (Parmentier *et al.*, 1995).

Another classification system categorises GPCRs into four groups on the basis of the mode of ligand binding to the receptor (Ji *et al.*, 1998). The first group includes rhodopsin and biogenic amine receptors in which the ligand binds to the transmembrane core of the receptor. Receptors which require both transmembrane region and exoloop as binding sites for the short-peptide ligands are classified as the second group, examples include formyl and angiotensin receptors. Alternatively, receptors for longer peptide ligands such as calcitonin and vasointestinal peptide which bind to multiple contact sites on both transmembrane core and exoloops of the receptor fall into the third group. The fourth group of GPCRs are receptors that are activated by initial binding of the ligand at the amino-terminal domain of the receptor followed by secondary contact of the amino-terminal domain with exoloops. Examples of GPCRs in this group are neurotransmitter receptors, glycoprotein hormone receptors, and a newly identified GPCR family termed proteinase-activated receptors (PARs).

1.1.2 G proteins

Membrane-associated G proteins are heterotrimeric signalling components that consist of three subunits, namely the α, β and γ-subunits (reviewed in Hermans, 2003). The Gα subunit contains the site for the binding of guanine-nucleotides, guanosine diphosphate (GDP) and guanosine triphosphate (GTP) and exhibits intrinsic GTPase

activity. The Gβ and Gγ subunits, on the other hand, exist as a tightly bound dimer. The terminology G proteins often refers to GPCR-bound G protein heterotrimers which are also known as 'large' G proteins. There are also 'small' G proteins which exist as a single subunit; such monomeric proteins for example $p21^{ras}$ are regularly classified as GTPases in order to distinguish them from heterotrimeric G proteins (Bar-Sagi & Hall, 2000).

A wide range of heterogeneity for G protein subunits has been demonstrated (reviewed in Hermans, 2003). To date, at least 23 types of Gα subunits derived from 17 different genes have been reported, all of which are categorised into the four classes namely $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$. Similarly, 6 Gβ and 12 Gγ subunits have been cloned and identified, signifying the complexity and specificity of GPCR signalling paradigm.

In the inactive state, Gα interacts with the Gβγ complex in close proximity to the GPCRs. Receptor activation causes conformational changes of the receptor domains leading to association of GPCRs with distinct classes of G proteins. This promotes the exchange of GDP for GTP on the Gα subunit, resulting in the dissociation of the heterotrimeric component (Hamm, 1998). The freed GTP-bound Gα subunit and Gβγ complex are able to interact with a diverse array of effector molecules, accounting for the vast variety of GPCR signalling transduction (see Section 1.1.3). Termination of the response occurs when GTP is hydrolysed to GDP, rendering the Gα subunit inactive and resuming its association with Gβγ complex (Hamm, 1998).

1.1.3 The Roles of G proteins in GPCR Signalling Paradigm

Generally, each subtype of Gα subunit activates distinct effector signalling pathways (reviewed in Milligan & Kostenis, 2006). Activation of $G\alpha_s$ leads to adenylyl cyclase stimulation and an increase in cyclic AMP (cAMP) production. In contrast, $G\alpha_{i/o}$ stimulation inhibits adenylyl cyclase, resulting in the reduction of cAMP synthesis (Hurley, 1999). Activation of $G\alpha_{q/11}$ results in the stimulation of phospholipase C

(PLC)-β, a key enzyme in phosphatidylinositol metabolism, to produce inositol triphosphate (IP_3) and diacyglycerol (DAG) (reviewed in Hubbard & Hepler, 2006). The effector molecules of $G\alpha_{12/13}$, however, are not as well characterised as other members of Gα subunits, with the exception of small GTP-binding proteins such as p115RhoGEF which are involved in the regulation of cytoskeleton (Riobo & Manning, 2005).

Whilst Gα subunits are known to selectively activate specific effector system, the Gβγ complex was classically thought to act as an inhibitory component to prevent the activation of Gα. Recently it has become apparent that the Gβ and Gγ subunits can bind to and modulate the activity of a variety of effector molecules including adenylyl cyclase, PLCβ, intermediary components of mitogen-activated protein kinases and PI3-kinase (reviewed in Hur & Kim, 2002). Thus it appears that the G protein subunits can contribute to dual intracellular signalling through a single heterotrimeric G protein, for example, $G\alpha_{i/o}$–mediated adenylyl cyclase activity and Gβγ-dependent PLCβ stimulation (Morris & Scarlata, 1997; Rhee & Bae, 1997).

1.1.4 Diversity and Complexity of GPCR Signalling

GPCR signalling is one of the most complex paradigms amongst cell surface receptors, determined by the aforementioned large number of subtypes identified for each G protein subunit. Various biochemical studies have indicated the specificities of certain Gβ and Gγ subtypes to interact with specific Gα subunits, giving rise to diverse yet unique signal transduction pathways for each receptor (reviewed in Cabrera-Vera *et al.*, 2003). In addition, a large body of literature has indicated that a single GPCR is able to functionally couple to more than one subtype of G protein; for instance the α_2-adrenergic receptor was reported to associate with both $G\alpha_s$ and $G\alpha_i$ (Eason *et al.*, 1992). Moreover, there is also evidence of secondary regulation of effector molecules, for example the activation of adenylyl cyclase by a $G\alpha_{q/11}$–mediated protein kinase C (PKC) pathway, further adding to the complexity of GPCR–mediated signalling (Hur & Kim, 2002).

It has now emerged that GPCRs form dimers and/or oligomers in a manner similar to other receptor families such as growth hormone receptors (reviewed in Hansen & Sheik, 2004; Terrillon & Bouvier, 2004). Such phenomenon has been regarded as a mode of cross-talk between receptors and has great impact in various aspects including cell surface expression, function and signalling specificities. Heterodimerisation of GPCRs can occur between the same or different subtypes of receptor and could account for the changes in signalling properties. For example, the κ and δ opioid receptor heterodimer exhibits a discrete signalling profile compared to the individual receptors (Jordan & Devi, 1999). Alternatively, the heterodimer formed between dopaminergic receptor and somatostatin receptor has been shown to produce enhanced signalling to either receptor agonist stimulation (Rocheville *et al.*, 2000). Cross-talk between two different GPCRs at the level of second messenger system has also been documented (Hur & Kim, 2002). For instance, it has been shown that PLC-coupled $P2Y_2$ receptor selectively inhibited β_2–adrenergic receptor–stimulated cAMP production (Suh *et al.*, 2001).

Activation of certain GPCRs can also lead to the stimulation of other signalling components such as ion channels, mitogen-activated protein kinase (MAPK) cascades and nuclear factor kappa B (NFκB) activation. For example, the β_2–adrenergic receptor has been shown to be physically associated with the L-type calcium channel to modulate its activity (Davare *et al.*, 2001). The regulation of MAPK by GPCRs has been extensively studied and will be further discussed in Section 1.3.2. However, little is known about GPCR-induced activation of NFκB and this will be further elaborated in Section 1.4.6. Notably, there is also increasing evidence showing G protein-independent signal transduction induced by certain GPCRs such as metabotropic glumate receptors (Heuss *et al.*, 1999), M_3-muscarinic and H_1-histamine receptor (Mitchell *et al.*, 1998).

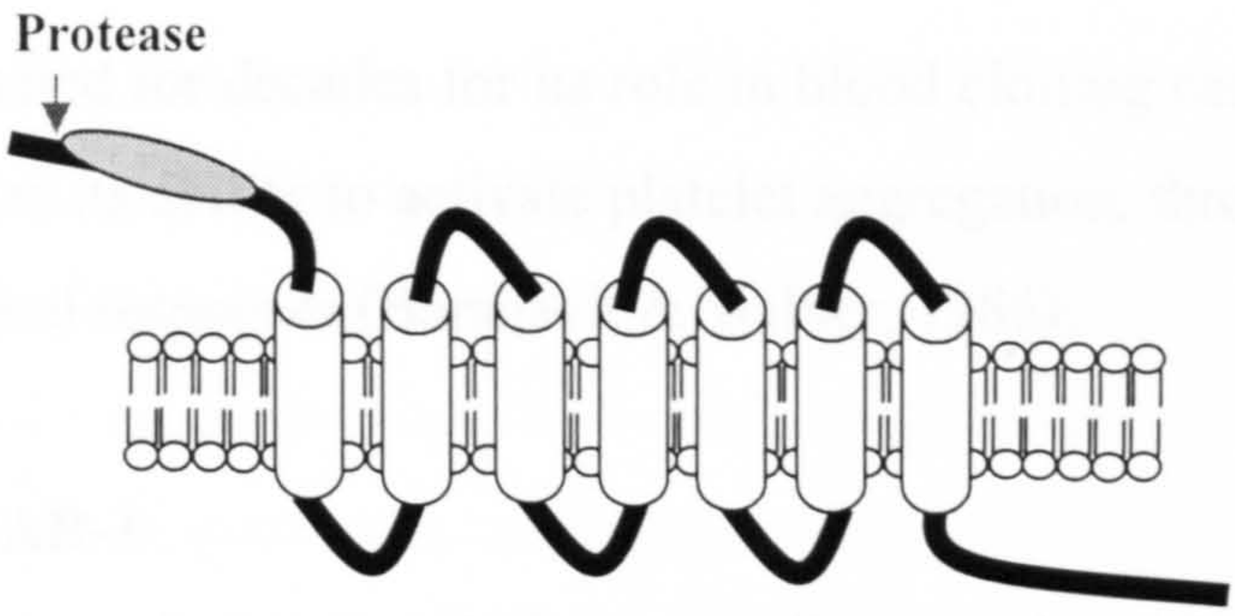
Thus overall, the classical linear downstream signalling of GPCRs appears to be far more complex than initially thought, involving both G protein-dependent and independent transduction. The overall outcome of a particular GPCR stimulation will thus reflect the sum of its diverse signalling cascades.

1.2 PROTEINASE-ACTIVATED RECEPTORS (PARs)

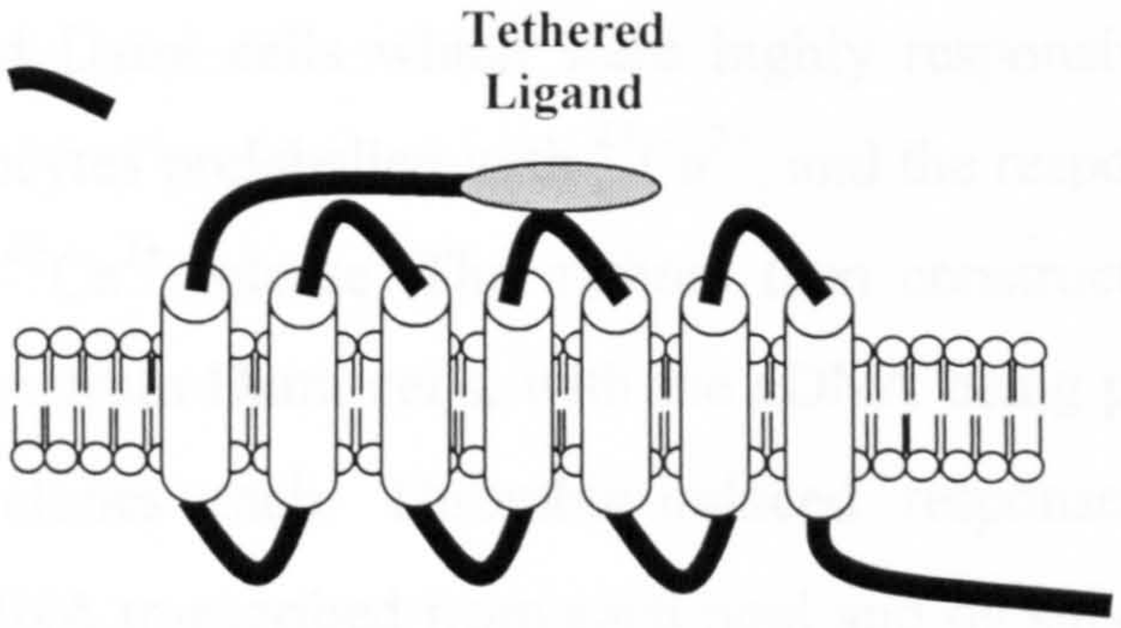
Proteases or peptidases are proteolytic enzymes that catalyse the hydrolysis of covalent peptidic bonds, and their classical main actions were thought to be helping in food digestion. To date there are ~ 700 proteases identified within the mammalian proteome; which are currently categorised into five groups; metallo, serine, cysteine, threonine and aspartyl (Rawlings *et al.*, 2004). Such a nomenclature is derived on the basis of the mechanism of peptide bond hydrolysis catalysed by the enzyme, i.e. for serine proteases, the nucleophilic attack of the targeted peptidic bond is facilitated by a serine residue within the active site of the enzyme.

The relative abundance of proteases has clearly indicated that they might not function solely as digestive enzymes, but could be important mediators for regulating cellular behaviour. In fact, early studies reported the effects of proteases in mediating cellular responses dependent on their enzymatic properties (Sefton & Rubin, 1970; Pohjanpelto, 1977). Eventually this led to the discovery of the first protease-related receptor, termed proteinase-activated receptor-1 (PAR-1) (Vu *et al.*, 1991). Subsequently three subtypes of this family receptor were identified, namely PAR-2, PAR-3 and PAR-4 (reviewed in Macfarlane *et al.*, 2001). Rather than being activated through a conventional ligand-binding process, PARs were found to be stimulated by serine proteases through cleavage of the receptor's amino-terminal exodomain, leading to the unmasking of a new terminal which then serves as a tethered ligand to effect intramolecular receptor activation (see Figure 1.1). As PARs are activated by the endoproteolytic mechanism, the terminology proteinase, which is often referred to as an endopeptidase, has been designated to describe this novel group of receptors (Hollenberg & Compton, 2002). The family of PARs belongs to the superfamily of GPCRs, and the endogenous serine proteinase thrombin activates three of four PARs, namely PAR-1, PAR-3 and PAR-4 respectively, whilst trypsin or other related proteinase, activates PAR-2.

a)



b)



c)

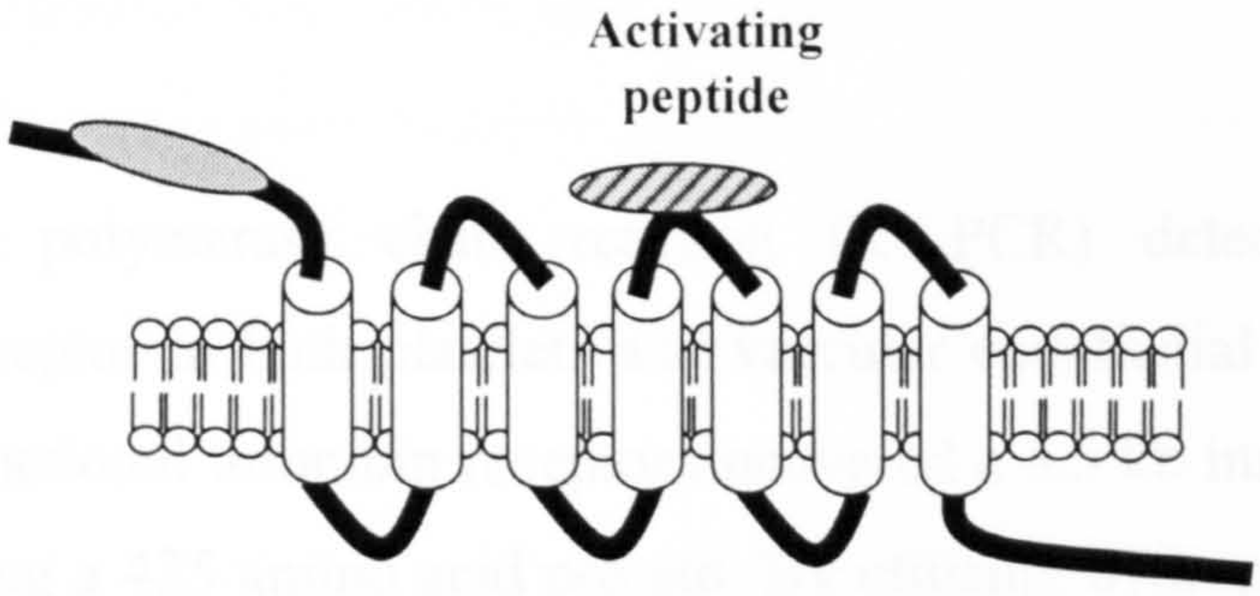


Figure 1.1 Mechanism of activation for PARs.

(a) and (b) illustrate the receptor activation by proteolytic cleavage. (c) shows the binding of an activating peptide to the receptor.

1.2.1 PAR-1, PAR-3 and PAR-4

Thrombin has been studied for decades for its role in blood clotting cascade. It was then shown that in addition to its ability to activate platelet aggregation, thrombin also served to elicit diverse biological responses (Barshavit & Wilner, 1986).

1.2.1.1 Discovery of PAR-1

The first proteinase-activated receptor, PAR-1, was discovered in 1991 when Coughlin and colleagues isolated the complementary DNA (cDNA) encoding the human thrombin receptor using an expression cloning approach (Vu *et al.*, 1991). Messenger RNA (mRNA) from HEL and Dami cells which were highly responsive to thrombin was injected into *Xenopus* oocytes prelabelled with $^{45}Ca^{2+}$, and the responses were measured as thrombin-stimulated $^{45}Ca^{2+}$ release. The authors then constructed a cDNA library using fractionated mRNA from Dami cells, with the cDNA being plated in 50 pools of approximately 20,000 clones each. Thrombin-induced responses were assayed in oocytes injected with cRNA transcribed from each pool and by subdividing the positive pools progressively, a single cDNA encoding a thrombin receptor was eventually identified.

Reverse-transcription polymerase chain reaction (RT-PCR) detected the mRNA expression of this receptor in both platelets and vascular endothelial cells. Nucleotide sequencing of this functional thrombin receptor uncovered a 3.5 kb insert, with an open reading frame encoding a 425 amino acid protein. By utilising hydropathy analysis, the inferred amino acid sequence of this receptor showed resemblance to the seven-transmembrane-domain GPCRs. The extracellular amino-terminal of this thrombin receptor, or PAR-1, was found to consist of 75 amino acids, including an anion-binding site known as hirudin-like domain. A later study proposed that the receptor's hirudin-like domain induces a conformational change in thrombin's active moiety to effect receptor activation (Ishii *et al.*, 1995). Through the generation of receptor chimeras and receptor point mutagenesis, the authors concluded that proteolytic cleavage of the receptor amino-terminal caused by thrombin at $LDPR^{41}/S^{42}FLL$ was essential for

activation (see Figure 1.2). Thrombin was found to cleave at Arg^{41} of PAR-1 to expose a tethered ligand which then binds intramolecularly to activate the receptor, representing a novel mode of receptor activation for GPCR. Interestingly, synthetic peptides which mimicked the newly exposed amino-terminal appeared to be able to stimulate the receptor without the need for proteolysis, thus confirming the tethered ligand model of activation for the receptor. A gene mapping study revealed that the human PAR-1 gene was located in chromosomal region 5q13 (Bahou *et al.*, 1993).

At the same time, similar findings were reported in another study in which a functional hamster thrombin receptor was successfully cloned and expressed in *Xenopus* oocytes (Rasmussen *et al.*, 1991). The hamster thrombin receptor shared 79% amino acid sequence identity with the human thrombin receptor, and included a thrombin consensus cleavage site and a negative charge cluster in the extracellular amino-terminal.

1.2.1.2 Pharmacology and structure-activity relationship (SAR) for PAR-1 activating peptides (APs)

Synthetic activating peptides (APs) which have an amino acid sequence that corresponds to the tethered ligand, can cause receptor activation, thus by-passing the need for receptor cleavage by enzyme (see Figure 1.1). The advantage of using APs in experimental design is that the unspecific proteolytic actions of the enzyme on other receptors can be excluded. In a series of studies based on the tethered ligand sequence of PAR-1, SFLLRN, the structure-activity relationship (SAR) for PAR-1 APs were derived and a series of moderate to high affinity agonist peptides were developed (Scarborough *et al.*, 1992; Nose *et al.*, 1993, Feng *et al.*, 1995). This approach was also facilitated by the use of site-directed mutagenesis to identify amino acid residues essential for ligand binding (Nanevicz *et al.*, 1995). In addition, extracellular loop (ECL)-2 has been identified as the important domain in binding of PAR-1 APs to the receptor (Lerner *et al.*, 1996).

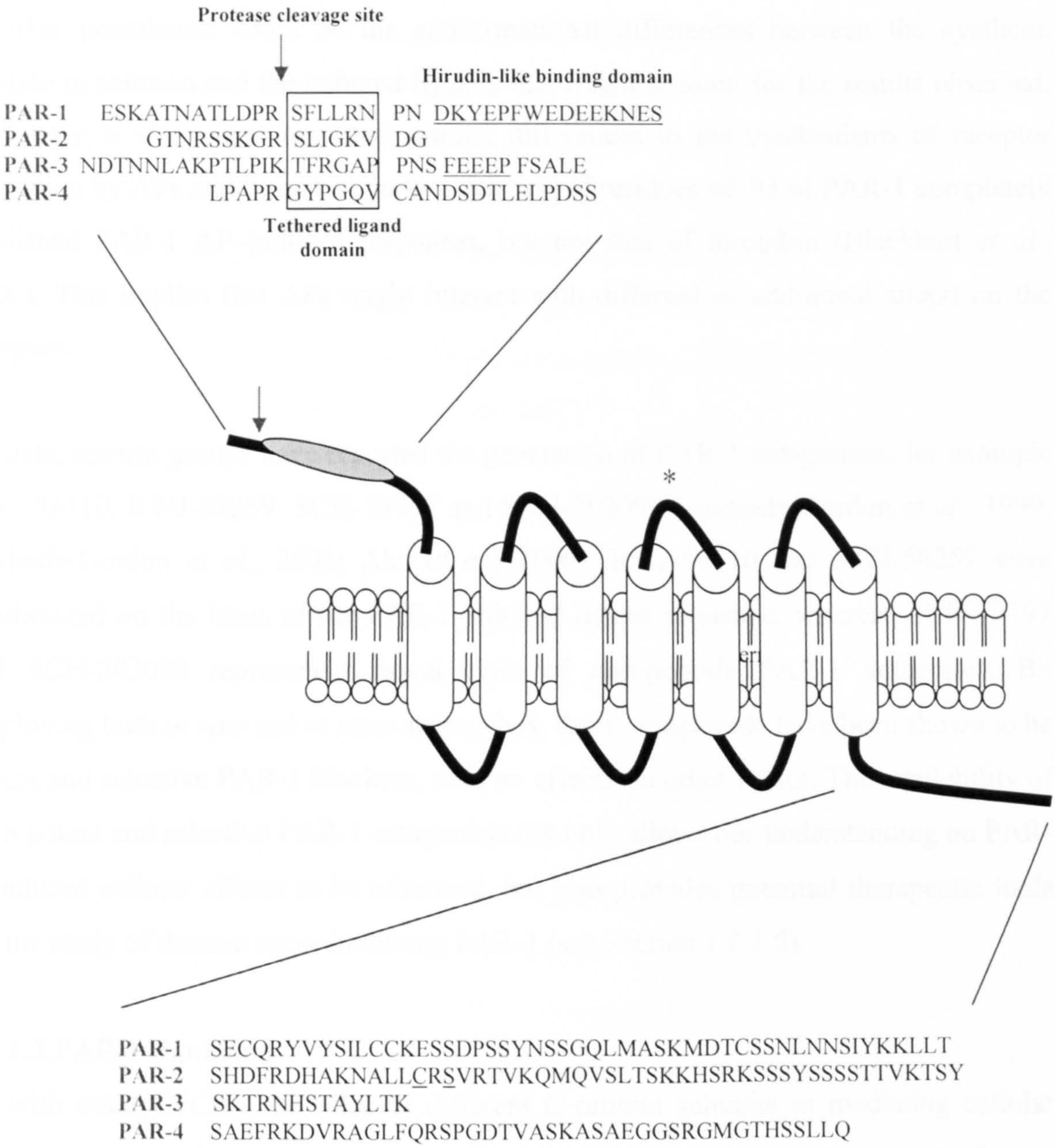


Figure 1.2 Protein structure of PAR-1, PAR-2, PAR-3 and PAR-4.

Important features of the structure are highlighted. * denotes the extracellular loop 2 (ECL-2) which is crucial in binding of agonist to the receptor. (Adapted from Macfarlane *et al.*, 2001)

Generally, APs showed lower potency than thrombin in activating the receptor. One possible explanation is that an enzyme molecule could activate several receptors. Another postulation could be the conformational differences between the synthetic peptide in solution and the tethered ligands that might account for the results observed. Strikingly, a study has suggested distinct differences in the mechanisms of receptor activation by APs and thrombin, in that deletion of residues 68-93 of PAR-1 completely abolished PAR-1 AP–induced responses, but not that of thrombin (Blackhart *et al.*, 2000). This implies that APs might interact with different or additional site(s) on the receptor.

To date, several groups have reported the generation of PAR-1 antagonists, for example RWJ-56110, RWJ-58259, SCH-79797 and SCH-203099 (Andrade-Gordon *et al.*, 1999; Andrade-Gordon *et al.*, 2001; Ahn *et al.*, 2000). RWJ-56110 and RWJ-58259 were synthesized on the basis of the PAR-1 tethered ligand sequence, whereas SCH-79797 and SCH-203099 represent a novel class of non-peptide PAR-1 antagonist. By employing both *in vivo* and *in vitro* approaches, these compounds have been shown to be potent and selective PAR-1 blockers, with no effects on other PARs. The availability of such potent and selective PAR-1 antagonists not only allows our understanding on PAR-1–induced cellular effects to be advanced, but also provides potential therapeutic tools for the study of disease states involving PAR-1 (see Section 1.2.1.5).

1.2.1.3 PAR-1 signalling

As with other GPCRs, the roles of different G-protein subunits in mediating cellular signalling of PAR-1 have been implicated including $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$ (Macfarlane *et al.*, 2001). In addition, lipid enzymes and protein kinases have also been implicated as part of the PAR-1 signalling paradigm.

Stimulation of PAR-1 activates $G\alpha_{q/11}$, leading to PLC-induced phosphoinositide hydrolysis to produce IP_3 and DAG as well as subsequent calcium mobilization (Brass *et al.*, 1991, Hung *et al.*, 1992a, Hung *et al.*, 1992b). $G\alpha_q$ coupling represents the major

pathway of PAR-1 signalling in platelet activation, as platelets derived from $G\alpha_q$–deficent mice showed attenuated thrombin-induced aggregation (Offermanns *et al.*, 1997). In addition, Hung and colleagues have characterised the coupling of PAR-1 to $G\alpha_i$, resulting in cAMP inhibition (Hung *et al.*, 1992a). PAR-1 also couples to $G\alpha_{12/13}$ and it was shown that thrombin regulates endothelial barrier function and hyper-permeability through the downstream effectors of $G\alpha_{12/13}$, p115RhoGEF, RhoA and Rho-associated coiled-coil forming kinase (ROCK) (Majumdar *et al.*, 1999; Vouret-Craviari *et al.*, 1998; Carbajal *et al.*, 2000, Nguyen *et al.*, 2002). In addition to PLC, PAR-1 has also been shown to stimulate phospholipase A_2 (PLA_2) to release arachidonic acid (Lan *et al.*, 2001; Rickard & McHowat, 2002).

PAR-1 was one of the first GPCRs to be investigated for kinase signalling as its endogenous activator, thrombin, is a well-known mitogen (Seuwen *et al.*, 1990; McNamara *et al.*, 1993). Activation of MAPKs by PAR-1 was revealed when PAR-1 AP was found to stimulate $p21^{ras}$, the upstream activator of extracellular signal-regulated kinase (ERK) cascade, shedding light on the role of PAR-1 in mitogenesis via the tyrosine kinases pathway (Vancorven *et al.*, 1993). Interestingly, ERK activation was found to exhibit a biphasic profile, with the early phase being PTX-sensitive (Trejo *et al.*, 1996). Further studies reported that in various cell lines, PAR-1 was found to induce the activation of ERK, c-Jun amino-terminal kinases (JNKs), p38 MAPK and NFκB via multiple pathways which will be further discussed in Sections 1.3.2 and 1.4.6 (Madamanchi *et al.*, 2001; Buresi *et al.*, 2002; Sabri *et al.*, 2000; Rahman *et al.*, 2002; Sabri *et al.*, 2002; Wang *et al.*, 2002; Tantivejkul *et al.*, 2005).

1.2.1.4 Identification of additional PARs: PAR-3 and PAR-4

i) PAR-3

The potential for a second thrombin receptor was uncovered in studies using PAR-1 deletion mice (Connolly *et al.*, 1996). Although fibroblasts derived from PAR-1 deficient mice failed to elicit response upon thrombin stimulation, platelet activation as well as calcium mobilization induced by thrombin were analogous in both knockout and

wild type mice, strongly suggesting the presence of a novel receptor activated by thrombin other than PAR-1. This novel receptor, designated as PAR-3, was cloned by Ishihara and colleagues, and was found to share 27% structural identity with PAR-1 (Ishihara *et al.*, 1997).

PAR-3 was also found to share the same mechanism of activation as PAR-1, with thrombin-mediated cleavage exposing a new amino-terminal, TFRGAP. Substitution of Thr^{39} with Pro rendered the receptor inactive to thrombin stimulation, confirming that the cleavage site lies at residues Lys-38/Thr-39 of PAR-3's exodomain. As with PAR-1, the amino-terminal of PAR-3 was found to encompass a hirudin-like domain and residue Phe^{40} of the tethered ligand domain was confirmed to be important in receptor activation, as deletion of this residue resulted in diminished signalling responses (Ishihara *et al.*, 1997).

Amongst all the proteases tested, thrombin was the only serine protease capable of activating PAR-3, having an EC_{50} of 0.2 nM, approximately twice as low as that reported for PAR-1 (Ishihara *et al.*, 1997). Surprisingly, unlike PAR-1, APs that mimic the sequence of PAR-3 tethered ligand were found to be inactive even at concentrations up to 100μM. Notably, recent studies have shown that APs derived from PAR-3 do not act on the native receptor but instead possess agonistic actions on PAR-1 and PAR-2, indicating high cross-reactivity of PAR activating peptides between each receptor (Hansen *et al.*, 2004; Kaufmann *et al.*, 2005) (see Section 1.2.2.2).

The expression of PAR-3 was not confined to platelets, but was also detected in human bone marrow and mouse splenic megakaryocytes (Ishihara *et al.*, 1997, Schmidt *et al.*, 1998). Notably, flow cytometry revealed the relative expression of PAR-3 on platelet to be only 10% of that for PAR-1, thus PAR-3 may be less significant in mediating platelet activation (Schmidt *et al.*, 1998). The PAR-3 gene was mapped to chromosome 5q13, sharing a similar genomic localization with that of PAR-1 (Schmidt *et al.*, 1998).

ii) PAR-4

As outlined above, Connolly and co-workers demonstrated that PAR-1 played no role in mediating mouse platelet activation; in fact such effects were thought to be mediated by PAR-3 (Connolly *et al.*, 1996). However, further study has revealed that in PAR-3 deficient platelets, whilst aggregation was delayed, maximal responses to thrombin were still being obtained, highlighting an additional thrombin receptor in this context (Kahn *et al.*, 1998). This led to the discovery of the fourth member of the PAR family, PAR-4 (Kahn *et al.*, 1998, Xu *et al.*, 1998).

PAR-4 was found to share 33% overall sequence identity with other PARs (Xu *et al.*, 1998). Nevertheless, vast differences were observed in the extracellular amino-terminal, intracellular carboxyl tail and ECL-2 of PAR-4 compared to other PAR family members (Xu *et al.*, 1998) and in contrast with PAR-1 and PAR-3, PAR-4 does not contain a fibrinogen-binding exosite. Notably, PAR-4 was sensitive to both thrombin and trypsin, with an EC_{50} of 5nM for both agonists (Xu *et al.*, 1998).

By utilising site-directed mutagenesis, the PAR-4 cleavage site was confirmed to lie at Arg-47/Gly-48. As with PAR-1, GYPGQV, the AP derived from the putative tethered ligand of PAR-4, was found to be functionally capable of activating the receptor, albeit with a relatively low potency (EC_{50} = 100μM) (Xu *et al.*, 1998). The synthetic peptide AYPGKF which has multiple residue substitutions, including an alanine in place of the glycine, was found to possess 10-fold higher potency than that of the parent peptide (Faruqi *et al.*, 2000). Notably, substitution of Tyr^2 of the PAR-4 parent peptide with Phe or *para*-Phe conferred PAR-1 activating ability to the AP, suggesting that the second residue of the peptide sequence might play a critical role in determining receptor selectivity (Faruqi *et al.*, 2000, Kahn *et al.*, 1998). Recently, low potency antagonists have also been developed for PAR-4 such as peptide mimetic trans-cinnamoyl-YPGKF-NH_2 (Hollenberg & Saifeddine, 2001), and novel cell-penetrating molecules known as pepducins that disrupt the association of PAR-4 with downstream effectors (Covic *et al.*, 2002). However, a recent study has proposed that peptides derived from the PAR-4

tethered ligand sequence might act on other non-PAR-4 receptors and thus the specificity of these antagonists remains to be unequivocally established (Hollenberg *et al.*, 2004).

Northern blot analysis revealed PAR-4 expression in lungs, pancreas, thyroid, testis and small intestine (Xu *et al.*, 1998). In addition, Xu and colleagues have mapped the PAR-4 gene to chromosomal region 19p12, not in accordance with other family of PARs. PAR-4 has been shown to associate with $G_{q/11}$ and $G_{i/o}$ proteins in various studies, nonetheless the signalling paradigm for this receptor remains largely unknown (Faruqi *et al.*, 2000; Momota *et al.*, 2006).

1.2.1.5 Physiological roles of PAR-1, PAR-3 & PAR-4

With the discovery of PARs, it is now known that thrombin influences cellular behaviour via PAR-1, PAR-3 or PAR-4, all of which are widely expressed. With the design and synthesis of potent agonists and antagonists, the work to identify receptor subtype-specific responses has been made plausible.

Although both PAR-1 and PAR-4 were found to be co-expressed in human platelets, the action of thrombin in causing platelet aggregation has been shown to be predominantly mediated by PAR-1 (Kahn *et al.*, 1999). PAR-4 has a much lower affinity for thrombin compared to that of PAR-1 and only responds to high concentration of the enzyme. However PAR-4 has been implied in maintaining the sustained effect of thrombin, thereby contributing to the late phase of platelet aggregation (Covic *et al.*, 2000). Interestingly, in murine platelets, thrombin only signals through PAR-4 despite the fact that PAR-3 expression has also been detected, and the latter functions as the coactivator for the former (see Section 1.2.2.6) (Nakanishi-Matsui *et al.*, 2000).

In the vasculature, PAR-1 stimulation causes endothelium-dependent nitric oxide-mediated vasorelaxation in vessels isolated from various species including human pulmonary artery and rat aorta (Hamilton *et al.*, 2001a; Magazine *et al.*, 1996). In

addition, PAR-1 can also signal to contract vascular smooth muscle in endothelium-denuded preparations (Laniyonu & Hollenberg, 1995). Just recently, PAR-4 has been shown to induce nitric oxide (NO) production in vascular endothelial cells, denoting a possible role for this thrombin receptor subtype in mediating vasodilation (Momota *et al.*, 2006). The mitogenic effects of thrombin in endothelial and vascular smooth muscle cells have been reported to be mediated by PAR-1 and PAR-4 (McNamara *et al.*, 1993; Mirza *et al.*, 1996).

PAR-1 expression has been widely detected in the brain, denoting its vital roles in the nervous system (Weinstein *et al.*, 1995). Both PAR-1 and PAR-4 are responsible for thrombin–induced $[Ca^{2+}]_i$ elevation in human astrocytomas, contributing to the excitability and survival of the cells (Kaufmann *et al.*, 2000). In addition, activation of PAR-1 leads to morphological changes in neurons and astrocytes as well as shielding the cells from environmental stresses (Jalink *et al.*, 1994; Vaughan *et al.*, 1995).

PAR-1 has a role in neurogenic inflammation, as intraplantar injection of PAR-1 agonists causes oedema of rat paw through the release of substance P from peripheral afferent neurons (de Garavilla *et al.*, 2001). Not only that, PAR-1 has been implicated in hyperalgesia although the underlying mechanism has yet to be defined (Asfaha *et al.*, 2002). Notably, the ability of thrombin to induce leukocyte rolling and adherence was found to be mediated solely via PAR-4 rather than PAR-1 as previously thought (Vergnolle *et al.*, 2002). PAR-4 has also been shown to have a proinflammatory role in a rat paw oedema model and, unlike PAR-1, the underlying mechanism appears to be non-neurogenic but possibly through neutrophil recruitment activation and the kallikrein-kinin system (Hollenberg *et al.*, 2004, Houle *et al.*, 2005).

In the gastrointestinal tract, PAR-1 has been shown to mediate ion transport, electrolyte secretion, intestinal motility, contraction of longitudinal muscle and in contrast, relaxation of colonic circular muscle (Buresi *et al.*, 2002; Saifeddine *et al.*, 1996; Mule & Baffi, 2002; Corvera *et al.*, 1999). Alternatively, PAR-4 is implicated in relaxing pre-

contracted rat oesophagus, in contrary to PAR-1 which causes further contraction (Kawabata *et al.*, 2000b). Such findings highlighted the bi-functional roles of thrombin in the same tissue, depending on the amount of enzyme available and the relative levels of PAR-1 and PAR-4 expression.

The mitogenic property of thrombin is also exemplified via actions of PAR-1 on human airway smooth muscle cells and keratinocytes which might contribute to pulmonary fibrosis and wound healing respectively (Tran & Stewart, 2003; Howell *et al.*, 2002; Santulli *et al.*, 1995). PAR-1 is also a potential oncogene as it is associated with metastasis and invasion in a model of breast carcinoma xenograft (Even-Ram *et al.*, 1998; Boire *et al.*, 2005).

Taken together, these studies suggest that the intricate and diverse biological effects of thrombin can be mediated through individual or combinations of PAR-1, PAR-3 and PAR-4 in a species and/or tissue-specific manner. The unavailability of an active PAR-3 synthetic ligand has left this thrombin receptor subtype less well characterised. Further studies are needed to further elucidate the specific role of each receptor subtype in mediating the wide array of thrombin–induced cellular effects.

1.2.2 PAR-2

1.2.2.1 Discovery of PAR-2

Another subtype of PAR which was discovered concomitantly with PAR-3 and PAR-4 was PAR-2. Through the work of screening a mouse genomic library with moderate stringency using two oligonucleotides corresponding to regions of bovine substance K receptor, a cosmid clone containing a 3.7-kb *Pst* I fragment was revealed (Nystedt *et al.*, 1994). Further work showed an open reading frame encoding a protein of 395 amino acids. Similar to PAR-1, hydropathy analysis of the deduced protein sequence disclosed seven transmembrane regions. Through a database search, it was found that this new receptor, now termed PAR-2, was closely related to PAR-1, sharing a 30% sequence

homology, despite little similarity in the amino-terminal and the intracellular carboxyl-terminal sequences (Nystedt *et al.*, 1994).

The PAR-2 amino-terminal was found to be 29 amino acids shorter than that of PAR-1, without the presence of an anion-binding exosite which is thought to mediate high-affinity thrombin-receptor activation (see Figure 1.2) (Nystedt *et al.*, 1994). Notably, from a PAR-1 and PAR-2 sequence alignment, ECL-2 was identified as a highly conserved region, suggesting a common and important feature in PAR activation (see Section 1.2.2.3). Mouse PAR-2 was found to share a similar mechanism of activation to PAR-1, in that it was activated by proteolytic cleavage. It was then shown that PAR-2 remained relatively insensitive to thrombin but could be activated by trypsin and the peptide derived from its amino-terminal, SLIGRL. Human PAR-2 which was revealed to consist of 397 residues was then cloned and the deduced sequence was found to share 83% identity with the mouse isoform (Nystedt *et al.*, 1995b, Böhm *et al.*, 1996b). The cleavage site of human PAR-2 was found to lie at $SKGR^{36}\downarrow S^{37}LIGKV$, unmasking a new terminus with SLIGKV as the putative tethered ligand sequence (Nystedt *et al.*, 1995b). The authors detected the location of human PAR-2 gene at chromosome 5q13, the same region previously described for PAR-1 and PAR-3 (Nystedt *et al.*, 1995b). Northern blotting revealed the expression of PAR-2 to be in highly vascularised tissues, such as stomach, pancreas, liver, kidney, small intestine and colon (Nystedt *et al.*, 1994, Nystedt *et al.*, 1995b, Böhm *et al.*, 1996b).

1.2.2.2 PAR-2 activating peptides (APs)

i) Structure-activity relationship (SAR) for PAR-2 agonists

A comprehensive understanding of SAR for PAR-2 APs is essential as it will form the fundamental basis for the development of potent PAR-2 agonists to serve as pharmacological tools. In addition, given that PAR-2 has been implicated in various patho-physiological conditions (see Section 1.2.2.8), SAR for PAR-2 agonists will facilitate the design of novel PAR-2 antagonists which might be therapeutically useful. To date, multiple assays have been utilised to evaluate the SAR of PAR-2 agonist

peptides including; stable PAR-2 expressing cell lines derived from Kirsten virus-transformed rat kidney cells (KNRK) and NCTC-2544, *Xenopus* oocytes expressing PAR-2, radioligand-binding, *in vitro* vascular tissue preparations and *in vivo* salivation (Nystedt *et al.*, 1994; Blackhart *et al.*, 1996; Al-Ani *et al.*, 1999a; Al-Ani *et al.*, 1999b; Hollenberg *et al.*, 1996, Kawabata *et al.*, 2004).

Early SAR studies were based on the use of hexapeptide sequences SLIGKV, the human tethered ligand sequence, and the murine variant, SLIGRL. Notably, such short peptide sequences appeared to be sufficient to activate the receptor. A disparity between the human and murine isoforms was observed within the extracellular amino-terminal, SLIGKV being less potent than SLIGRL (Nystedt *et al.*, 1994; Nystedt *et al.*, 1995a). Trypsin was found to activate PAR-2 at a very low concentration, with an EC_{50} of 1nM, whilst SLIGKV and SLIGRL activate PAR-2 with micromolar concentrations, with EC_{50} of 12 μM and 5-6 μM respectively, showing that the peptides were less potent than trypsin at human PAR-2 (Nystedt *et al.*, 1994; Nystedt *et al.*, 1995a; Maryanoff *et al.*, 2001). Such a discrepancy in potency was also noted in parallel to that observed for thrombin and APs acting at PAR-1 (discussed in Section 1.2.1.2).

Several groups have performed studies, including alanine scanning, to evaluate the importance of each amino acid residue of the peptide sequences (Hollenberg *et al.*, 1997; Maryanoff *et al.*, 2001, Al-Ani *et al.*, 2004). Carboxy-terminal amidation of the peptides increases the potency of the agonists compared to the non-amidated peptides, i.e. SLIGKV-OH vs SLIGKV-NH_2. The role of the free amino group on the serine residue has been explored by both acetylation and removal of this component from the peptide sequence (Blackhart *et al.*, 1996; Maryanoff *et al.*, 2001, Santagada *et al.*, 2002). The acetylated peptide was shown to be inactive, whereas the potency of the peptide lacking the amino group was only reduced by 4 fold, indicating that the free amino group is not absolutely crucial for agonist potency, but an amino-terminal amide is detrimental. In general, several lines of evidence have supported the critical roles of Leu^2, Ile^3 and Arg^5

in PAR-2 peptide sequence in activating the receptor (Hollenberg *et al.*, 1996; Hollenberg *et al.*, 1997).

Ferrell *et al.* has reported the synthesis of a more potent agonist peptide, 2-furoyl-LIGKV-OH (2f-LIGKV-OH), in which the amino-terminal serine is replaced with a furoyl group (Ferrell *et al.*, 2003). By using multiple *in vitro* and *in vivo* assay systems, 2f-LIGRL-NH_2, which has the incorporation of a 2-furoyl group into the amidated mouse sequence, was found to be the most potent PAR-2 AP so far (Kawabata *et al.*, 2004). The authors also showed that the substitution of the amino-terminal serine with a furoyl group rendered the peptide more resistant to aminopeptidase degradation. Similar findings were reported independently by a different group in which another furoylated compound, 2f-LIGRL-ornithine-NH_2, was also found to exhibit high PAR-2 agonistic activity (McGuire *et al.*, 2004). Al-Ani *et al.* (1999b) has reported the generation of N-*trans*-cinnamoyl-LIGRL-ornithine-NH_2 that can be radiolabelled at the free ornithine amino group with [^{3}H]-propionic anhydride to function as a radioligand-binding probe. To date the shortest functioning peptide reported for PAR-2 was shown to be the dipeptidyl analogue derived from N^{α}-benzoyl-R(NO_2)-L-NH_2, which possesses similar potency to that of a full length AP, suggesting the potential development of a small molecule as a PAR-2 AP (Santagada *et al.*, 2002).

ii) Ligand cross-reactivity of PAR-2 with other PARs

One possible problem is the potential ligand cross-reactivity between the PAR family members (Blackhart *et al.*, 1996; Kawabata *et al.*, 1999). Blackhart *et al.* (1996) provided information that the PAR-1 AP, SFLLRNP-NH_2, is capable of activating PAR-2 with a similar potency to that of PAR-2 AP. Moreover, mercaptopropionyl-FChaCha-RKDNPKY-NH_2, a peptide previously described to act as a PAR-1 antagonist, was found to exert full agonistic activity on PAR-2 (Kawabata *et al.*, 1999). Additionally, PAR-3–derived synthetic ligands were found to be able to stimulate PAR-2 in Jurkat T cells (Hansen *et al.*, 2004). These findings suggest that careful interpretation of data

obtained with the synthetic ligands for PARs is required in order to provide a clearer picture in this context.

iii) PAR-2 antagonists

Unlike PAR-1, relatively little information is available regarding a potent PAR-2 antagonist. An early study has described the ability of two peptides, LLRY-NH_2 and LSIGRL-NH_2, in antagonising the non-proteolytic agonistic action of trypsin on the PAR-2 but not that of PAR-2 APs, revealing an as yet identified distinct mechanism(s) of interaction between the tethered/soluble ligand and the receptor (Al-Ani *et al.*, 2002a). Together with the information obtained from receptor pharmacology (see Section 1.2.2.3), these lines of evidence strongly suggest that the putative tethered ligand and synthetic peptide may act on different docking sites of PAR-2, giving rise to different degrees of receptor activation and distinct intracellular signalling mechanisms (Al-Ani *et al.*, 1999b; Al-Ani *et al.*, 2002a; Al-Ani *et al.*, 2002b).

A recent report has illustrated a novel compound, N^1-3-methylbutyryl-N^4-6-aminohexanoyl-piperazine (ENMD-1068), as a PAR-2 antagonist (Kelso *et al.*, 2006). ENMD-1068 has been shown to inhibit calcium signalling induced by PAR-2 agonists *in vitro* without any effects on that of PAR-1; nor platelet aggregation mediated by PAR-3 or PAR-4. In *in vivo* studies, although ENMD-1068 did not affect the knee joint swelling mediated by thrombin; it significantly reduced the inflammatory responses in the model of carrageenan/kaolin-induced arthritis. This suggests that ENMD-1068 might serve as a potential target as an anti-arthritic therapy. However, the authors did not provide the direct evidence of PAR-2 antagonism by this compound *in vivo*; whether or not this is the exact underlying mechanism for the protective role of ENMD-1068 in this autoimmune disease model remains to be investigated.

From these limited studies, it is clear that more work is needed in order to obtain a comprehensive picture of PAR-2 peptide pharmacology. The observed ligand cross-reactivity as well as the distinct interactions of the soluble peptide and the tethered

ligand with the receptor have to be taken into account for the design and synthesis of high affinity agonists and antagonists for PAR-2.

1.2.2.3 Receptor Pharmacology of PAR-2

The identification of specific receptor domain(s) involved in interacting with the tethered ligand is essential for the understanding of the transmembrane signal transduction mechanism. An early study highlighted the role of ECL-2 in governing PAR-2 agonist activity using chimeric receptors in which the individual extracellular domains were being substituted (Lerner *et al.*, 1996). Further studies have pointed out the roles of residues Pro-231/Glu-232/Glu-233 (PEE) and Phe-240 of ECL-2 in governing PAR-2 activity and agonist specificity respectively (Al-Ani *et al.*, 1999a; Compton *et al.*, 2000).

Notably, PAR-2 has been shown to be glycosylated at both ECL-2 and the amino-terminal on Ile-223 and Arg-31 respectively (Compton *et al.*, 2002a). The authors concluded that these glycosylation sites are important determinants for efficient cell surface receptor expression and the ability of tryptase in activating PAR-2. Such an observation is consistent with the findings that glycosylation of PAR-2 has been shown to impede the access of tryptase to the receptor's cleavage site, thus accounting for the varied ability of tryptase to activate PAR-2 (Section 1.2.2.7) (Compton *et al.*, 2002b).

1.2.2.4 PAR-2 signalling

Compared to PAR-1, considerably less is known about PAR-2–mediated signalling. Thus far PAR-2 has been shown to induce a wide array of signalling molecules including G proteins, MAPK pathways and NFκB. The regulation of MAPK and NFκB signalling cascades that form the basis of this thesis will be discussed in more detail in Section 1.3 and 1.4 respectively.

Concomitant with its discovery, PAR-2 was shown to induce calcium mobilization and IP_3 production in *Xenopus* oocytes, keratinocytes and epithelial cells, suggesting the

coupling of PAR-2 to $G\alpha_{q/11}$ and PLC isoforms (Nystedt *et al.*, 1994, Santulli *et al.*, 1995, Böhm *et al.*, 1996b). Activation of PTX-insensitive IP_3/Ca^{2+} signalling by PAR-2 has also been observed in myocytes, astrocytes, enterocytes, neurons and tumour cell lines (Corvera *et al.*, 1997, Corvera *et al.*, 1999, Kong *et al.*, 1997, Ubl *et al.*, 1998; Bushell *et al.*, 2006). Apart from $G\alpha_{q/11}$, a study has reported that the calcium signalling induced by PAR-2 was associated with PTX-sensitive G proteins, indicating the potential role of $G\alpha_{i/o}$ in this context (Schultheiss *et al.*, 1997). In addition, PAR-2 has also been documented to be associated with Rho activation, a member of the small GTPases Ras superfamily, presumably via a PTX-insensitive mechanism (Scott *et al.*, 2003, Klarenbach *et al.*, 2003, Greenberg *et al.*, 2003). However, to date there is no direct evidence of PAR-2 coupling to $G\alpha_{q/11}$ or any other G proteins, further investigation such as coimmunoprecipitation studies might shed some light on this matter.

As with thrombin, trypsin has also been observed to activate components of the MAPK cascades. Trypsin and PAR-2 APs have been shown to activate ERK, p38 MAPK and JNK in several different models (Belham *et al.*, 1996; DeFea *et al.*, 2000; Stalheim *et al.*, 2005; Kanke *et al.*, 2001). Direct evidence of NFκB as a downstream effector of PAR-2 was demonstrated when both trypsin and PAR-2 AP caused a significant, time-dependent nuclear translocation of NFκB in smooth muscle cells and keratinocytes (Bretschneider *et al.*, 1999; Kanke *et al.*, 2001). The mechanisms which PAR-2 utilises to mediate MAPK and NFκB activation will be further described in Section 1.3.2 and 1.4.6 respectively. Other signalling components that have been shown to be activated by PAR-2 agonists include the focal adhesion kinase, proline-rich tyrosine kinase-2 (PYK-2), and the tyrosine phosphatase, SHP-2 (DeFea *et al.*, 2000; Yu *et al.*, 1997).

Taken together, the ability of PAR-2 to signal to a wide range of effector components enables this receptor to elicit diverse cellular effects through multiple signalling (see Section 1.2.2.8).

1.2.2.5 Desensitisation and resensitisation of PARs

i) Desensitisation as a mode of signal transmission termination

As PARs are proteolytically irreversibly activated, 'shut off' mechanisms involved in controlling the magnitude and extent of signal transduction are of particular importance in order to prevent persistent activation.

In general, two different groups of serine/theorine kinases are involved in desensitisation (reviewed in Shenoy & Lefkowitz, 2003). The first group includes second-messenger-regulated kinases such as PKA and PKC that mediate heterologous desensitisation, an event that is associated with receptor phosphorylation even in the absence of agonist (Shenoy & Lefkowitz, 2003). Alternatively, GPCR kinases (GRKs) represent the second group of kinases which phosphorylate only agonist-stimulated receptor thus homologous desensitisation. To date, seven isoforms of GRKs are known (GRK 1-7), all of which have been shown to exhibit receptor-specific kinase activity (Reiter & Lefkowitz, 2006). Notably, phosphorylation of the receptor would lead to the recruitment of β–arrestins and subsequently uncoupling of the receptor from its cognate G-protein.

For PAR-1, several studies have reported that the desensitisation of this receptor which occurs within minutes of receptor stimulation involves components such as GRK-3, GRK-5 and β–arrestins (Iaccarino *et al.*, 1998; Tiruppathi *et al.*, 2000; Paing *et al.*, 2002). Relatively little is known about events governing desensitisation of PAR-3 and PAR-4. Desensitisation of PAR-2 which shares similar kinetics to PAR-1, on the other hand, is associated with PKC (Böhm *et al.*, 1996a). In addition, a recent study by Stalheim *et al.* (2005) has demonstrated the involvement of β–arrestins in mediating PAR-2 desensitisation in that cells lacking β–arrestins exhibited enhanced total inositol phosphate accumulation in response to PAR-2 stimulation. However, the role of GRK(s) in regulating PAR-2 desensitisation has yet to be demonstrated.

ii) Intracellular trafficking and resensitisation of receptor

Following desensitisation, receptors undergo intracellular trafficking and processing of the respective receptors (reviewed in Shenoy & Leftkowitz, 2003). The general paradigm of GPCR internalisation involves several integral proteins such as β–arrestins, dynamin and clathrin-coated pit (Shenoy & Leftkowitz, 2003). The endocytosed receptor will then be transported to endosomes and subsequently be targeted for either degradation by lysosomes or recycling back to the cell surface.

PAR-1 endocytosis has been well-studied, and several lines of evidence have suggested that upon receptor activation, PAR-1 is rapidly internalised through dynamin-mediated clathrin-coated pit formation with the majority of the receptors targeted to lysosomes for degradation (Hoxie *et al.*, 1993; Trejo *et al.*, 2000). Thus resensitisation of PAR-1 which occurs within 60 minutes requires mobilisation of the receptor from Golgi stores and protein synthesis. Interestingly, in contrast to other GPCRs, β–arrestins have been shown to have no role in regulating PAR-1 endocytosis in a study using cells derived from β–arrestin knockout mice (Paing *et al.*, 2002). Again, the resensitisation process for PAR-3 and PAR-4 remains largely undefined.

Similar intracellular trafficking and resensitisation mechanisms have been reported for PAR-2. PAR-2 stimulation causes rapid internalisation and co-localisation of the receptor with β–arrestins into endosomes (Böhm *et al.*, 1996a; Dery *et al.*, 1999). Thus unlike PAR-1, arrestins are important mediators in modulating PAR-2 endocytosis (Dery *et al.*, 1999; Stalheim *et al.*, 2005). Additional evidence also suggested the involvement of a GTPase, rab5a, in regulating PAR-2 internalisation (Roosterman *et al.*, 2003). Subsequently, PAR-2 is transported to lysosomes and replenishment of the receptor at the cell surface requires receptor mobilisation from Golgi apparatus and the synthesis of new protein (Böhm *et al.*, 1996a; Dery *et al.*, 1999). In addition, Roosterman *et al.* (2003) reported that the trafficking of PAR-2 from Golgi store involves rab11a, a GTPase which has been found to co-localise with PAR-2 in migrating vesicles from the Golgi to the cell membrane.

1.2.2.6 Functional Interactions of PARs

In several studies, PARs have been identified to be present in the same cell line, leading to further investigations of the functional interactions between PARs (Molino *et al.*, 1997a; Hollenberg *et al.*, 1999). The fact that a ligand can serve as an agonist for several receptors adds to the complexity of the functional interactions of PARs.

i) PAR-2 with other PARs

Extensive studies have illustrated the functional interactions between PAR-1, PAR-3 and PAR-4 and considerably less is known regarding PAR-2. O' Brien *et al.* (2000) has described the intermolecular signalling involving PAR-1 and PAR-2. The authors reported that a truncated PAR-1 which was unable to signal and a wild type PAR-2, when expressed together, responded to thrombin, suggesting transactivation of PAR-2 by PAR-1. In addition, in endothelial cells that naturally express both PAR-1 and PAR-2, a PAR-1 antagonist blocked only 75% of the response mediated by thrombin; with the remaining 25% being abolished following PAR-2 desensitization. Although transactivation of PAR-2 by PAR-1 might not be the main mechanism which accounts for thrombin signalling in endothelial cells, this result highlighted the fact that effective antagonism of thrombin response can be complicated in the presence of PAR-2 and possibly *vice versa* and considering that the same phenomenon might exist in other cell lines.

ii) PAR-1, PAR-3 and PAR-4

A large body of evidence has proposed the functional interactions between PAR-1, PAR-3 and PAR-4. An initial study has indicated the role of PAR-1 and PAR-4 in mediating the activation of human platelets (Kahn *et al.*, 1999). The same study also demonstrated a role for PAR-1 in mediating platelet activation at low thrombin concentrations, whilst in contrast PAR-4 was shown to be effective only at high concentrations of thrombin. Notably, PAR-4 is not made redundant by the presence of PAR-1, as a further study reported that both PAR-1 and PAR-4 have distinct kinetics upon activation by thrombin in which PAR-4 induced a more prolonged response

compared to that of PAR-1 (Shapiro *et al.*, 2000). This PAR-4 response was important in regulating the late phase of platelet aggregation process (Covic *et al.*, 2000b). These results indicate that both PAR-1 and PAR-4 can account for the observed thrombin responses, with distinct kinetics that would lead to different signalling mechanisms. Recently, PAR-1 has been shown to form heterodimers with PAR-4 in human fibroblast and platelets (Leger *et al.*, 2006). Such a dual-receptor paradigm might provide important implications in generating useful therapeutic tools for anti-coagulant and anti-thrombotic purposes.

Interestingly, mouse PAR-3 (mPAR-3) was found to be a cofactor for mPAR-4 activation in mediating platelet activation (Nakanishi-Matsui *et al.*, 2000). The authors showed that co-expression of mPAR-3 and mPAR-4 greatly enhanced thrombin signalling response in platelets, and it was deduced that the amino-terminal exodomain of mPAR-3 played the key role in promoting mPAR-4 activation by localising thrombin to the cell membrane via its hirudin-binding site, a motif that is absent in PAR-4. However, the extrapolation of this finding to a human model is limited, as PAR-3 expression was not detected in human platelet (Kahn *et al.*, 1999). In addition, an interaction between the same receptor subtypes has also been implicated (Chen *et al.*, 1994). Through generation of mutant receptors, the authors showed that for PAR-1, a cleaved receptor is able to activate another uncleaved receptor. Collectively, these data provide new insight into the mechanism(s) of activation of PARs, whether or not cofactors play a direct role in human involving PAR-2 requires further exploration.

1.2.2.7 Protease activators of PAR-2

Several endogenous activators have been identified for PAR-2 (see below). An early study identified the serine protease, trypsin, as the endogenous activator for PAR-2 especially in the gastrointestinal system where the enzyme is secreted and the receptor is highly expressed (Böhm *et al.*, 1996b). Soon it was realised that trypsin is in fact widely distributed in the human body, further supporting the role of trypsin as the predominant

activator for PAR-2 even outside the gastrointestinal environment (Koshikawa *et al.*, 1997; Koshikawa *et al.*, 1998; Cocks *et al.*, 1999b).

To date, four subtypes of trypsin have been identified; trypsin I, trypsin II, trypsin III and trypsin IV (Emi *et al.*, 1986; Nyaruhucha *et al.*, 1997; Wiegand *et al.*, 1993). Trypsin I-III are pancreatic enzymes whereas trypsin IV is found in the brain. Trypsin II isolated from a carcinoma cell line was found to stimulate PAR-2 expressed in an insect cell line (Alm *et al.*, 2000). Another study also reported that trypsin IV was able to activate both PAR-2 and PAR-4 expressed in KNRK cells (Cottrell *et al.*, 2004). Unlike pancreatic trypsin, trypsin IV was shown to be resistant to inhibition by polypeptide inhibitors such as soybean trypsin inhibitor, suggesting that trypsin IV might be capable of producing a more prolonged response *in vivo*. In addition, a trypsin-like serine protease designated as P22, purified from rat brain, was found to be able to stimulate PAR-2 activation in glioblastoma cells (Sawada *et al.*, 2000).

Several other proteases in the vasculature have been reported to activate PAR-2, for example tryptase secreted from human mast cells, is the first protease other than trypsin shown to be able to activate PAR-2, albeit with a lower potency (Molino *et al.*, 1997b; Fox *et al.*, 1997; Mirza *et al.*, 1997). However, another study has failed to detect PAR-2 activation by tryptase (Huang *et al.*, 2001). It was later concluded that glycosylation of PAR-2 would impede receptor activation by tryptase, although the mechanisms involved remain unclarified (Compton *et al.*, 2002b). Interestingly, the coagulation protease factor Xa (Xa) which normally acts upstream of thrombin has been shown to activate PAR-2 along with PAR-1, albeit at high non-physiological concentrations (~10nM) (Camerer *et al.*, 2000). Further studies revealed that Xa retains high affinity for its activator, the tissue factor (TF)-factor VIIa (VIIa) complex, forming a ternary complex, TF-VIIa-Xa, that is able to activate both PAR-1 and PAR-2 (Baugh *et al.*, 2000, Riewald & Ruf, 2001). These data described a novel function for the upstream coagulation mediators, which were previously thought to play pivotal roles solely in zymogen cleavage. Proteases secreted by leukocytes are also capable of activating PARs. For example, the

neutrophil degranulation products proteinase 3 (PR3) and cathepsin G, have been shown to signal via PAR-2 and PAR-4 respectively (Uehara *et al.*, 2002; Sambrano *et al.*, 2000).

Other miscellaneous PAR-2 activators include a recently cloned transmembrane protein with an extracellular protease domain, termed membrane-type serine protease 1 (MT-SP1), which resulted in calcium signalling when exposed to PAR-2 expressing oocytes (Takeuchi *et al.*, 2000). In addition, acrosin, a serine protease found in the acrosomal vesicle of mammalian spermatozoa has been shown to activate PAR-2 (Fox *et al.*, 1997). Such findings contribute to the postulation that PAR-2 may play a part in the reproductive system, in addition to the fact that high levels of receptor expression were found in the prostate and ovary (Nystedt *et al.*, 1995b, Böhm *et al.*, 1996b). In the brain, B-50/GAP-43, a neuronal growth associated protein, was found to serve as the precursor for PAR-2 agonist (Hollenberg *et al.*, 2000). Other studies have also reported that certain non-mammalian proteases are potential PAR activators. RgpB, an arginine-specific cysteine protease produced by *Porphyromonas gingivalis*, the causative agent of periodontal disease, caused calcium mobilization and interleukin (IL)-6 secretion in an oral epithelial cell line that is, at least in part, mediated via PAR-1 and PAR-2 (Lourbakos *et al.*, 2001). In addition, Der p3 and Der p9, two major serine proteases produced by dust mite, have been shown to signal to PAR-2 in epithelial cells (Sun *et al.*, 2001).

These findings highlight the fact that there are several potential activators for PAR-2 in different types of tissues, suggesting the role of this receptor in a wide range of biological processes (see Section 1.2.2.8). The implication is that within a given tissue, the extent of PAR-2 activation may be a composite of the actions of several different proteases and thus will reflect the overall role of PAR-2 in normal physiology and disease conditions. However, more studies are required.

1.2.2.8 Physiological roles of PAR-2

PAR-2 has been shown to be widely expressed in various tissues (reviewed in Macfarlane *et al.*, 2001). As with other PARs, PAR-2 has been implicated in diverse biological functions including haemostasis, inflammation, lung physiology, nervous system, digestive system, pain sensation and dermatological processes as discussed below.

i) Cardiovascular and circulatory system

Early studies reported the presence of PAR-2 in highly vascularised tissues such as kidney, small intestine and stomach, proposing its role in mediating haemodynamic effects (Nystedt *et al.*, 1994, D'Andrea *et al.*, 1998). Since then the role of PAR-2 in the modulation of vascular tone has been investigated extensively using classical organ bath pharmacology and also *in vivo*.

The expression of PAR-2 in the vasculature has been detected in endothelial and smooth muscle cells (Mirza *et al.*, 1996; D'Andrea *et al.*, 1998; O'Brien *et al.*, 2000). Activators of PAR-2 have been shown to cause endothelium-dependent relaxation of blood vessels of isolated tissues from various species including human, porcine coronary arteries and rat pulmonary arteries (Al-Ani *et al.*, 2002a; Hamilton *et al.*, 1998; Hwa *et al.*, 1996). Such effects were thought to be mediated via both NO-dependent and independent mechanisms. The relaxant effect of PAR-2 activation was then confirmed in studies monitoring blood pressure changes in intact animals and in PAR-2 knockout mice (Cheung *et al.*, 1998, Emilsson *et al.*, 1997, Damiano *et al.*, 1999a; Damiano *et al.*, 1999b). Evidence for the activation of PAR-2 resulting in a vasorelaxant effect in humans *in vivo* has been provided by Robin and colleagues (Robin *et al.*, 2003). Apart from the predominant hypotensive effect, PAR-2 APs have been shown to cause contraction of mouse renal arteries in endothelium-denuded preparations, suggesting direct smooth muscle contraction stimulated by PAR-2 (Moffatt & Cocks, 1998).

Thus far there is limited evidence confirming the role of PAR-2 in regulating vascular tone under normal physiological condition, in fact in mice lacking PAR-2 there was no obvious cardiovascular abnormalities (Damiano *et al.*, 1999b). Nevertheless, PAR-2 may play a role during inflammation given the fact that inflammatory agents such as tumour necrosis factor (TNF)-α, IL-1 and lipopolysaccharide (LPS) have been shown to cause the upregulation of PAR-2 mRNA in cultured endothelial cells within 4-20 hours (Nystedt *et al.*, 1996). Similar findings were also reported by several other *in vitro* studies (Cicala *et al.*, 1999; Hamilton *et al.*, 2001b; Damiano *et al.*, 1999a). It is possible that the effects of these inflammatory mediators on the blood vessels might be mediated via PAR-2. However, this idea would be contingent on the concomitant release of a PAR-2 activator. Also, by using a transgenic *in vivo* model, a recent study has provided evidence for PAR-2 mediating vasodilation in diabetes, under conditions in which the vessels were unresponsive to acetylcholine (Roviezzo *et al.*, 2005). Taken together, these results indicate that PAR-2 might have a pathological and/or compensatory role during inflammation and certain cardiovascular disorders.

PAR-2 activation may also lead to certain vascular protective mechanisms. For example, PAR-2 was found to mediate cerebral artery vasodilation, implying a potential protective role in cerebral circulation in the event of chronic hypertension (Sobey & Cocks, 1998; Sobey *et al.*, 1999). Also, Napoli and colleagues have documented a role of PAR-2 in ischemia-reperfusion injury, in that receptor activation improved efficiency of ischemic preconditioning and enhanced myocardial functional recovery (Napoli *et al.*, 2000, Napoli *et al.*, 2002). In addition, the mitogenic property of PAR-2 has been shown to promote angiogenesis and thus haemodynamic recovery in a mouse model of unilateral hindlimb ischemia (Milia *et al.*, 2002).

PAR-2 activation in immune cells is also vital in mediating inflammation. This is supported by the findings of PAR-2 expression in human neutrophils and eosinophils (Howells *et al.*, 1997, Miike *et al.*, 2001). Further studies revealed that PAR-2 may be involved in early inflammatory events, in that it promotes leukocytes rolling and

adhesion as well as chemotaxis, presumably through the release of platelet-activating factor (PAF) (Vergnolle, 1999). Lindner and co-workers showed that inflammatory responses were delayed but not abolished in PAR-2 deficient mice (Lindner *et al.*, 2000). The direct effect of PAR-2 in inflammation was demonstrated when injection of trypsin and PAR-2 AP into rat hindpaw was found to enhance vascular permeability and induce oedema formation, at least partly mediated through mast cell degranulation (Kawabata *et al.*, 1998). This was consistent with the work of Vergnolle *et al.* (1999), in which administration of PAR-2 APs into rat paw was found to result in an acute inflammatory reaction, exemplified by oedema and granulocyte infiltration. In contrast to Kawabata *et al.* (1998), the latter study proposed the involvement of a mast cell-independent mechanism. Furthermore, PAR-2 has also been shown to play a role in chronic inflammation. Intra-articular injection of PAR-2 AP induced persistent joint oedema and synovial hyperemia, suggesting PAR-2 as a proinflammatory factor in arthritis (Ferrell *et al.*, 2003). Furthermore, in PAR-2 knockout mice, the chronic inflammatory responses were not observed. Taken together, these findings point to the fact that PAR-2 plays a fundamental role in both the acute and chronic phases of inflammation.

ii) Respiratory System

PAR-2 is expressed by various cells types in the airway including ciliated and non-ciliated epithelial cells, airway smooth muscle cells, pulmonary endothelial cells and pulmonary vascular smooth muscle cells of human and other species (D'Andrea *et al.*, 1998; Cocks *et al.*, 1999b; Schmidlin *et al.*, 2001; Ricciardolo *et al.*, 2000; Miotto *et al.*, 2002).

There is controversial evidence as to whether PAR-2 mediates protective bronchodilatation or detrimental bronchoconstriction. Evidence favouring the protective role of PAR-2 was provided by Cocks and co-worker who showed that PAR-2 activation mediated an epithelium and prostanoid-dependent relaxation in isolated bronchi from several species precontracted with carbachol (Cocks *et al.*, 1999b). In contrast,

activation of PAR-2 in isolated human bronchial rings caused contraction (Chambers *et al.*, 2001; Schmidlin *et al.*, 2001). Thus, the effects of PAR-2 activation in the airway may depend on the type of tissue and species of the experimental model.

The effects of PAR-2 activation in the airways have also been investigated in intact animals. Again, various groups have shown both smooth muscle relaxant and contractile effects mediated by PAR-2 in this context. Aerosol administration of PAR-2 agonists to anaesthetised rats protected the animals against the bronchoconstriction effects induced by 5-hydroxytryptamine (5-HT) (Cocks *et al.*, 1999b). Similarly, intravenous injections of PAR-2 AP significantly inhibited histamine-induced increase in airway resistance in guinea pigs (Cicala *et al.*, 2001). On the contrary, another *in vivo* study reported that intravenous or intratracheal administration of PAR-2 agonists produced bronchoconstriction in guinea pigs, mediated in part by prostanoids and a neurogenic mechanism involving neurokinin receptors (Ricciardolo *et al.*, 2000).

Apart from regulating airway resistance, PAR-2 has been implicated in other aspects of the pulmonary system including airway remodelling (Akers *et al.*, 2000; Chambers *et al.*, 2001; Vliagoftis *et al.*, 2000), ion transport (Danahay *et al.*, 2001; Oshiro *et al.*, 2002) and airway inflammation (Cocks & Moffatt, 2001; Moffatt *et al.*, 2002; Asokananthan *et al.*, 2002b; Schmidlin *et al.*, 2002). Interestingly, house dust mite allergens such Der p3 and Der p9, are capable of activating PAR-2 to induce the release of proinflammatory cytokines such as IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in human lung epithelial cells (Sun *et al.*, 2001). Together with the fact that PAR-2 expression is upregulated in the bronchial epithelium of asthmatics, these findings imply a role for PAR-2 in immunity development and allergic inflammation of the airway (Knight *et al.*, 2001; Asokananthan *et al.*, 2002a).

iii) Skin

One of the earliest organs in which the expression of PAR-2 has been detected was skin, thus implying a role of PAR-2 in mediating dermatological processes (Santulli *et al.*, 1995). Further studies revealed the expression of PAR-2 in the basal, spinous and granular cell layers of the epidermis, as well as in the inner root sheath of hair follicles and myoepithelial cells of sweat glands (D'Andrea *et al.*, 1998; Steinhoff *et al.*, 1999).

The proinflammatory role of PAR-2 in skin was revealed when the receptor expression was found to be markedly enhanced in atopic dermatitis and psoriatic skin (Steinhoff *et al.*, 1999). This was further confirmed by *in vivo* studies, in that compared with the control group, PAR-2 knockout mice demonstrated reduced inflammatory responses including; attenuation of oedema and inflammatory cell infiltration induced by topical application of sensitizers. These results suggest a role for PAR-2 in type IV allergic dermatitis (Kawagoe *et al.*, 2002). Similar findings were reported by another study in which PAR-2 deletion mice showed reduced inflammation responses in a model of experimentally-induced contact dermatitis (Seeliger *et al.*, 2003). The proinflammatory effects of PAR-2 in *in vivo* may be mediated partly by the direct effects upon dermal microvascular endothelial cells since trypsin was shown to stimulate cytokine expression and NFκB activation (Shpacovitch *et al.*, 2002). In addition, PAR-2 activation induces the release of IL-6, IL-8 and GM-CSF in cultured human keratinocytes (Hou *et al.*, 1998; Wakita *et al.*, 1997). In inflamed skin, the number of tryptase-containing mast cells was found to be increased in the dermis layer as well as at the dermal-epidermal border. This led to the postulation that during skin inflammation tryptase released from mast cells might activate PAR-2 in keratinocytes, causing receptor hyperactivation and the release of various proinflammatory cytokines, giving rise to various pathological responses (Steinhoff *et al.*, 1999). Taken together, these findings suggest that PAR-2 might serve as the key factor underlying certain dermatological disorders.

Another important dermatological process mediated by PAR-2 is pigmentation. Melanocytes are melanosomes-containing cells that are responsible for skin

pigmentation. Although melanocytes do not express PAR-2, activation of PAR-2 in keratinocytes has been shown to facilitate the phagocytosis of melanosomes thus resulting in skin darkening (Seiberg *et al.*, 2000; Sharlow *et al.*, 2000). A role for PAR-2 in pigmentation also correlates well with the fact that PAR-2 expression in keratinocytes is upregulated after ultraviolet exposure (Scott *et al.*, 2001). Interestingly, Babiarz-Magee and co-workers showed that the expression of PAR-2 and trypsin were enhanced in the pigmented skin compared to the control group in an *in vitro* human study (Babiarz-Magee *et al.*, 2004). This led to the postulation that PAR-2 might contribute to ethnic skin colour phenotypes.

PAR-2 also represents a potential candidate for therapeutic intervention in pruritus when PAR-2 AP was found to induce prolonged itch sensation, highlighting a novel role for this receptor (Steinhoff *et al.*, 2003). This is consistent with a recent study which showed that intradermal injection of tryptase caused pruritogenic response which was thought to be mediated via PAR-2 (Ui *et al.*, 2006).

iv) Gastrointestinal System

Trypsin serves as one of the principle endogenous activators for PAR-2, thus it is not surprising to anticipate the role of the receptor in the gastrointestinal tract where this enzyme is abundantly expressed. Strong expression of PAR-2 has been detected in the small intestine, colon, pancreas and liver, and to a lesser extent in the stomach (Nystedt *et al.*, 1994; Nystedt *et al.*, 1995a; Böhm *et al.*, 1996b). At the cellular level, significant immunoreactivity of PAR-2 was detected at the apical and basolateral membrane of enterocytes, neurons of the myenteric and submucosal plexus, as well as vascular smooth muscle cells and endothelial cells (D'Andrea *et al.*, 1998; Kong *et al.*, 1997; Green *et al.*, 2000; Reed *et al.*, 2003; Corvera *et al.*, 1999).

Early studies have shown that PAR-2 stimulation causes the contraction of gastric longitudinal muscle (Al-Ani *et al.*, 1995; Saifeddine *et al.*, 1996). A later study reported the biphasic effects of PAR-2 AP in inducing relaxation followed by contraction in

mouse gastric fundus (Cocks *et al.*, 1999a). In contrast, PAR-2 activation in rat colon was found to inhibit rhythmic contraction (Corvera *et al.*, 1997). Further studies also revealed that PAR-2 exerts differential effects on circular and longitudinal muscle in rat colonic tissues, with a reduction in spontaneous contraction and a contractile response observed in the former and latter respectively (Mule & Baffi, 2002). Evidence from *in vivo* studies indicated that the net effect of PAR-2 activation is to promote gastrointestinal transit via apamin-sensitive K^+ channels and L-type Ca^{2+} channels (Kawabata *et al.*, 2001a).

Another important function of PAR-2 in the gastrointestinal system is in intestinal ion transport in that the activation of PAR-2 stimulates the secretion of Cl^- ions from the intestinal mucosa (Vergnolle *et al.*, 1998; Green *et al.*, 2000; Cuffe *et al.*, 2002). Also, PAR-2 APs stimulate Ca^{2+}-activated Cl^- and K^+ channels when added to the basolateral but not apical surface of dog pancreatic duct epithelial cells (Nguyen *et al.*, 1999). The functional implication proposed by these findings is that PAR-2 activation might lead to diarrhoea and increased ductal secretion that can be protective to promote toxin clearance.

PAR-2 is also involved in modulating salivary, pancreatic and gastric secretions. Stimulation of PAR-2 in isolated rat salivary glands and in intact animals causes an increase in mucin secretion (Kawabata *et al.*, 2000a; Kawabata *et al.*, 2000c). Similar results were obtained in both *in vitro* and *in vivo* models whereby PAR-2 activation enhances pancreatic exocrine secretion (Böhm *et al.*, 1996b; Kawabata *et al.*, 2000a). Gastric, but not duodenal secretion, was stimulated upon PAR-2 activation; such effects were thought to be modulated by the release of neuropeptides from sensory nerves (Kawabata *et al.*, 2001b; Kawabata *et al.*, 2002). PAR-2 expression has been detected in pepsin-secreting chief cells of the stomach, and PAR-2 APs have been shown to stimulate pepsin secretion *in vitro*, but contradicting results were obtained *in vivo* (Kawao *et al.*, 2002, Nishikawa *et al.*, 2002).

v) Nervous System

PAR-2 has been detected in the central nervous system whereby it is located in the neurons and the astroglia of the hypothalamus (D'Andrea *et al.*, 1998; Smith-Swintosky *et al.*, 1997). It is now known that PAR-2 has important functions in several aspects of the nervous system including neurodegeneration and neuroprotection, neurogenic inflammation as well as hyperalgesia (see below).

Contradicting results have been reported for the role of PAR-2 in mediating neurodegeneration or the opposing effect neuroprotection. Smith-Swintosky *et al.* (1997) showed that stimulation of PAR-2 in rat hippocampus causes neuronal cell death. In contrast, other studies supported a protective role of PAR-2 during acute focal ischaemia and HIV dementia (Jin *et al.*, 2005; Noorbakhsh *et al.*, 2005).

PAR-2 is coexpressed with the neuropeptides Substance P (SP) and calcitoxin gene-related product (CGRP) in peripheral sensory neurons, providing clue for its role in neurogenic inflammation and hyperalgesia (Steinhoff *et al.*, 2000). PAR-2-induced neuropeptide release and this effect has been shown to underlie the oedema resulting from intraplantar injection of PAR-2 AP (Steinhoff *et al.*, 2000). In addition, sub-inflammatory doses of PAR-2 AP caused thermal and mechanical hyperalgesia, which involves nociceptors activation and fos expression in the dorsal horn (Vergnolle *et al.*, 2001). The same study also illustrated the role of neurokinin receptor and mast cell degranulation as the underlying mechanisms of PAR-2–induced hyperalgesia. Further studies using transgenic mice has implicated the role of the non-selective cation channel, transient receptor potential (TRP), in PAR-2–mediated thermal hyperalgesia (Amadesi *et al.*, 2004).

Taken together, PAR-2 is vital in mediating diverse patho-physiological processes. Not only that, the presence of PAR-2 in various cells involved in inflammatory responses and the activation of this receptor leading to the release of multiple cytokines and inflammatory mediators further support PAR-2 as a potential therapeutic target.

1.3 MITOGEN-ACTIVATED PROTEIN (MAP) KINASES (MAPKs)

A large body of evidence has indicated that certain GPCRs are able to generate mitogenic signals, a phenomenon that cannot be fully defined by the stimulation of classical GPCR signalling effectors (reviewed in Hur & Kim, 2002). It is now known that several GPCRs including PAR-1, PAR-2, bombesin, bradykinin, muscarinic and adrenergic receptors are associated with MAPK cascades activation to modulate cell growth and differentiation (Hur & Kim, 2002, DeFea *et al.*, 2000; Kanke *et al.*, 2001).

To date, five distinct subfamilies of MAP kinases have been reported in mammalian, namely extracellular signal-regulated kinase (ERK)-1 and 2, c-Jun amino-terminal kinases (JNKs), p38 MAP kinase, ERK-3/4, and ERK-5 or big MAP kinase-1 (BMK-1) (Pearson *et al.*, 2001). JNKs, p38 MAP kinase and BMK-1 form the group of mammalian stress-activated protein kinases (SAPKs). However, ERK-3/4 and BMK-1 cascades are less well characterised thus this section will mainly focus on ERK-1/2, JNKs and p38 MAP kinase.

1.3.1 General activation paradigm of MAP kinases

All MAP kinases are regulated by phosphorylation cascades that involve the central three-tiered components, namely the MAP kinase; the MAP kinase activator called MAP kinase kinase (MAPKK or MEK) and a diverse group of MEK activator known as MAP kinase kinase kinase (MAPKKK or MEKK) (see Figure 1.3) (Pearson *et al.*, 2001). MAP kinases are activated by MEK through dual phosphorylations on tyrosine/theorine residues; whereas MEKK stimulates MEK activity via serine/theorine phosphorylations. A wide variety of substrates have been identified to serve as downstream effectors for the MAPK cascades, including membrane proteins, cytosolic and cytoskeletal components, protein kinases collectively termed MAPK-activated protein kinases (MK), as well as transcription factors (reviewed in Chen *et al.*, 2001). Numerous studies have delineated the interactions between the MAP kinase cascades and the components regulating each part of the pathway (see Figure 1.3) (Kyriakis & Avruch, 2001).

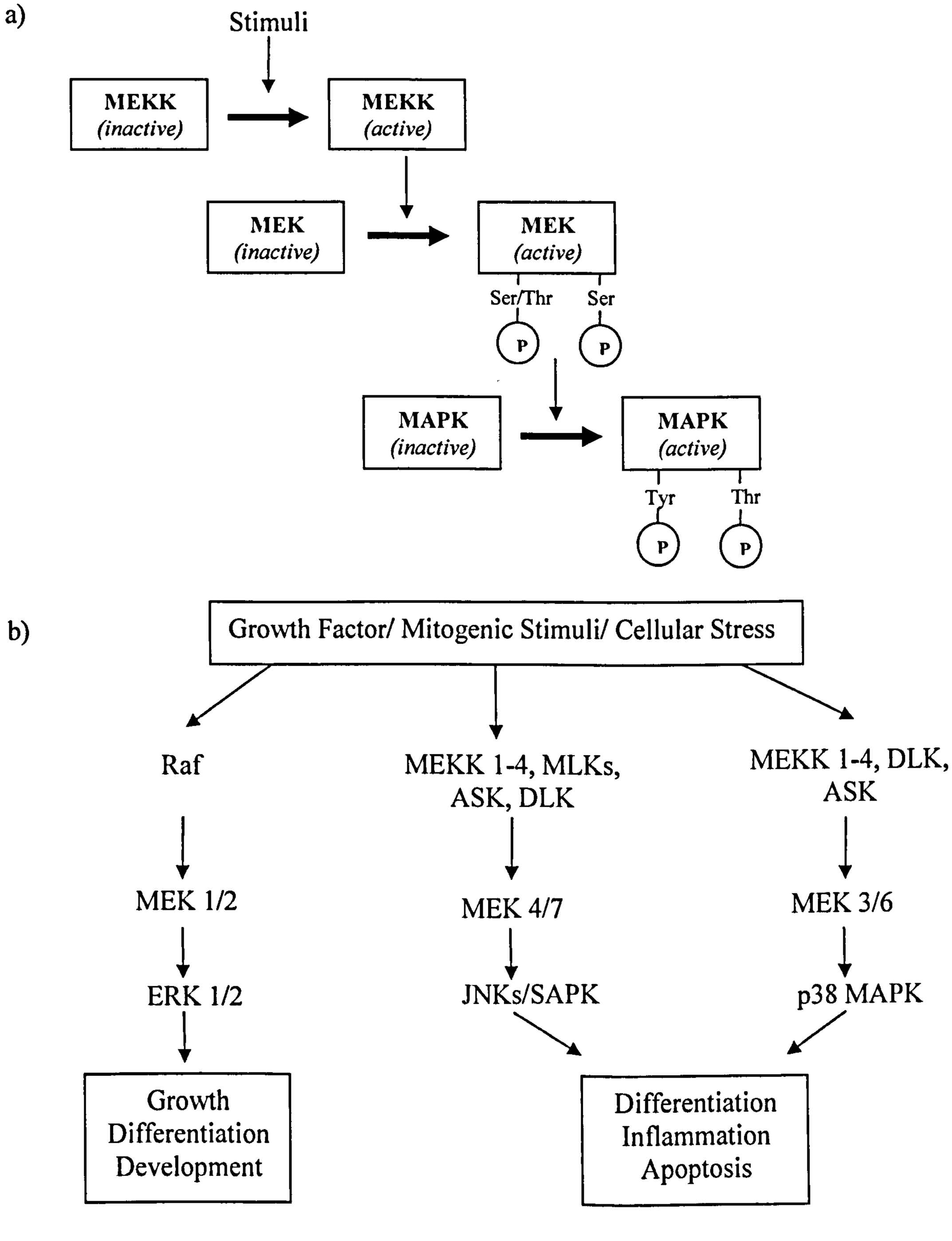


Figure 1.3 The activation of MAPKs. a) Schematic diagram illustrates the components involved in MAPK signalling paradigm. b) The upstream effectors of MAPK cascades. (Modified from Pearson *et al.*, 2001). Abbreviations: *MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MEKK, MEK kinase; MLK, mixed lineage kinase; ASK, apoptosis signal-regulating kinase; DLK, dual leucine zipper kinase; ERK, extracellular signal-regulated kinase; JNK, c-jun amino-terminal kinase; SAPK, stress-activated protein kinase.*

Classically, the general activation paradigm of a MAPK cascade by extracellular stimuli involves stimulation of the cell surface receptor and the subsequent activation of small G proteins such as $p21^{ras}$, Cdc42, Rac and Rho, through which the signal will be relayed to activate specific downstream MEKK components (reviewed in Chen *et al.*, 2001). For example, in response to growth factor receptor stimulation, receptor tyrosine kinase activation causes phosphorylation of receptor tyrosine motifs which then bind the adaptor protein Shc to recruit and activate the growth-factor-receptor-bound protein (GRB)-2 (Malarkey *et al.*, 1995). The archetypal Ras exchange factor, son of sevenless (SOS), is in turn towed to the membrane by GRB-2 to activate the GTPase, $p21^{ras}$, leading to the targeting and activation of a MEKK component in the ERK cascade, Raf-1 (see below), thus initiating the response. A number of variations exist on this model with several forms of Shc adaptor proteins and different subtypes of docking proteins such as Grb-10 and Gab-1 (Chen *et al.*, 2001). A similar paradigm holds for activation of the SAP kinases, however not all of the upstream intermediates have been established (Kyriakis & Avruch, 2001).

i) Extracellular signal-regulated kinase (ERK)-1 and 2

Extracellular signal-regulated kinase (ERK) -1 and 2, which are also known as the classical mitogen kinases, represent the first and best studied cascade amongst all MAP kinases (Pearson *et al.*, 2001). ERK-1/2 are proteins of 42 and 44 kDa respectively, sharing 83% of overall amino acid identity (Boulton *et al.*, 1990). The direct upstream mediators of ERK-1/2 are two high homology proteins called MEK-1 and MEK-2; whereas the MEKKs that lie above MEK-1/2 are Raf proteins (Pearson *et al.*, 2001).

The ERK pathway is well-known for its role as the key regulator for cell survival and proliferation, exemplied by the fact that ERK inhibitors are potential anticancer agents (Kohno & Pouyssegur, 2003). The cellular effects of ERK are mediated by a large number of substrates which could be found in various cellular compartments including Syk (Zhang *et al.*, 1996), p90 risobosomal S6 kinases (RSKs) (Sassone-Corsi *et al.*, 1999), mitogen and stress kinases (MSKs) (Thomson *et al.*, 1999), signal transducer and

activator of transcription (STAT) (Pircher *et al.*, 1999), heat shock factor (HSF)-1 (Dai *et al.*, 2000), and nuclear factor of activated T cell (NFATc) (Porter *et al.*, 2000) .

ii) c-Jun amino-terminal kinases (JNKs)/ Stress-activated protein kinases (SAPKs)

Another module of MAP kinase that was discovered soon after the identification of ERK is c-Jun amino-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs) (reviewed in Kyriakis & Avruch 2001). JNKs are encoded by three genes namely JNK-1/SAPKγ, JNK-2/SAPKα and JNK-3/SAPKβ. JNKs 1-3 share up to 85% of protein homology with more than ten alternative spliced forms identified to date. JNK-1 and JNK-2 are proteins of 46 and 54 kDa respectively, whereas the molecular weight of JNK-3 isoforms range from 45-57 kDa (Kyriakis & Avruch, 2001).

MEK-4 and MEK-7 have been implicated to lie at the direct upstream of the JNK/SAPK pathway; whereas several other kinases have been reported to act at MEKK level, amongst those are MEKK 1-4, mixed lineage kinase (MLK)-2 and -3, Tpl-2/Cot, dual leucine zipper kinase (DLK) and apoptosis signal-regulating kinase (ASK)-1 (reviewed in Pearson *et al.*, 2001). The JNK/SAPK module is strongly activated in response to UV radiation, growth factor deprivation and inflammatory cytokines such as TNFα and IL-1; but to a lesser extent by serum and GPCRs (Kyriakis & Avruch, 2001).

Similarly to ERK, the downstream substrates of activated JNKs are distributed in different cellular compartments. These include insulin receptor-substrate-1 (IRS-1) (Aguirre *et al.*, 2000), Bcl-2 (Fan *et al.*, 2000), and transcription factors such as c-Jun, activating transcription factor (ATF)-2, STAT-3, HSF-1 and NFATc (reviewed in Chen *et al.*, 2001).

iii) p38 MAP kinase

The p38 MAPK isoforms represent the second mammalian stress-activated MAP kinase subfamily. Four genes have been identified to encode the family of p38 MAPK, namely p38α, p38β, p38γ and p38δ (reviewed in Kyriakis & Avruch, 2001). Protein kinases that

have been shown to directly activate p38 isoforms are MEK-3 and MEK-6, whereas several MEKKs have been documented to act as the first tier of this MAPK module, including MEKKs 1-4, DLK, ASK-1, Tpl2/Cot and Tak-1 (Kyriakis & Avruch, 2001). As with JNKs, p38 isoforms are strongly activated by physical and chemical stresses, UV radiation, and cytokines for example TNFα and IL-1, but minimally affected by mitogenic stimuli (reviewed in Chen *et al.*, 2001). Notably, most stimuli that activate the NK pathways also trigger p38 MAPK cascade, highlighting the fact that both SAPK pathways work in parallel to integrate extracellular changes (Kyriakis & Avruch, 2001).

The downstream substrates of p38 MAPK include enzymes such as phospholipase A_2 (Kramer *et al.*, 1996), protein kinases such as MAPKAP kinases 2 and 3, ATF (Tan *et al.*, 1996), Elk-1 (Whitmarsh *et al.*, 1995), p53 (Huang *et al.*, 1999) and CREB homologous protein (CHOP) (Wang & Ron, 1996). The p38 MAPK also shares several effector molecules with ERK and JNKs, for example MNKs, MSKs, NFATc and HSF-1 (Chen *et al.*, 2001).

1.3.2 Regulation of MAPKs by GPCRs

Generally, the mechanisms by which GPCRs regulate MAPKs that have been elucidated to date include; activation of the intermediates of the classical MAP kinase cascade through G proteins–generated second messengers such as PKA and PKC, transactivation of epidermal growth factor (EGF) receptor, and the involvement of β-arrestins (reviewed in Luttrell, 2002).

Classical downstream effectors of G proteins that have been implicated in GPCR-induced MAPK activation include cAMP, PKA, PLC, intracellular Ca^{2+} and PKC isoforms (Luttrell, 2002). Generally, these signalling components converge at the level of ras or Raf activation. For example, evidence has suggested that $G\alpha_s$ stimulates ERK activity through direct phosphorylation of ras (Vossler *et al.*, 1997; Grewal *et al.*, 2000), whereas $G\alpha_{q/11}$ activates the same pathway through PKC and Raf-dependent but ras-independent mechanism (Hawes *et al.*, 1995).

Another distinct signalling mechanism linking GPCRs to MAPK cascades is the cross-talk between GPCRs and the receptor tyrosine kinase, epidermal growth factor (EGF) receptor (Luttrell, 2002). GPCRs are able to transactivate EGF receptor via both ligand-dependent and independent mechanisms. The former paradigm involves ectodomain shedding, a process by which the EGF receptor ligands are cleaved from the transmembrane precursor (Sanderson *et al.*, 2006). Literature has suggested that activation of specific GPCRs induces metalloprotease activity which cleaves the heparin-binding EGF-like growth factor (HB-EGF) precursor which in turn binds to the extracellular domain of the EGF receptor leading to receptor stimulation and MAPK activation (Koch *et al.*, 1994; Faure *et al.*, 1994; Prenzel *et al.*, 1999). Alternatively, ligand-independent transactivation mechanism which involves intracellular elements have also been proposed (Daub *et al.*, 1996; Zwick *et al.*, 1999). Tyrosine kinases such as PKC and $pp60^{src}$ which act downstream of GPCRs have been postulated to be able to phosphorylate thus activate EGF receptor directly to stimulate the MAPK cascades.

Additionally, endocytosis-mediated MAPK activation has been implied for GPCRs including the β_2–adrenergic and angiotensin receptor (Ahn *et al.*, 1999; Luttrell *et al.*, 2001). This is thought to be mediated via the scaffolding property of β-arrestins in which signalling molecules such as $pp60^{src}$, Raf, ERK and JNK-3 are brought to close proximity of GPCRs thus activating the MAPK cascades (Luttrell, 2002; Werry *et al.*, 2005). In fact several studies have provided the evidence of the formation of a multiprotein complex which consists of the activated receptor, β-arrestins and other signalling components (DeFea *et al.*, 2000; Luttrell *et al.*, 2001).

The regulation of MAPK cascades induced by PARs, however, is complex and not fully understood, with apparent features of all the three aforementioned modes of activation. PAR-1 has been shown to couple to all the three MAPK pathways in several models (see Section 1.2.1.3). Several upstream mediators have been identified for PAR-1-stimulated MAPK activity including Gβγ and PKC (Wang *et al.*, 2002), ras, Raf and Janus-activated kinase-2 (JAK-2) (Madamanchi *et al.*, 2001), as well as Src (Darmoul *et al.*,

2004). In addition, a large body of evidence has indicated the role of EGF receptor transactivation in linking PAR-1 to ERK, p38 MAPK and JNK (Kanda *et al.*, 2001; Buresi *et al.*, 2002; Sabri *et al.*, 2002; Darmoul *et al.*, 2004). Notably, the implication of β-arrestin in this context has yet to be reported. Relatively little is known regarding the substrates of PAR-1/MAPK pathways, the components that have been identified to date include STAT and HSP (Madamanchi *et al.*, 2001).

As with PAR-1, PAR-2 has also been observed to activate components of the MAP kinase cascades. Trypsin and PAR-2 APs have been shown to activate c-Raf-1, MEK-1 and 2 as well as ERK and p38 MAPK in rat aortic smooth-muscle cells (Belham *et al.*, 1996). In human keratinocyte cell line NCTC-2544, PAR-2 activation caused JNK and p38 MAPK activation, shown to be mediated at least in part via PKC (Kanke *et al.*, 2001). Unlike PAR-1, ERK activation mediated by PAR-2 has been shown to be arrestin-dependent (DeFea *et al.*, 2000; Stalheim *et al.*, 2005). Interestingly, such stable complex formation has been reported to be able to retain the activated ERK in the cytosol, rendering the subsequent transcriptional activation and cell mitogenesis unfeasible (DeFea *et al.*, 2000). Notably, compared to PAR-1, relatively little is known regarding the role of EGF receptor transactivation in the regime of PAR-2-induced MAPK stimulation except a recent report which has described such interaction in colon cancer cells (Darmoul *et al.*, 2004).

The downstream effectors of PAR-2-induced MAPK pathways remain largely undefined. Studies have indicated the activation of the component of activating-protein (AP)-1, c-jun and c-fos, in response to PAR-2 stimulation (Temkin *et al.*, 2002; Yu *et al.*, 1997). Further studies indicated the role of c-jun activation domain-binding protein (Jab)-1 as an important mediator for c-jun activation induced by PAR-2 (Luo *et al.*, 2006). Nevertheless, the precise mechanisms underlying PAR-2/MAPK signalling paradigm remain largely unknown and thus more investigation is required.

1.4 Nuclear Factor Kappa B (NFκB)

Nuclear factor kappa B (NFκB) was originally identified as a constitutively active nuclear factor that was vital for immunoglobulin kappa light chain transcription in B lymphocytes (Sen and Baltimore, 1986). Since then it was quickly recognized to be a ubiquitously expressed cytoplasmic signalling component that upon activation will translocate into the nucleus to regulate specific target genes transcription.

1.4.1 Structure of NFκB

NFκB is a group of structurally related proteins called Rel. To date, five members have been identified in mammals, namely p50 (NFκB-1), p52 (NFκB-2), p65 (Rel A), Rel B and c-Rel (reviewed in Ghosh *et al.*, 1998), all of which can be divided into two distinct groups. The first group includes NFκB members that are synthesised as the functional proteins, namely p65, Rel B and c-Rel. The members of the second group are p50 and p52, both of which are produced *in vivo* as their precursor proteins, p105 and p100 respectively, requiring proteolytic cleavage to produce the active molecules. NFκB exist as homo- or heterodimers, sharing a highly conserved 300 amino acid long amino terminus domain known as Rel Homology Domain (RHD), within which lies DNA-binding and dimerization domains as well as nuclear localization signal (NLS) (Ghosh *et al.*, 1998). The heterodimer p65/p50 was the first NFκB molecule described which is also the most abundant form, thus it is classically referred to as NFκB despite the identification of other isoforms.

The specific DNA binding sites for NFκB have the decameric sequence 5'-GGGRNNYYCC-3' (where R is purine and Y is pyrimidine), referred to as κB sites (Kunsch *et al.*, 1992; Parry & Mackman, 1994). Such sequence has been identified in the promoter region of numerous inducible genes, giving rise to the various patho-physiological roles of NFκB (see Section 1.4.5). Each individual NFκB dimer has distinct DNA-binding specificity and transactivation potential (Ghosh *et al.*, 1998). Rel-A or p65, the most common NFκB member, contains two transactivation domains

(TADs), with TAD1 being confined to the terminal 31 amino acids (521-551) and TAD2 enclosed in the adjacent 91 residues (430-520) (Schmitz *et al.*, 1995). Notably, a large body of evidence has suggested that the phosphorylation of p65 in the TADs serves as a mode of post-translational modification of this transcription factor to regulate its transcriptional activation (reviewed in Viatour *et al.*, 2005). Thus far, there are at least nine inducible phosphorylation sites identified in p65 in response to a wide range of stimuli.

1.4.2 Inhibitory Kappa B (IκB)

NFκB is usually present in an inactive form in the cytosol, being physically associated with its inhibitory protein called inhibitory kappa B (IκB). IκB masks the NLS of NFκB, rendering this transcription factor unable to translocate into the nucleus (Beg *et al.*, 1992). Similar to NFκB, IκB represents a group of evolutionary conserved proteins which include IκBα, IκBβ, IκBε, IκBγ and Bcl-3 (reviewed in Ghosh *et al.*, 1998).

All the IκBs contain a domain encoding multiple copies of ankyrin motifs termed ankyrin repeats, which are responsible for protein-protein interaction thus the binding of IκB to RHD of NFκB (Ghosh *et al.*, 1998). Different subtypes of IκB proteins bind preferentially to specific NFκB heterodimers, i.e. IκBα and IκBβ interact predominantly with p50/p65 and p50/c-Rel; IκBε associates mainly with p50 and p52 homodimers (Thompson *et al.*, 1995; Whiteside *et al.*, 1997).

IκBα was the first IκB protein identified and still remains as the best characterised (Ghosh *et al.*, 1998). The general paradigm for IκB activation in response to specific extracellular stimuli involves phosphorylation, ubiquitination and degradation by the proteosome. In summary, the following events have been identified for IκBα activation; i) phosphorylation of IκBα at specific serine residues of 32 and 36 (Traenckner *et al.*, 1995), ii) Ubiquitination of IκBα on two neighbouring amino-terminal lysine residues at 21 and 22 (Scherer *et al.*, 1995), iii) Subsequent rapid degradation of IκBα by

proteosome 26S, iv) Translocation of NFκB into the nucleus to regulate the transcription of target gene(s). Notably, phosphorylation or ubiquitination alone is not sufficient to cause IκBα degradation.

1.4.3 The Upstream of NFκB Pathway: IκB Kinase (IKK) and IKK Kinase

The upstream mediator of IκB responsible for activating NFκB is the IκB kinase (IKK) complex which consists of three subunits: IKKα (IKK1), IKKβ (IKK2) and IKKγ (Ghosh *et al.*, 1998).

IKKα (85kDa) and IKKβ (87kDa) are catalytic subunits of the complex, sharing 52% overall homology (Mercurio *et al.*, 1997; Woronicz *et al.*, 1997; Zandi *et al.*, 1997). Both subunits are found to be ubiquitously expressed; each protein contains an amino-terminal kinase domain flanked by a leucine zipper (LZ) region followed by a C-terminal helix-loop-helix (HLH) domain (Ghosh *et al.*, 1998). IKKα and IKKβ are able to form homo- or heterodimers through interactions at the LZ motif (Mercurio *et al.*, 1997; Woronicz *et al.*, 1997; Zandi *et al.*, 1997). The HLH domain, on the other hand, is vital for IKK complex activation (Zandi *et al.*, 1997).

IKKγ, also known as NFκB essential modulator (NEMO), IKK-associated protein 1 (IKKAP1) or 14.7 interacting protein (FIP-3) is the regulatory component of IKK complex (Rothwarf *et al.*, 1998; Yamaoka *et al.*, 1998; Mercurio *et al.*, 1999; Li *et al.*, 1999). Further study has reported the isolation of a new IκB kinase designated as IKKi/ε (Peters *et al.*, 2000). IKKi/ε is distinct from IKKα or IKKβ in that it is inducible and has been postulated to act upstream of the classical IKK complex (Peters & Maniatis, 2001).

To date, several protein kinases have been reported to be able to activate IKK complex, including various PKC isoforms (Sanz *et al.*, 1999; Lallena *et al.*, 1999; Tojima *et al.*, 2000), members of MEKK family such as NFκB-inducing kinase (NIK) (Malinin *et al.*, 1997) and MEKK-1-3 (Mercurio *et al.*, 1997; Zhao & Lee, 1999), AKT/Protein Kinase

B (Ozes *et al.*, 1999) and transforming growth factor-beta-activated kinase-1 (TAK-1) (Takaesu *et al.*, 2003; Yun *et al.*, 2005).

1.4.4 Three Distinct NFκB-activating Pathways

It has now become apparent that there are three distinct signalling mechanisms which mediate NFκB activation, namely the classical, alternative and atypical pathways; all of which depend on successive kinase activation (Viatour *et al.*, 2005) (see Figure 1.4).

i) The classical pathway

This pathway, also known as the canonical pathway is utilised by proinflammatory cytokines such as TNFα, IL-1 and LPS to stimulate NFκB activity. TNFα regulates NFκB activation through TNF receptor-1 (TNFR-1), TNF receptor-associated death domain protein (TRADD), receptor-interacting protein (RIP) and TNF receptor–associated factor-2 (TRAF-2) (Hsu *et al.*, 1995). This is then followed by IKK complex activation, IκBα degradation and eventually NFκB activation (Devin *et al.*, 2000). Alternatively, IL-1 signals to NFκB through IL-1 receptor-1 (IL-1R1), accessory protein (ACP), MyD88, IL-1R-activated kinase (IRAK) and TRAF-6 (Greenfeder *et al.*, 1995; Muzio *et al.*, 1997).

ii) The alternative pathway

The second, non-canonical pathway requires the proteolytic processing of the NFκB precursor protein, p100 (Viatour *et al.*, 2005). Stimuli including viruses such as Epstein-Barr virus and human T-cell leukaemia virus; cytokines such as B-cell activating factor (BAFF) and CD40 ligand trigger the activation of TRAF proteins and NIK, leading to IKKα homodimer-induced site-specific phosphorylation and ubiquitination of the precursor protein (Claudio *et al.*, 2002; Coope *et al.*, 2002; Xiao *et al.*, 2001a & b; Eliopoulos *et al.*, 2003; Senftleben *et al.*, 2001). The resultant product, p52, can then translocate into the nucleus as a heterodimer with rel-B to regulate gene expression.

iii) The atypical pathway

This novel axis, unlike the other two aforementioned pathways, involves IKK-independent NFκB activation. Stimuli such as hypoxia, pervanadate and ultraviolet radiation have been shown to activate NFκB pathway through phosphorylation of IκBα at novel and yet to be identified site(s) other than Ser-32 and 36 (Beraud, 1999; Schoonbroodt *et al.*, 2000; Li & Karin, 1998; Kato *et al.*, 2003).

1.4.5 Biological Importance of NFκB

NFκB has now been recognised to induce the transcription of an exceptionally large number of genes including cytokines such as TNFα, IL-1, IL-6, IL-8, beta interferon (IFNβ); adhesion molecules such as VCAM-1, ICAM-1 and E-selectin; cytokine receptors such as IL-2α receptor chain receptor (IL-2Rα), transcription factors for example c-myc, p53 as well as NFκB itself (reviewed in Baldwin, 2001). Thus it is not surprising to speculate the diverse patho-physiological roles exhibited by NFκB and its implications in human disorders.

i) Immune and inflammatory responses

Evidence from p50 and c-Rel knockout mice has indicated that certain NFκB members are mandatory for immune and inflammatory responses as null mice developed abnormal T and B cells, and failed to defend against bacterial infection (reviewed in Ghosh *et al.*, 1998). In addition, several studies have postulated that NFκB is involved in inflammatory conditions such as asthma, arthritis and inflammatory bowel disease (Barnes & Larin, 1997; Marok *et al.*, 1996; Neurath *et al.*, 1996).

ii) Cell growth and apoptosis

Interestingly, mice lacking p65 developed hepatocyte apoptosis, suggesting that this transcription factor is important for cell survival and/or anti-apoptotic events (Beg *et al.*, 1995). In contrast, the pro-apoptotic effects of NFκB have also been demonstrated in certain cells such as B-cells, T-cells and endothelial cells (reviewed in Shishodia &

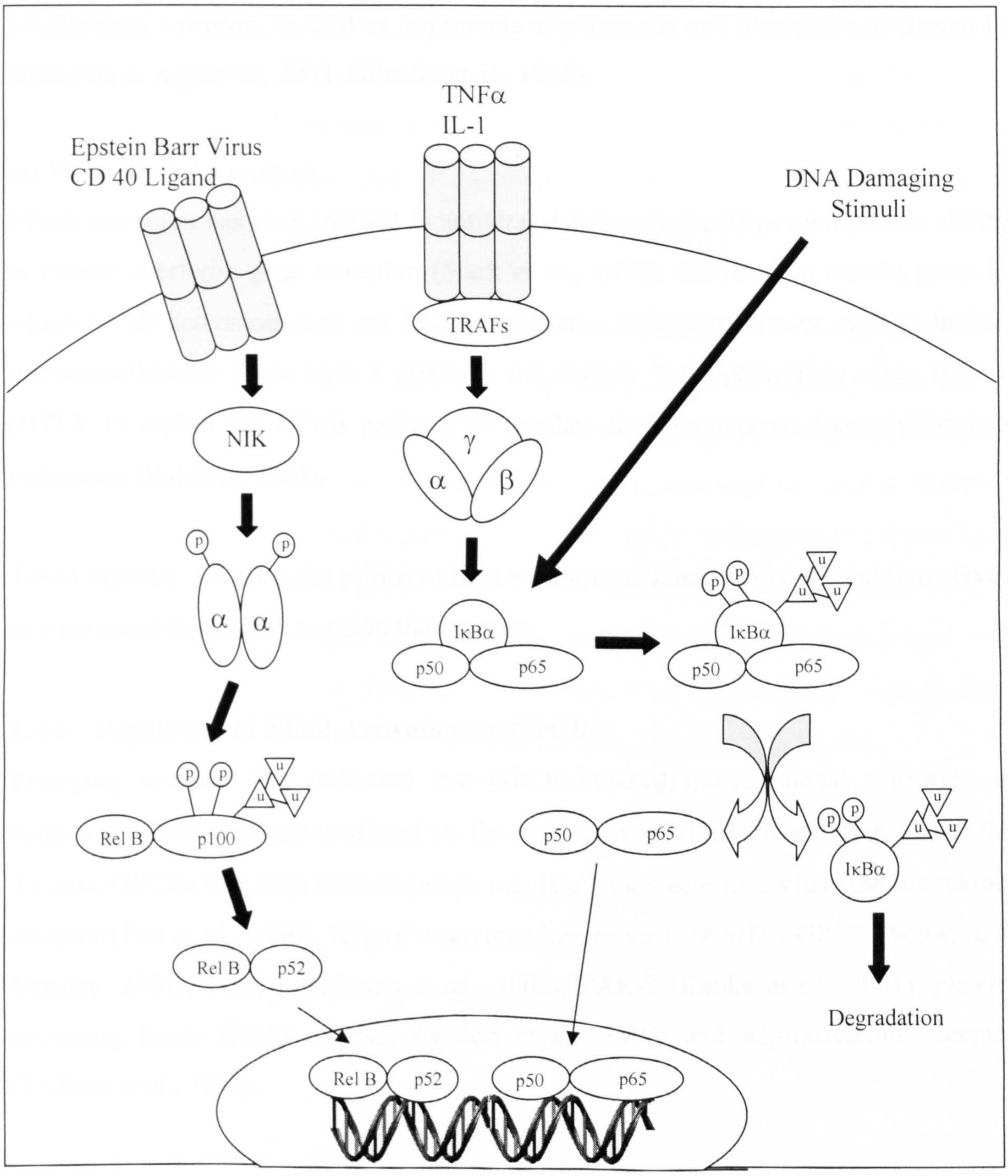


Figure 1.4 Schematic diagram showing the alternative, classical and atypical pathways for NFκB activation.

Aggarwal, 2004). NFκB proteins are also oncogenic and have been shown to be involved in multiple myeloma due to its ability to induce cellular transformation, proliferation, invasion, as well as to promote angiogenesis and metastasis (reviewed in Shishodia & Aggarwal, 2004; Gilmore *et al.*, 1996).

iii) Virus-related responses

NFκB activation has been implied in antiviral defence mechanisms owing to its ability to induce interferon gene induction (Stark *et al.*, 1998). However in certain cases in which NFκB activation does not lead to interferon induction, viruses such as human immunodeficiency virus type 1 (HIV-1) and human T-lymphotrophic virus type 1 (HTLV-1) exploit the NFκB pathway to regulate their own genes transcription and replication (Baldwin, 2001).

Taken together, NFκB is the primary effector of several human diseases and thus serves as a potential therapeutic target in this context.

1.4.6 Regulation of NFκB Activation by GPCRs

Emerging evidence has indicated that GPCR–induced transcriptional activation in various cell lines is in part mediated via the regulation of NFκB (reviewed in Ye, 2001). To date, GPCRs that have been shown to regulate NFκB activity include the bradykinin receptor (Pan *et al.*, 1996), Kaposi's sarcoma herpes virus (KSHV)-GPCR (Schwarz & Murphy, 2001), PAR-1 (Rahman *et al.*, 2002), PAR-2 (Kanke *et al.*, 2001), platelet activating factor (PAF) receptor (Mutoh *et al.*, 1994) and M_3-muscarinic receptor (Todisco *et al.*, 1999).

The signalling cascades that are utilised by GPCRs to signal to NFκB activation are distinct from the aforementioned pathways described for classical cytokines such as TNFα and IL-1; in that GPCRs employ heterotrimeric G proteins for initiation of the response which have been shown to converge at the level of IKK complex activation

and/or IκB loss. For example, KSHV-GPCR, bradykinin and M_3-muscarinic receptors have been shown to activate IKK complex (Dadke *et al.*, 2003; Xie *et al.*, 2000; Todisco *et al.*, 1999) whilst bradykinin, lysophosphatidic acid, PAF and endothelin receptors have been reported to induce cellular IκB degradation (Zhu *et al.*, 2003; Raj *et al.*, 2004; Kravchenko *et al.*, 1995; Gallois *et al.*, 1998). Collectively, both Gα subunits and the Gβγ complex have been implicated in activating NFκB, and at least one member in each class of Gα protein has been reported to play a role in this paradigm.

$G\alpha_s$ and its primary effectors PKA and cAMP have been shown to negatively control the activity of NFκB mediated via the β-adrenergic receptor (Parry & Mackman, 1997; Farmer & Pugin, 2000). Parry and Mackman (1997) showed that in endothelial cells, PKA–induced phosphorylation of cAMP-response element-binding protein (CREB) leads to recruitment of the transcription coactivator, CREB binding protein; rendering it unavailable to other transcription factors including NFκB thus inhibiting NFκB-mediated transcriptional activation. Additionally, another study has provided evidence of NFκB inhibition by β–adrenergic agonists via increasing cytoplasmic IκBα concentration to prevent NFκB translocation (Farmer & Pugin, 2000).

Alternatively, $G\alpha_{i/o}$ subunit has also been reported to regulate NFκB activity, as suggested by the fact that the activation of this transcription factor induced by GPCRs was strongly inhibited by PTX pre-treatment, for example in the case of PAF receptor, lysophosphatidic acid receptor and leukotriene B4 receptor (Brach *et al.*, 1992; Shahrestanifar *et al.*, 1999; Kravchenko *et al.*, 1995). However, there is limited evidence supporting the role of $G\alpha_{i/o}$–mediated cAMP inhibition as the underlying mechanism for this signalling paradigm, and it is widely accepted that the Gβγ subunits released upon $G\alpha_{i/o}$ activation are in fact the key components in this context. It has then been shown that Gβγ can signal to NFκB via PI3K and Akt, as exemplified by the B_2-bradykinin receptor; but relatively little is known about Gβγ-induced PLCβ in this regime (Xie *et al.*, 2000).

The members of $G\alpha_{q/11}$ have been shown to couple to NFκB stimulation induced by GPCRs such as endothelin and bradykinin receptor (Gallois *et al.*, 1998; Xie *et al.*, 2000). These studies suggest that $G\alpha_{q/11}$ subunits employ multiple effectors including intracellular Ca^{2+}, PKC as well as PI3 kinase as the intermediary components in this signalling axis. It is likely that PLCβ serves to activate PKC which then leads to a convergent point in the classical paradigm at IKK complex activation.

Several GPCRs agonists such as sphingosine-1-phosphate (S1P), KSHV and carbachol have been associated with NFκB activation via their respective receptors which can signal through $G\alpha_{12/13}$ (Shi *et al.*, 2001; Shepard *et al.*, 2001; Siehler *et al.*, 2001). Shepard and co-workers proposed that KSHV couples to NFκB pathway to produce IL-8 secretion via $G\alpha_{12/13}$ and RhoA; whereas other studies did not address the possible downstream mechanisms involved.

As with other GPCRs, the regulation of NFκB activation mediated by PAR-1 and PAR-2 remains largely unknown. A study has proposed that PAR-1-induced NFκB activation in endothelial cells is mediated by $G\alpha_q$/PKCδ in parallel to Gβγ/PI3 kinase, both of which then converge at the level of Akt (Rahman *et al.*, 2002). For PAR-2, activation of both IKKα and IKKβ in response to receptor stimulation has been reported (Kanke *et al.*, 2001). The same study also pointed out the possible involvement of typical and atypical PKC isoforms in this context. Further studies provided the evidence of the vital role of intracellular calcium in the PAR-2/NFκB pathway (Macfarlane *et al.*, 2005).

Taken together, the regulation of NFκB activity by GPCRs can be multifaceted involving distinct mechanisms. A large body of evidence has suggested that a GPCR can utilise multiple G proteins concurrently to transactivate the NFκB pathway, as exemplified by KSHV-GPCR and lysophosphatidic acid receptor (Shahrestanifar *et al.*, 1999; Shepard *et al.*, 2001). Nevertheless, the links between G proteins and NFκB activation remain largely unknown, urging more investigation.

1.5 AIMS

Given the fact that PAR-2 is involved in diverse patho-physiological conditions, a good understanding of the mechanisms by which PAR-2 utilises to exert such effects would be beneficial in order to provide novel therapeutic options. MAP kinases together with NFκB signalling represent the major pathways that are employed by eukaryotic cells to react to a wide array of external stimuli and thus controlling important cellular effects such as cell proliferation and differentiation, cytokine release as well as apoptosis. However, the signalling paradigm linking PAR-2 to the MAPK pathways and NFκB is poorly defined.

The aims of this study were to characterise PAR-2–mediated MAP kinases and NFκB activation using a model of NCTC-2544 skin cell line stably expressing PAR-2. In order to extend previous findings reported by Kanke *et al.* (2001) and Macfarlane *et al.* (2005) regarding the regulation of NFκB mediated by PAR-2, this study sought to further delineate the roles of $G\alpha_{q/11}$, PKC, intracellular calcium and IKK complex in this context. The working hypothesis for this project was that $G\alpha_{q/11}$ serves as the upstream effector of PKC and calcium mobilisation to effect NFκB activation including phosphorylation of p65 NFκB in response to PAR-2 stimulation.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1 General Reagents

All materials used were of highest grade available and were purchased from Sigma-Aldrich Co Ltd. (Poole, Dorset, U.K.) unless otherwise stated. PD 098059, SB 203580, SP 600125, rottlerin and BAPTA-AM were reconstituted in dimethyl sulfoxide (DMSO).

Amersham International PLC (Aylesbury Buckinghamshire, U.K.)

ECL Detection reagents

Biorad Laboratories (Hertfordshire, U.K.)

BioRad AG® 1-X8 Resin, pre-stained SDS-PAGE molecular weight markers

Boehringer Mannheim (East Sussex, U.K.)

Bovine serum albumin (Fraction V), Dithiothreitol (DTT)

Calbiochem (Nottingham, U.K.)

PD 098059

SB 203580

SP 600125

Rottlerin

Costar (Buckinghamshire, U.K.)

Nitrocellulose membranes

Molecular Probes Europe (Netherlands)

BAPTA-AM

Santa Cruz Biotechnology Inc. (CA, U.S.A.)

Recombinant human TNFα

Wallac UK (Milton Keynes, U.K.)

Optiphase Hi-safe^{TM} scintillant

Whatman (Kent, U.K.)

3MM paper

2.1.2 Reagents for Cell Culture and Transfection

Corning B.V. (Netherlands)

Cell culture plasticware

Life Technologies Ltd. (Paisley, U.K.)

Antibiotics (penicillin, streptomycin), Foetal calf serum (FCS), Geneticin (G418) Lipofectamine ™, PLUS ™ reagent, Medium 199 with Earl's salts (M199), Versene (0.2% EDTA/PBS)

Cascade Biologics ™ (Nottinghamshire, U.K.)

Human epidermal keratinocytes -neonatal (HEKn), splitting solution (dispase), Epilife ® media, human keratinocyte growth supplement (HKGS), defined trypsin inhibitor (sterile and purified soybean trypsin inhibitor in PBS) and coating matrix kit (sterile recombinant human type-1 collagen).

2.1.3 Antibodies

Amersham Pharmacia Biotech, Inc. (NJ, U.S.A.)

Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG, HRP-conjugated donkey anti-rabbit IgG

Santa Cruz Biotechnology Inc. (CA, U.S.A.)

Anti-ERK2 (Rabbit monoclonal, C-14)

Anti- phospho-ERK (Mouse monoclonal, E-4)

Anti- p38 (Rabbit polyclonal, N-20)

Anti- phospho-JNK (Rabbit polyclonal, KM-1)

Anti- JNK-1 (Rabbit polyclonal, FL)

Anti- $G_{\alpha q/11}$ (Rabbit polyclonal, C-19)

Anti- p65 (Rabbit polyclonal, C-20)

Anti- IκBα (Rabbit polyclonal, C-21)

Anti- IKKα (Rabbit polyclonal, H-744)

Anti- IKKα/β (Rabbit polyclonal, H-470)

Biosource Europe SA (Belgium)

Anti- phospho-p38 (Rabbit polyclonal, 44684-G)

New England Biolab (England, U.K.)

Anti- phospho-p65 (Rabbit polyclonal, Ser-536)

Sigma Chemical Company (Poole, Dorset, U.K.)

Anti-flag (mouse monoclonal)

2.1.4 PAR-2 Agonist Peptides

All the PAR-2 APs were purchased from Kowa Company, Ltd., Japan.

2.1.5 Radiochemicals

NEN Dupont (Hertfordshire, U.K.)

$\gamma[^{32}P]$-ATP (3000Ci/mmol) and myo-[2-$^{3}H(N)$]-inositol (10-25Ci/mmol)

2.2 CELL CULTURE

All cell culture work was performed in a class II cell culture hood under aseptic conditions. All the cells were grown in 75cm^2 flasks unless otherwise stated.

2.2.1 Human Keratinocytes NCTC-2544

Human keratinocyte cell line NCTC-2544 was maintained at 37° C under a humidified atmosphere of 5% CO_2 in medium M199 containing Earl's salt supplement, penicillin 100units/ml, streptomycin 100µg/ml, and 10% (v/v) foetal calf serum. Cells were passaged using trypsin-EDTA solution (0.25% trypsin, 1mM EDTA) (see below).

NCTC-2544 cells stably expressing PAR-2 (clone G) (Kanke *et al.*, 2001) were maintained in the above medium supplemented with geneticin (400µg/ml) to maintain selection pressure. Cells were passaged using Versene (0.2g/L EDTA with sterile PBS) (see below).

NCTC-2544 cells stably expressing both PAR-2 and NFκB, CRE or AP-1 reporter plasmid were maintained in the above medium supplemented with both geneticin (400µg/ml) and blastocidin (5µg/ml) to maintain selection pressure. Cells were passaged using Versene (0.2g/L EDTA with sterile PBS) (see below).

Cells were subcultured upon reaching ~90% confluency. The medium was removed and the cells washed twice with either trypsin-EDTA solution or Versene. The solution was then removed and the flask placed in an incubator at 37^{0}C, 5% CO2, for 2-5 min until the cells began to change shape, indicating that they had begun to detach from the flask. The flask was then gently tapped, to completely detach the cells from the surface, and then M199 medium was then added to the flask. The cells were then seeded into fresh flask(s) or plate(s) as appropriate. Cells were maintained in 37^{0}C in an incubator and the media replaced every 2 to 3 days.

2.2.2 Human Embryonic Kidney (HEK) 293

Low passage human embryonic kidney (HEK) 293 cells were maintained in modified Eagle's medium (MEM) supplemented with penicillin (250units/ml), streptomycin (100μg/ml), glutamine (27mg/ml) and 10% (v/v) foetal calf serum. Cells were passaged using trypsin-EDTA solution (0.25% trypsin, 1mM EDTA) as described for NCTC-2544 cells.

2.2.3 Human Epidermal Keratinocytes

Human epidermal keratinocytes were purchased from Cascade Biologics ™ (Nottinghamshire, U.K.).

2.2.3.1 Subculturing of human epidermal keratinocytes

Cells were subcultured upon reaching ~70% confluency. The medium was aspirated and the cells washed once with 2 to 3 mls of trypsin-EDTA (0.25% trypsin, 1mM EDTA). The solution was then removed, and 1-2 mls of fresh trypsin-EDTA was reintroduced into the flask and left to incubate at 37^0C, 5% CO_2, for 2-5 min until the rounding of cells was observed (indicative of cell detachment from the flask). Once cells were able to move freely, 500μl of defined trypsin inhibitor was added. Following this, 3mls of fresh Epilife medium was added to wash cells off the flask, and the solution was transferred into a fresh 15ml centrifuge tube which was centrifuged for 7 minutes at 1200 r.p.m. At the same time, coating matrix was added into fresh flask(s) or plate(s) where appropriate; ensuring that the full surface area was covered. Excess coating matrix was removed after 2 minutes, and the surface area was left to dry for 2 minutes. After centrifugation, the supernatant was discarded and the pellet resuspended in 2-5 mls of Epilife medium. Following this, the cell solution was distributed as appropriate, and the cells were maintained at 37° C under a humidified atmosphere of 5% CO_2 with the medium replaced every 2 to 3 days.

2.3 TRANSFECTION OF SMALL INTEFERING RNA (siRNA)

The double-stranded small interfering RNAs (siRNAs) were purchased from Dharmacon, U.K. The siRNA sequence targeting $G\alpha_{q/11}$ and firefly luciferase as a non-specific control relative to their start codons were 5'-AAGATGTTCGTGGACCTGAAC-3' and 5'-CGUACGCGGAAUACUUCGAAA-3' respectively (Barnes *et al.*, 2005; Kravchenko *et al.*, 2003). Cells were grown to 50% confluency and transfected with appropriate concentrations of the respective siRNAs. An appropriate volume of siRNA was diluted in 50µl of serum-free/antibiotic-free medium and incubated for 5 min at room temperature. At the same time 12µl of oligofectamine (Invitrogen, U.K.) was mixed with 48µl of serum-free/antibiotic-free medium for 5 min. The two solutions were then allowed to mix together for a period of 20 min at room temperature.

The growth medium of cells ready for transfection was replaced with 1ml of antibiotic-free medium per well. The transfection solution was then dropped evenly over the cells and allowed to incubate at 37 ^{0}C for 6 hours. Following this, the transfection medium was replaced with normal growth medium for 48 hours. The cells were then quiesced in serum-free medium for 18 hours before being assayed.

2.4 PREPARATION OF RECOMBINANT ADENOVIRUSES

Recombinant replication-deficient adenoviral vectors encoding RGS4 (Adv. RGS4), Lsc (Adv. Lsc), dominant-negative inhibitory kappa B kinase (IKK)α (Adv. $IKK\alpha^{+/-}$) and Adv. $IKK\beta^{+/-}$ were employed in this project. The propagation of these viruses was carried out in the HEK 293 packaging cell line. The adenoviral constructs were purified by ultracentrifugation in a caesium chloride gradient. Following that, the end-point dilution method was used for the titration of the viral stock (Nicklin & Baker, 1999). The adenoviral constructs encoding siRNA targeting $G\alpha_{q/11}$ tagged with GFP which was also utilised in this study was purchased from Vector Biolabs (Philadephia, U.S.A.).

2.4.1 Generation of Crude Adenoviral Lysates

Crude adenoviral lysates were generated in HEK 293 cells by infection of a 75 cm^2 flask with 0.5µl of viral stock when the cells had reached approximately 70% confluency. The flasks were then incubated at 37^{0}C, 5% CO_2 for 5-7 days until the cytopathic effect had occurred and cells were completely detached from the flask. Cells were removed from the flasks and pelleted by centrifugation at 1500g for 5 min. The supernatant was removed; the pellet was washed twice with PBS and subjected to centrifugation again (1500g for 5 min). The pellet was then resuspended in HE buffer (10mM HEPES pH 7.5, 1mM EDTA), frozen in liquid nitrogen and thawed in a 37^{0}C water bath. The freeze-thawing process was repeated for a total of three times. Cells were then centrifuged at 1500g for 5 min and the supernatant constituting the crude adenoviral lysate was transferred to a sterile tube and stored at -80^{0}C until use.

2.4.2 Generation of Pure High-Titre Stocks of Recombinant Adenoviruses

High-titre stocks of recombinant adenoviruses were generated by large scale amplification of the crude adenoviral lysates described in Section 2.4.1. Fifteen 225cm^2 flasks of HEK 293 cells were grown to approximately 70-80% confluency and each flask was infected with 100µl of crude adenoviral lysates. The flasks were then incubated at 37^{0}C, 5% CO_2 for 5-7 days until the cytopathic effect was apparent and the cells had detached from the flasks. The cells were removed together with the medium and were pelleted by centrifugation at 1500g for 5 min. The supernatant was aspirated off and the pellet was washed with 100ml PBS and pooled into two 50ml centrifuge tubes. The cells were again subjected to centrifugation (1500g for 5 min) and the supernatant was removed. The cells were washed again with 50ml of PBS and transferred to a 50ml centrifuge tube and pelleted with centrifugation at 170g for 5 min. The cells were resuspended with 5ml HE buffer (10mM HEPES pH 7.5, 1mM EDTA), freeze/thawed as described previously (Section 2.4.1). Subsequently, the cells were placed in a water bath sonicator for 2 x 45 seconds and centrifuged at 1500g for 5 min. Following this the supernatant constituting adenovirus was then collected and purified (see Section 2.4.3).

2.4.3 Purification of Adenovirus on a Caesium Chloride (CsCl) Gradient

As freeze-thawing is a crude method to extract adenovirus and the adenovirus preparations obtained might be contaminated with debris and cytotoxic components, the caesium chloride (CsCl) gradient purification technique employed was a simple and efficient means to purify and concentrate the recombinant adenoviral stocks.

A CsCl gradient was prepared by pipetting 2ml of CsCl (density 1.45) into a sterile 14 ml transparent centrifuge tube (Beckman, Ultraclear). Following this 3ml of CsCl (density 1.32) was added carefully onto the first layer, preventing the mixing of the solutions. Subsequently 2ml of 40% glycerol was gently layered on top of the CsCl solutions, and the crude adenovirus preparation was laid on top of the gradient in a drop-wise manner using a sterile glass pipette. Any extra space in the tube was then filled with Tris/saline. The tube was then subjected to centrifugation at 90,000g for 90 min at 4^0C in a Beckman ultracentrifuge. After centrifugation, the recombinant adenovirus could be observed as a discrete white/blue layer between the two CsCl solutions (Figure 2.1). A 21-gauge needle was used to pierce the tube just underneath the adenovirus layer; the virus was removed with a 1ml syringe with a gentle side to side sweeping motion. The adenovirus collected was then transferred into a sterile tube.

The adenovirus was then dialysed in the Collodion dialysis bag at 4^0C in 2 litre of dialysis buffer (10mM Tris pH 8, 1mM EDTA). The dialysis buffer was replaced every hour for three hours. Subsequently, the adenovirus was dialysed overnight against dialysis buffer containing 40% glycerol. The adenovirus obtained was then removed and aliquoted into sterile eppendorfs and stored at -80^0C until use.

2.4.4 Titration of Adenovirus by End-Point Dilution

The end-point dilution method was employed to determine the titre value of the adenovirus stocks obtained (Nicklin & Baker, 1999). HEK 293 cells were grown to approximately 80% confluency in a 96-well plate, serial dilutions of the adenovirus were

carried out and applied to the appropriate wells (Figure 2.2). The plate was incubated at 37^0C, 5% CO_2 overnight after which the medium containing adenovirus was replaced with fresh medium. The plate was then incubated at 37^0C, 5% CO_2 for 5-7 days until the cytopathic effect of the virus had reached a steady state. The wells containing plaques were then counted and the titre of the adenovirus stock in terms of plaque-forming units (pfu) was calculated as the example shown in Figure 2.3.

2.4.5 Infection of Cells With Adenovirus

Cells were grown to 80% confluency, and the cell number was determined using a haemocytometer. Following this, the cells were incubated with the appropriate multiplicity of infection (MOI) of the respective adenovirus constructs for 18 hours in normal growth medium. The cells were then quiesced for 18 hours prior to stimulation.

2.5 MEASUREMENT OF [^{3}H]-INOSITOL PHOSPHATE ACCUMULATION

Cells were grown to confluence on a 12-well plate and quiesced for 18 hours with serum-free M199 medium supplemented with myo-[2-^{3}H-(N)]-inositol (1 μCi/well; 1 Ci = 37 GBq).

Prior to the experiment, 1 volume of AGI-X8 Ion exchange resin (DOWEX) (Bio-Rad Laboratories, California) was suspended in 2 volumes of water. A 1 ml aliquot of the suspension was put into each scintillation vial and allowed to settle for 10 minutes. Water was aspirated off and resin was washed with 2.5ml of distilled water.

Cells were incubated with a final concentration of 10mM LiCl for 15 minutes before stimulation with agonists to inhibit the breakdown of inositol phosphates (IPs_{1-4}) (Oldham, 1990). The cells were incubated with appropriate agonist for 60 minutes; the cells were then washed twice in ice-cold PBS and removed by scraping into 1ml of methanol. Choloform (0.5ml) was added to each vial to give a 2:1 methanol: chloroform.

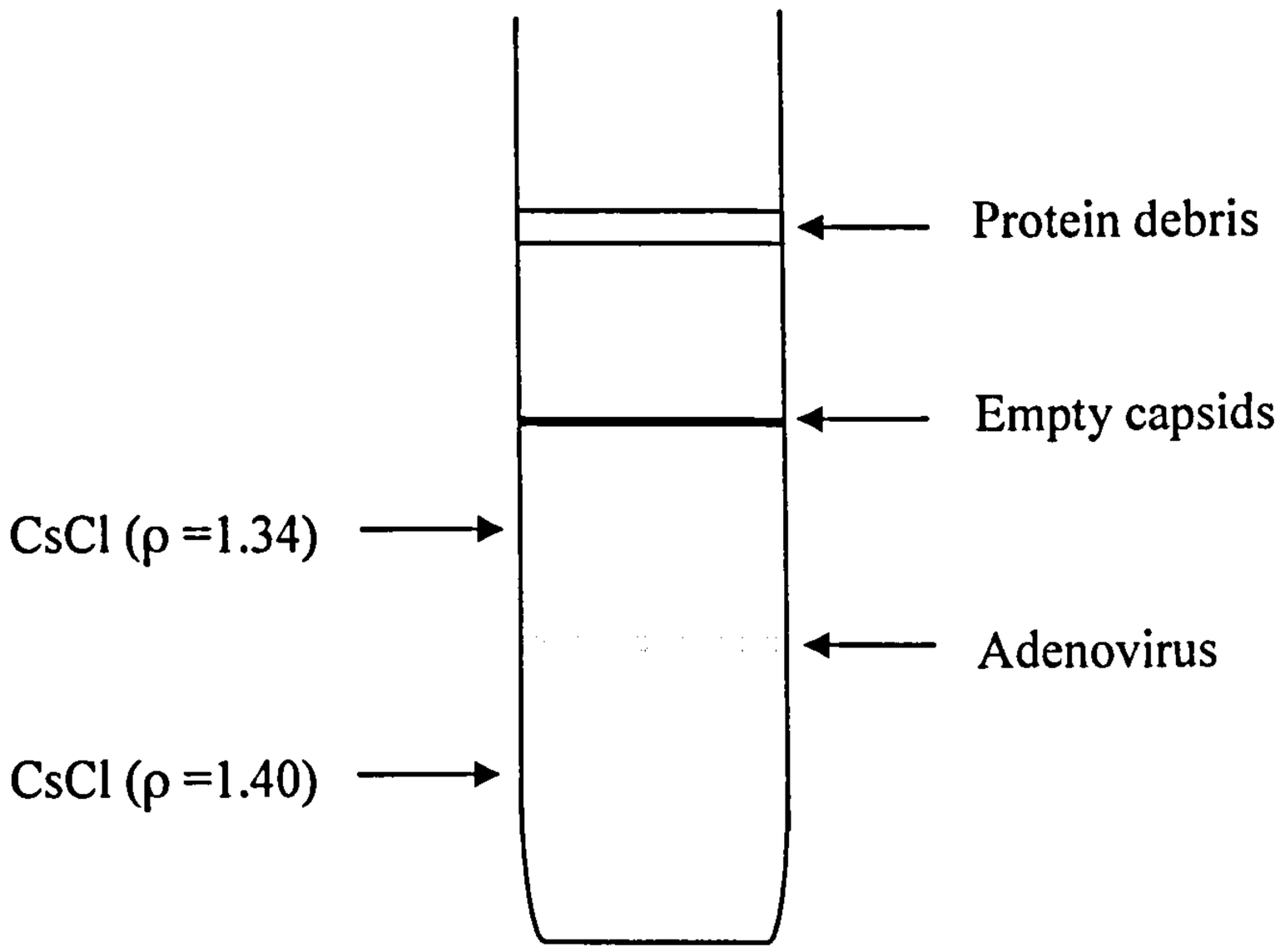


Figure 2.1. CsCl gradient for purification of recombinant adenovirus

Schematic diagram showing a CsCl gradient after centrifugation. The adenovirus lies between the two different densities of CsCl layers as denoted by the arrow. Empty adenoviral capsids are found above the CsCl (ρ = 1.34) whereas protein debris is deposited further above as indicated.

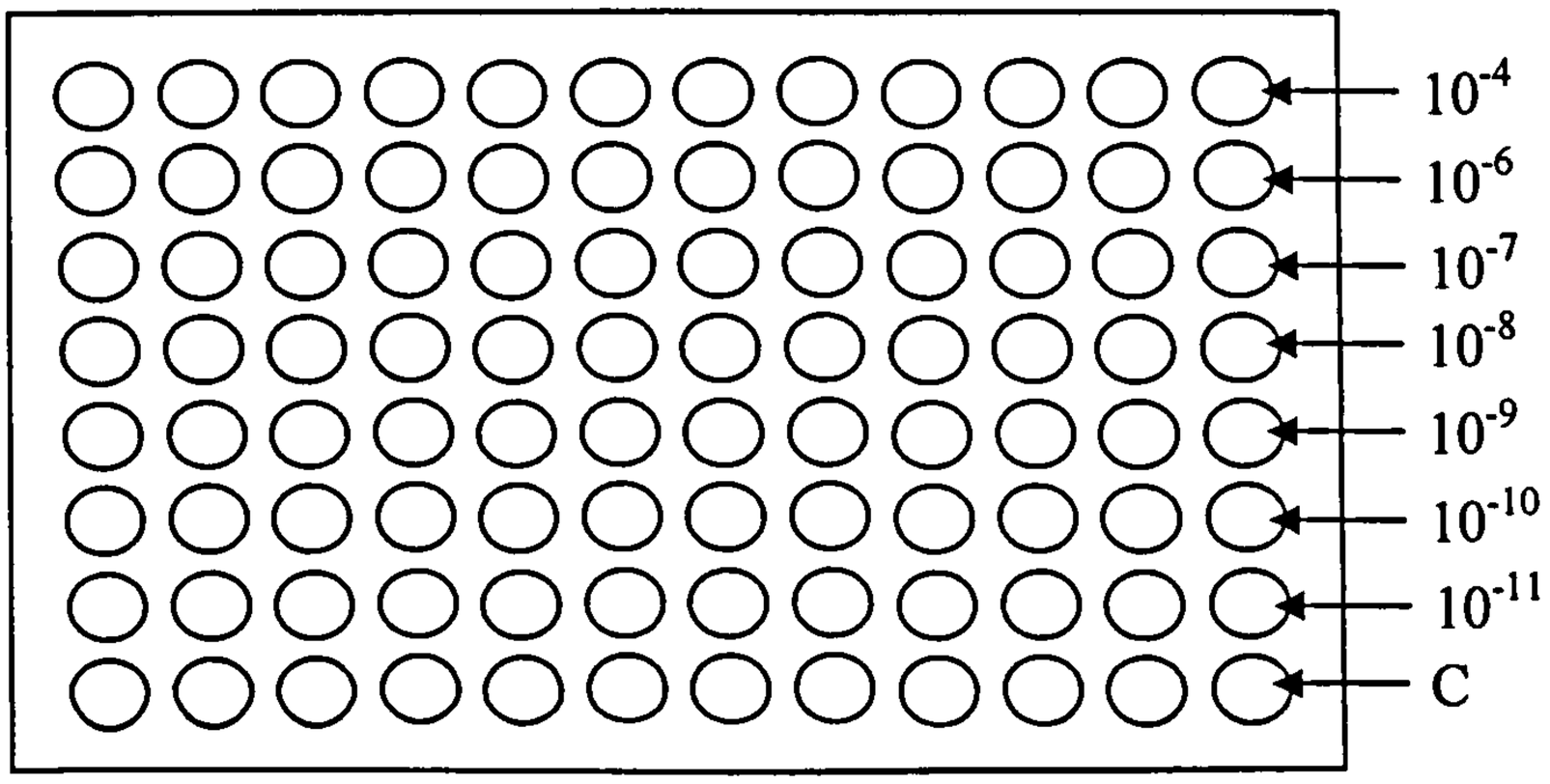


Figure 2.2. Layout of a 96-well plate for plaque purification and titration by end-point dilution

Adenovirus was added to the middle 10 wells on each row at the appropriate dilutions. In the bottom row medium without adenovirus was added to serve as control (C).

Number of wells containing plaques:

$10^{-4} = 10/10 = 100\%$

$10^{-6} = 10/10 = 100\%$

$10^{-7} = 10/10 = 100\%$

$10^{-8} = 10/10 = 100\%$

$10^{-9} = 10/10 = 100\%$

$10^{-10} = 5/10 = 50\%$

$10^{-11} = 2/10 = 20\%$

The proportionate distance = $\frac{\text{(\% wells positive > 50\%) - 50\%}}{\text{(\% wells positive > 50\%)-(\% wells positive} \leq \text{50\%)}}$

$= \frac{100-50}{100-50} = 1$

Log ID_{50} = log dilution factor at % wells positive above 50% + (proportionate distance x -1)

$= -9 + (-1) = -10$

ID_{50} $= 10^{-10}$

TCID (tissue culture infectivity dose)$_{50}$/100µl $= \frac{1}{10^{-10}} = 10^{10}$

$TCID_{50}$ /ml = 10^{10} x dilution factor

$= 10^{10} \times 10$

$= 1 \times 10^{11}$

1 $TCID_{50} \approx 0.7$ plaque forming units (pfu), therefore the final titre of adenovirus is

$1 \times 10^{11} \times 0.7 = 7 \times 10^{10}$ pfu/ml

$= 7 \times 10^{7}$ pfu/µl

Figure 2.3. Example calculation to establish titre of adenovirus

The samples were then vortexed and incubated on ice for 90 minutes. Following this incubation, 0.8ml dH_2O and 0.5ml chloroform were added to the vials which were again vortexed and allowed to settle. When the organic phase had clearly separated, 1ml of the upper aqueous phase was removed and added to the prepared DOWEX resin, and again the vials were vortexed and allowed to settle. The liquid was aspirated off and the vials were washed sequentially with 2.5ml of dH_2O, 2.5ml of sodium tetraborate solution [5mM $Na_2B_4O_7.10H_2O$, 60mM ammonium formate] and finally 2.5ml of dH_2O again. Following these washes 1ml of elution buffer [1M ammonium formate, 0.1M formic acid in distilled water] was used to elute total phosphoinositides ($[^3H]$-IP_{1-4}) from the resin (Berridge *et al.*, 1982). 1ml of the aqueous phase was removed to a fresh scintillation vial and mixed with 4 ml of scintillant fluid (Optiphase Hi-safe^{TM}). The vials were then read on liquid β-scintillation counter (Wallac, U.S.A.), for 2 minutes each.

2.6 WESTERN BLOTTING

2.6.1 Preparation of Whole Cell Lysate

Cells were grown to confluency in 6-well plates and quiesced for 18 hours in serum-free medium. After stimulation with appropriate agonists at indicated time-points, cells were washed twice in ice-cold PBS and lysed by adding 0.5ml of preheated (70^0C) Laemmli's sample buffer [63mM Tris-HCl (pH 6.8), 2mM $Na_4P_2O_7$, 5 mM EDTA, 10% glycerol, 2% SDS and 0.0007% (w/v) bromophenol blue, 50mM DTT]. The cells were then scraped into Eppendorf tubes and lysates were drawn repeatedly through 16G syringe-needle to shear chromosomal DNA. The tubes were boiled for 5 minutes to denature proteins and samples were stored at -20^0C until use.

2.6.2 SDS-polyacrylamide Gel Electrophoresis

The resolving gel was prepared by using the appropriate amount of acrylamide: [N,N'-methylenebis-acylamide (37.5:1), 0.375 M Tris (pH 8.8), 0.1% (w/v) SDS and 0.05% (w/v) ammonium persulfate]. The gel solution was prepared and polymerisation was initiated by the addition of 0.05% (v/v) N,N,N',N'-tetramethylethylenediamine

(TEMED). The gel solution was then poured into the assembled glass plates (Bio-Rad) according to the manufacturer's instructions. Gel was overlaid with 0.1% SDS solution to prevent drying. Once the gel was set, the 0.1% SDS solution was poured out and the stacking gel solution of the following composition: [5% (w/v) acrylamide, 125 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.1% (v/v) TEMED] was made and poured directly on top of the resolving gel. A teflon comb was then inserted into the stacking gel solution. The gel was then allowed to polymerise for 10 minutes before the comb was removed. Samples were then loaded into the wells, run concurrently with the pre-stained SDS protein markers of known molecular weights in the electrophoresis buffer [24.8mM Tris, 191.8mM glycine, 0.1% SDS] at 120V for 1 hour 30 minutes.

The resolved proteins on the gel were then transferred to nitrocellulose filters by electrophoretic blotting using a standard protocol (Towbin *et al.*, 1979). The gel was placed against a nitrocellulose membrane, sandwiched between two Whatmann 3MM paper porous pads. The cassette was then immersed in a BioRad Mini Trans-Blot electrophoresis tank containing transblotting buffer [25mM Tris, 192mM glycine, 20% (v/v) methanol] and was run for 2 hours at constant current, 250mA.

Upon the transfer of protein to the nitrocellulose membrane, the membrane was trimmed to the size of the gel, blocked in a 2% BSA/NATT [150mM NaCl, 20mM Tris-HCl (pH 7.4), 0.2% (v/v) Tween 20] solution for 2 hours, followed by probing overnight with a chosen antibody at an appropriate concentration in a 0.2% BSA/NATT solution. Equal protein loading was ensured by probing the membrane against total MAPK or p65 in parallel to the respective phospho-protein. On the following day, the membrane was washed every 15 minutes with NATT for one hour and further incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Again the membrane was washed every 15 minutes for one hour with NATT before being incubated in enhanced chemiluminescence (ECL) solution for 90 seconds. The membrane was mounted into an

exposure cassette and was exposed to X-ray film (Kodak X-OMAT LS) for the appropriate time and developed using a KODAK M35-M X-OMAT processor.

Primary Antibody	Dilution
p42/44	1:5000
p-p42/44	1:7500
p38	1:15000
p-p38	1:15000
p46	1:7500
p-p46	1:15000
p65	1:15000
p-p65	1:5000
IκBα	1:15000
Flag	1:15000
His	1:15000
$G\alpha_{q/11}$	1:15000
Secondary Antibody	**Dilution**
HRP-conjugated sheep anti mouse IgG	1:7500
HRP-conjugated donkey anti-rabbit IgG	1:7500

2.7 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

2.7.1 Preparation of Nuclear Extracts

Cells were grown in 6 well-plates to 100% confluency and rendered quiescent by replacing the growth medium with serum-free medium for 18 hours. The cells were stimulated with agonists for the appropriate durations before being washed twice in ice-cold PBS and scraped into 0.5ml PBS. The cells were harvested by centrifugation and the supernatant aspirated, followed by resuspension in 400µl of buffer 1 (10mM HEPES pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF,

0.5mg/ml leupeptin, 0.5mg/ml aprotinin, 0.5mg/ml pepstatin). The cells were incubated on ice for 15 minutes, followed by addition of 25µl 10% (v/v) NP-40 and vortexed at full speed for 10s. Cells were then pelleted by centrifugation and the pellet resuspended in 20µl of buffer 2 (20mM HEPES pH 7.9, 25% (v/v) glycerol, 0.4M NaCl, 1mM EDTA, 1mM DTT, 0.5mM PMSF, 0.5mg/ml leupeptin, 0.5mg/ml aprotinin, 0.5mg/ml pepstatin) at 4^0C on the shaking platform for 15 minutes. Cells were then sonicated in a bath-type sonicator for 2 x 30 seconds and the final extract was recovered by centrifugation at 13000 r.p.m. for 15 minutes. The supernatants were removed to sterile Eppendorf tubes and stored at -80^0C until use.

2.7.2 Determination of Protein Concentration by Bradford's Reaction

1µl protein sample of unknown concentration was mixed with 199µl Bradford solution diluted 5 times in distilled water and incubated at room temperature for 15 minutes. The mixed solution was then transferred to a 96-well plate and the absorbance at 595nm was assessed using a microtitre plate reader (Dynex Technology, U.S.A.). The protein concentration was calculated from a standard curve constructed using known concentrations of BSA (0 - 20µg).

2.7.3 Labelling of Oligonucleotides with ^{32}P-γ-ATP

Oligonucleotides containing the consensus binding sequence for the NFκB transcription factor (5'-AGTTGAGGGGACTTTCCCAGGC-3') were labelled at the 5'-end by incubation with T4 polynucleotide kinase and ^{32}P-γ-ATP at 37^0C for 30 minutes. The reaction was terminated by the addition of 0.5M EDTA and the labelled oligonucleotide diluted in TE buffer (10mM Tris base pH 8.0, 1mM EDTA). The efficiency of ^{32}P-phosphate incorporation into the oligonucleotides was determined by spotting aliquots of labelled primer onto four separate DE81 circular filters. Two filters were used to determine the total label in each aliquot, whilst the two remaining filters were washed in 0.5M Na_2HPO_4 (pH 6.8) to remove unincorporated label. All filters were then subjected to scintillation counting and the percentage of label incorporation was calculated. Labelled nucleotide was stored at 4^0C until use.

2.7.4 Transcription Factor Binding Assay

An aliquot containing 7μg of protein from a nuclear extract was made up to 7 μl with distilled H_2O. 2μl of 5X gel-shift binding buffer (2M Tris base pH 7.5, 80% glycerol (v/v), 1M $MgCl_2$, 0.5M EDTA, 5M NaCl, 1M DTT, 1 mg/ml poly[dI:dC]) was added and the mixture was incubated at room temperature for 30 minutes. 1μl of labelled oligonucleotide was then added and incubated for a further 30 minutes at room temperature. The reaction was terminated by the adding 1μl of 10x loading buffer (250mM Tris base pH 7.5, 0.2% bromophenol blue [w/v], 40% glycerol [v/v]). The sample was then loaded onto pre-run non-denaturing 4% acrylamide (w/v) gels containing N, N' methylenebisacrylamide, glycerol in TBE buffer (45mM Tris base pH 8.3, 1mM EDTA and 45mM boric acid) and resolved by electrophoresis at 100 V. The gels were then sandwiched between two cellophane sheets dried in the gel dryer (Hoefer Scientific Instruments, U.S.A.). Binding of transcription factors to the consensus oligonucleotides was determined by autoradiography.

2.8 LUCIFERASE REPORTER ACTIVITY ASSAY

Luciferase reporter plasmids generated with enhancer element containing NFκB-element (x3), AP-1-element (x7) or CRE-element (x4) were kind gifts from D. MacEwan (University of East Anglia, U.K.). NCTC-2544 cells stably expressing both PAR-2 and the respective reporter plasmid (reporter cells) were established by Toru Kanke (Kowa Ltd., Japan). The reporter cells were grown to confluency in 96-well plates (flat, white with clear bottom, Costa, Netherlands) and quiesced for 18 hours before stimulation with the appropriate agonists at the indicated time points. Stimulation was terminated by aspiration of the medium; followed by washing twice with ice-cold PBS. Subsequently, 100μl of lysis buffer [25 mM Tris phosphate (pH 7.8), 8mM $MgCl_2$, 1mM DTT, 1% Triton X 100, 15% (v/v) glycerol] containing 1mM ATP, 0.2 mM luciferin substrate and 1% (w/v) BSA was added into each well; and the relative light units were measured using the luminometer Wallac Trilux 1450 microbeta counter (Wallac Oy, Finland).

2.9 SCANNING DENSITOMETRY

Western blots and autoradiography from EMSA were scanned on a Hewlett Packard Scanjet using Hewlett Packard Scanjet Picture software. The captured images were then normalised to a control and quantified using Scion Image (Scion Corp., Maryland U.S.A.). An example of densitometry quantification is shown below.

	Density measured	Background control	Net density	Fold stimulation of basal control	% stimulated control
Unstimulated control	0.93	0.5	0.93 - 0.5 = 0.43	0.43/0.43 = 1	-
Unstimulated control + inhibitor	0.93	0.5	0.93 - 0.5 = 0.43	0.43/0.43 =1	-
Stimulated control	4.8	0.5	4.8 - 0.5 = 4.3	4.3/0.43 = 10	100%
Stimulated sample + inhibitor	2	0.5	2 - 0.5 = 1.5	1.5/0.43 = 3.49	$\frac{(3.49-1) \times 100}{(10-1)}$ = 27.67%

Therefore, % inhibition = 100 – 27.67 = 72.33%

2.10 STATISTICAL ANALYSIS

All data shown were expressed as mean $\pm$ s.e.m. Whenever applicable, statistical analysis was performed by one-way ANOVA with Dunnet's post-test or paired Student's t-test. Differences were considered significant at $P<0.05$.

CHAPTER 3

CHARACTERISATION OF PAR-2-MEDIATED SIGNALLING IN NCTC-2544 CELLS STABLY EXPRESSING PAR-2

3.1 Introduction

The expression of PAR-2 has been detected in a wide variety of tissues, signifying the vital roles of this receptor in several biological systems (discussed in Section 1.2.2.8). Amongst these, the skin which forms the largest organ of human body, has been found to highly express PAR-2 in three of the four layers in epidermis (D' Andrea *et al.*, 1998; Steinhoff *et al.*, 1999). Several studies have implicated that PAR-2 is in fact the key player in mediating skin inflammation, as PAR-2 activation causes the release of proinflammatory mediators such as IL-6, IL-8 and GM-CSF in cultured keratinocytes (Hou *et al.*, 1998; Wakita *et al.*, 1997). Moreover, further studies have pointed out that hyperactivation of this receptor could be the underlying cause of certain pathological responses in skin (Steinhoff *et al.*, 1999; Kawagoe *et al.*, 2002).

The signal transduction mechanisms of PAR-2 in skin, however, are not well characterised and the identification of PAR-2 downstream signalling components could shed some light on how does this receptor exert its biological actions. Therefore this study focused on the regulation of PAR-2–mediated signalling in skin, by utilising NCTC-2544 skin cell line that stably expresses PAR-2 (clone G). The signalling pathways that are of interest included mitogen-activated protein kinases such as ERK, p38 MAPK and JNK; as they represent the key components in controlling cell proliferation and inflammatory responses (see Section 1.3). Also, NFκB as one of the most important transcription factors in immune reactions and a major determinant of keratinocyte growth and differentiation, was investigated in relation to PAR-2 activation as well as activating protein-1 (AP-1) and cAMP-responsive element (CRE), the downstream components of MAP kinase cascades (Kaufman & Fuchs, 2000).

To date there has been no potent PAR-2 antagonist reported. Such compounds will serve as useful therapeutic agents given that this receptor has been implicated in various pathological conditions (see Section 1.2.2.8). This section has therefore included work on characterising potential novel PAR-2 antagonists, ENMD-1068 and K-14585, on PAR-2–induced signal transduction in keratinocytes. The effects of these compounds on

[^{3}H]-inositol phosphate accumulation, MAP kinases and NFκB activation were addressed.

3.2 Characterisation of PAR-2–Mediated Signalling in NCTC-2544 Cells Stably Expressing PAR-2 (clone G)

The kinetics of the aforementioned signalling components were assessed using the enzyme, trypsin; and the activating peptide (AP), 2f-LIGKV-OH (Ferrell *et al.*, 2003).

3.2.1 PAR-2 activation stimulated [^{3}H]-inositol phosphate accumulation

The time course and concentration-dependency of [^{3}H]-inositol phosphate accumulation induced by PAR-2 were investigated in clone G cells. Figure 3.1 illustrates the kinetics of the response to the stimulation with trypsin and the AP, 2f-LIGKV-OH, over a period of 120 min. Trypsin (50nM) stimulated a time-dependent accumulation of total [^{3}H]-inositol, which was evident at 5 min (4.3 $\pm$ 0.6 fold of basal control), and observed to be approximately 43 fold by 90-120 min. Stimulation of clone G cells with 30μM of 2f-LIGKV-OH also resulted in similar kinetics of inositol phosphate accumulation, which was found to be 4.5 $\pm$ 0.4 fold of basal control at 5 min and essentially linear over 120 min, with the maximal response quantified at this time point as approximately 40 fold above basal.

The effects of increasing agonist concentration in stimulating inositol phosphate formation were also assessed as depicted in Figure 3.2. Cells were stimulated with 1-100nM of trypsin or 1-200μM of 2f-LIGKV-OH and the response was determined 60 min after stimulation. Both trypsin and AP induced a concentration-dependent increase in inositol phosphate accumulation in clone G cells, and the maximum responses for each agonist were found to be similar at 50.5 $\pm$ 6.9 and 46.3 $\pm$ 4.8 fold of basal control respectively. The EC_{50} values obtained were 46.4 $\pm$ 2.5 nM and 23.5 $\pm$ 5.3 μM for trypsin and 2f-LIGKV-OH respectively.

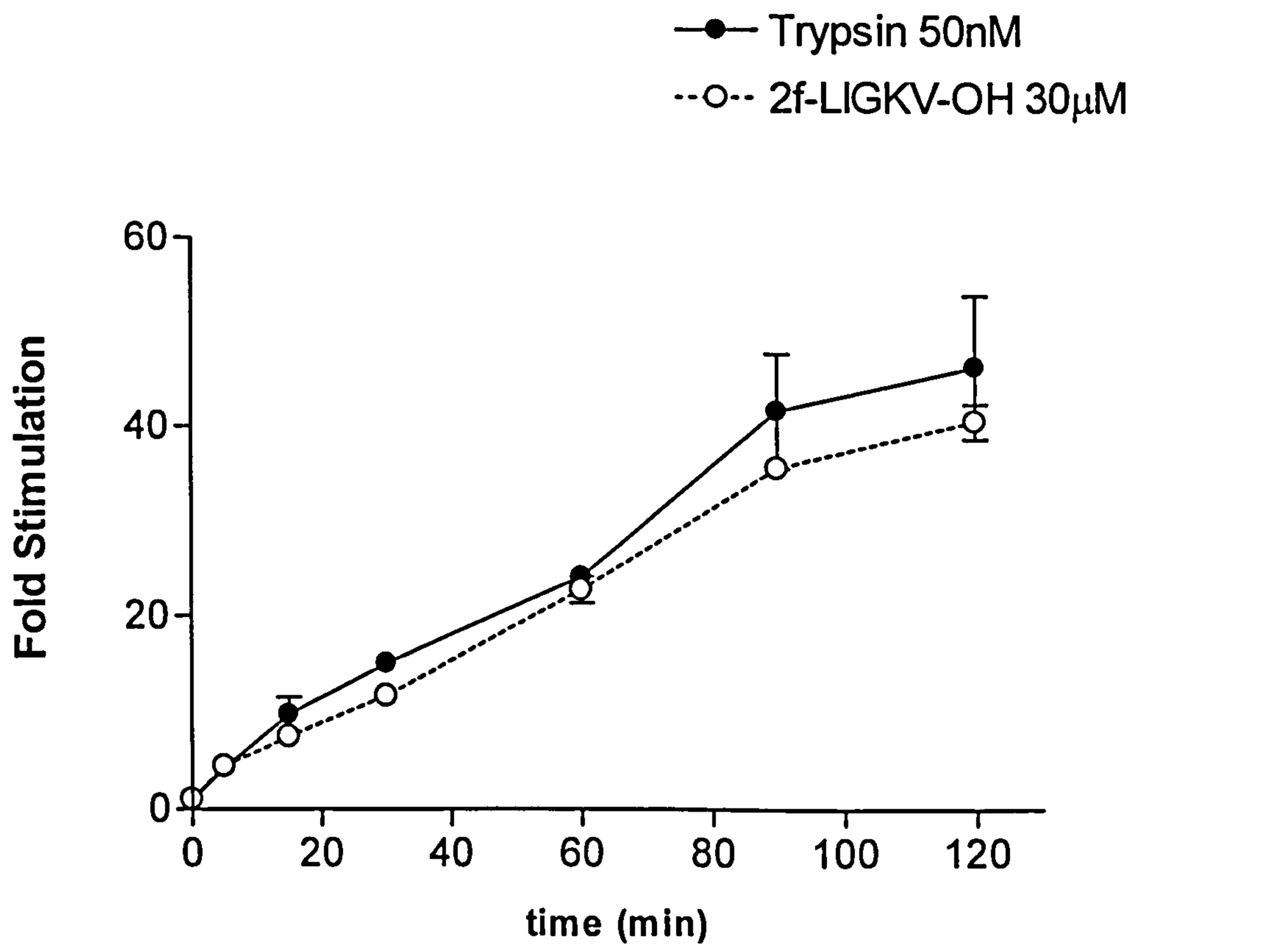
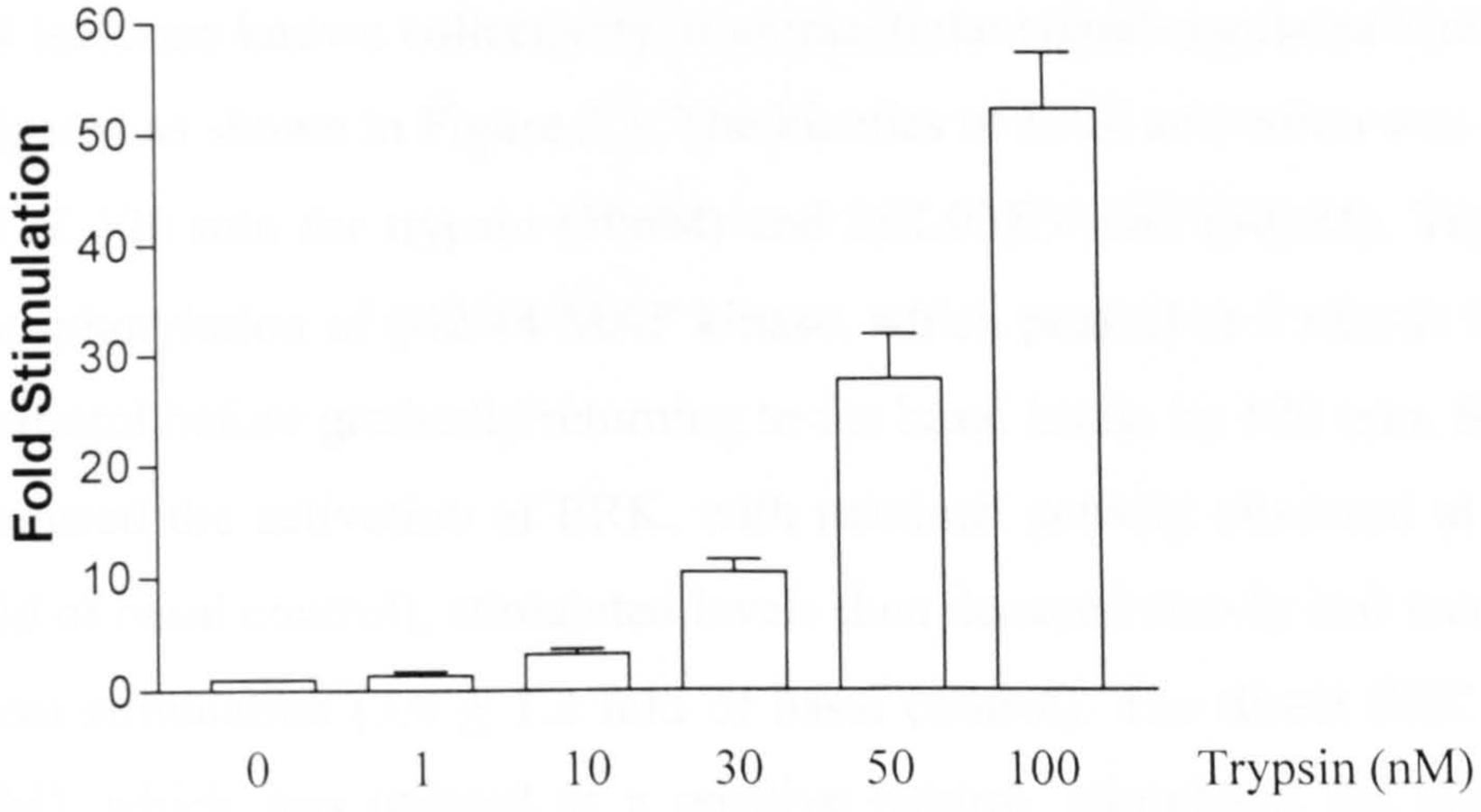


Figure 3.1. Time-course of trypsin and 2f-LIGKV-OH–mediated [^{3}H]-inositol phosphate accumulation in clone G cells

Cells were prelabelled with [^{3}H]-myo-inositol for 18 hours and incubated with LiCl (10mM) for 15 min. Cells were then stimulated with either trypsin (50nM) or 2f-LIGKV-OH (30μM) for various time points up to 120 min. Accumulation of total [^{3}H]-inositol phosphate was measured as described (Section 2.5). Each value represents the mean ± s.e.m. from three separate experiments performed in triplicate.

a)



b)

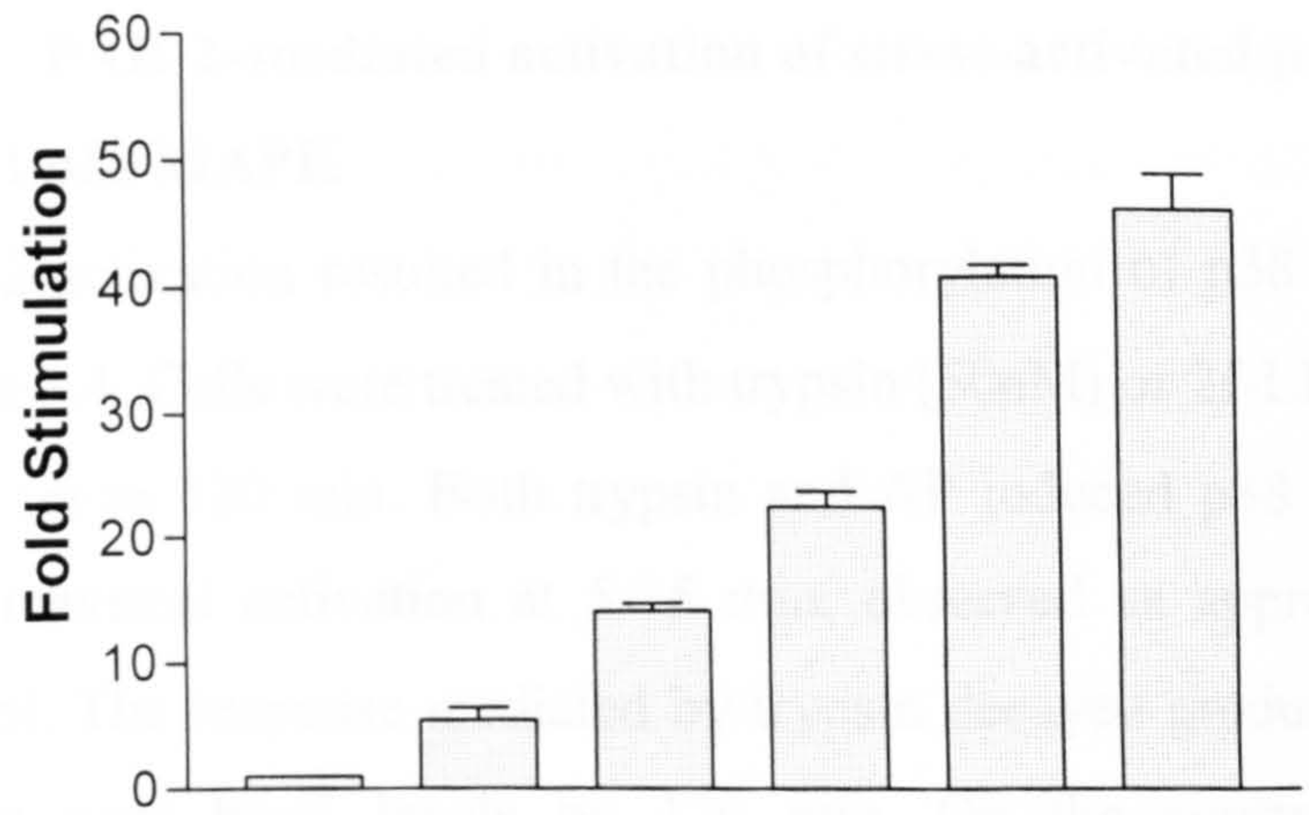


Figure 3.2. Concentration-dependent effects of trypsin and 2f-LIGKV-OH on PAR-2 –mediated [^{3}H]-inositol phosphate accumulation in clone G cells

Cells were prelabelled with [^{3}H]-myo-inositol for 18 hours in serum free media. LiCl (10mM) was added to the cells prior to stimulation with increasing concentrations of trypsin and 2f-LIGKV-OH for 60 min. Accumulation of total [^{3}H]-inositol phosphate was measured as described (Section 2.5). Each value represents the mean $\pm$ s.e.m. from three separate experiments performed in triplicate.

3.2.2 PAR-2 induced the activation of the classical MAP kinase

The effects of PAR-2 stimulation on the activation of the classical MAP kinase, the p42/44 isoforms known collectively as extracellular signal-regulated kinase (ERK), were investigated as shown in Figure 3.3. The kinetics of ERK activation was observed over a period of 120 min for trypsin (50nM) and 2f-LIGKV-OH (30μM). Trypsin stimulated the phosphorylation of p42/44 MAP kinase, which peaked at 5 min at 9.8 ± 0.7 fold of basal control before gradually returning to the basal levels by 120 min. Similarly, the AP also induced the activation of ERK, with maximal activity observed at 15 min (11.1 ± 0.7 fold of basal control), stimulated levels then decayed slowly and were evident at 120 min post stimulation (3.9 ± 1.2 fold of basal control). The direct PKC activator, PMA (100nM), which was utilised as a positive control, stimulated the phosphorylation of ERK by 7.3 ± 0.8 fold of basal control at 15 min.

3.2.3 PAR-2–mediated activation of stress-activated protein kinases (SAPKs)

3.2.3.1 p38 MAPK

PAR-2 activation resulted in the phosphorylation of p38 MAP kinase as illustrated in Figure 3.4. Cells were treated with trypsin (50nM) or 2f-LIGKV-OH (30μM) for various times up to 120 min. Both trypsin and AP induced p38 MAP kinase phosphorylation with maximal activation at 5-15 min, observed as approximately 10 fold over basal control. The response mediated by trypsin decayed gradually thereafter and returned to almost near basal levels by 120 min. On the contrary, AP-induced p38 MAPK phosphorylation remained sustained and still apparent at the longest time point, 120 min (4.8 ± 0.1 fold of basal control).

3.2.3.2 JNK

Figure 3.5 shows the effects of PAR-2 activation in stimulating phosphorylation of JNK-1 (p46). Again, cells were stimulated with either 50nM of trypsin or 30μM of 2f-LIGKV-OH over a period of 120 min. Trypsin–stimulated the phosphorylation of JNK-1, with the response peaking at 5-15 min, observed as approximately 3 fold of basal control. This response then slowly decreased to near basal values by 120 min. The PAR-

2 AP also stimulated a response of JNK-1 phosphorylation, evident at 5 min and peaking at a slightly later time point, 30 min, at 3.7 $\pm$ 0.7 fold of basal control. This response was still evident by 120 min (1.9 $\pm$ 0.2 fold of basal control).

3.2.4 Activation of the transcription factors, AP-1 and CRE, by PAR-2

This study also looked at PAR-2–stimulated activation of the distal transcription factors, AP-1 and CRE, in clone G cells.

3.2.4.1 AP-1

The time-dependent effects of various agonists on AP-1–driven gene transcription in clone G cells were assessed using trypsin (50nM), 2f-LIGKV-OH (100μM), PMA (100nM) and TNFα (20ng/ml) over a duration of 8 hours (Figure 3.6). Trypsin stimulated a time-dependent increase in AP-1 transcriptional activity, peaking at time 6-8 hours, with the maximal response quantified as 10.5 $\pm$ 0.4 fold of basal control. The PAR-2 AP, 2f-LIGKV-OH, displayed a similar activation profile, with a maximal stimulation of 13.8 $\pm$ 1.7 fold of basal control. PMA also induced AP-1 transcriptional activity in clone G cells, with the response peaking at time 8 hours, approximately 2 fold higher than that of PAR-2 agonists (29.6 $\pm$ 2.5 fold of basal control). The cytokine, TNFα, stimulated minimal AP-1 transcriptional activity in this assay system, with a consistently lower magnitude of response (up to 2 fold) observed throughout the time course tested.

3.2.4.2 CRE

Similarly, the time-dependent effects of CRE driven gene transcription in Clone G cells were investigated (Figure 3.7). Trypsin induced an increase in transcriptional activity over time, peaking at time 6 hours (8.5 $\pm$ 0.7 fold of basal control) and decaying thereafter. The AP, 2f-LIGKV-OH, also stimulated reporter activity in a time-dependent manner, reaching maximal at 6-8 hours, with the response observed to be approximately 4.6 $\pm$ 1.1 fold of basal control. The PKC activator, PMA, also stimulated an increase

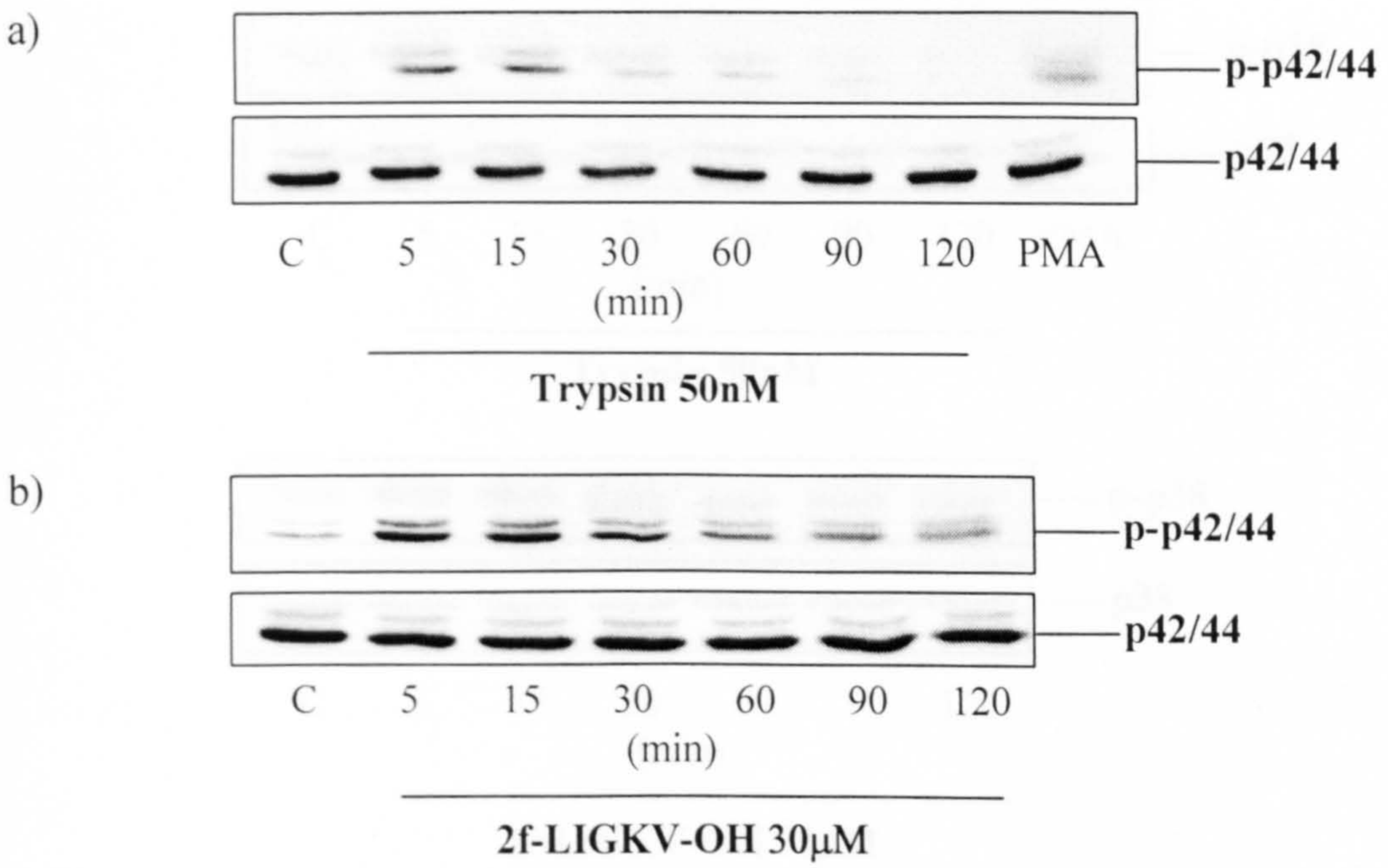
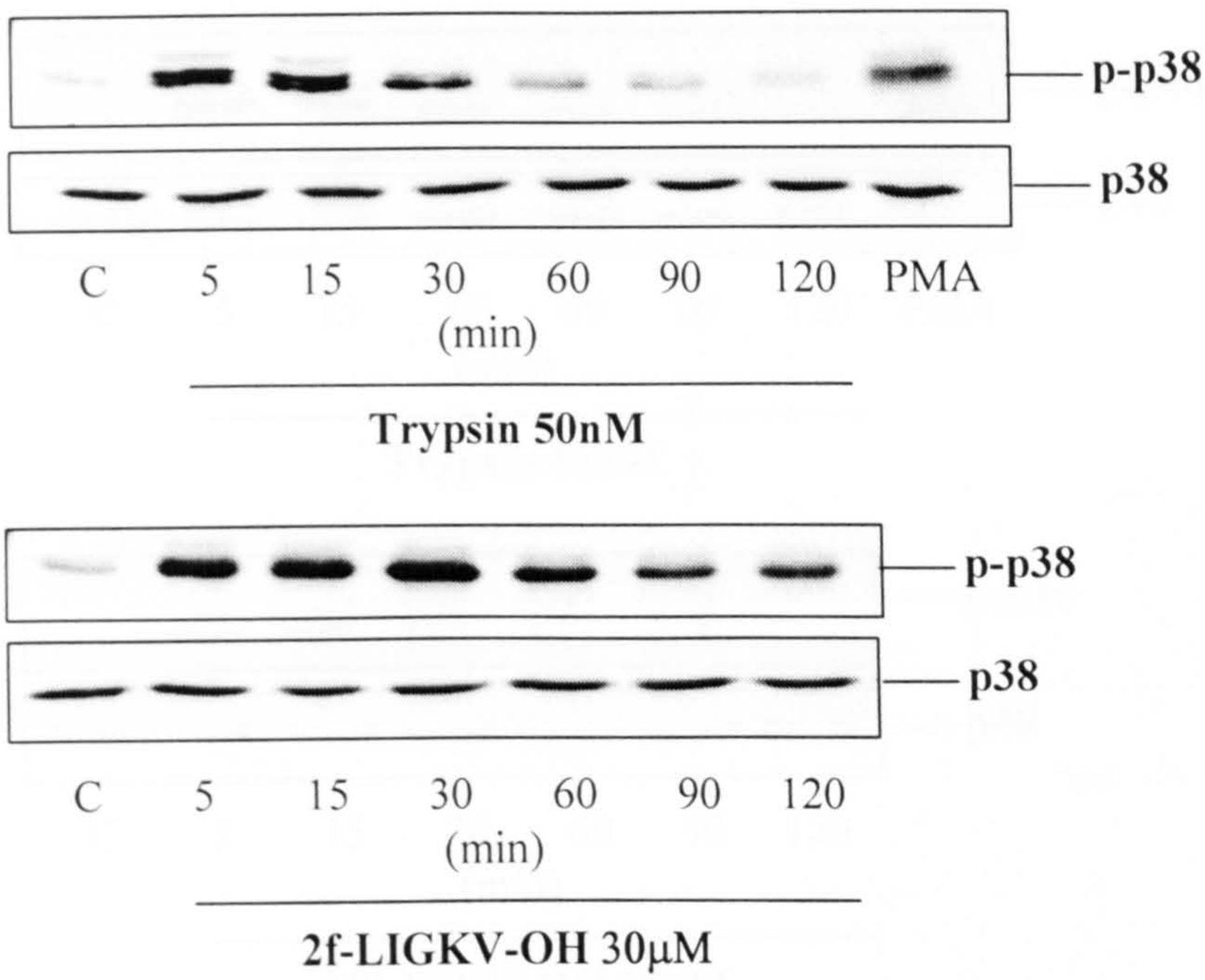


Figure 3.3. PAR-2–mediated phosphorylation of ERK in clone G cells

Cells were rendered quiescent for 18 hours prior to stimulation with 50nM of trypsin or 30μM of the PAR-2 AP, 2f-LIGKV-OH, for the times indicated. PMA (100nM) was used as a positive control (t = 15 min). Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2). (a) and (b) Blots shown are representative of two others. Equal protein loading was ensured by probing the membrane against total p42/44 (lower panel) in parallel to p-p42/44 (top panel). (c) Blots were quantified by densitometry and expressed as mean ± s.e.m. (fold stimulation).

a)



b)

c)

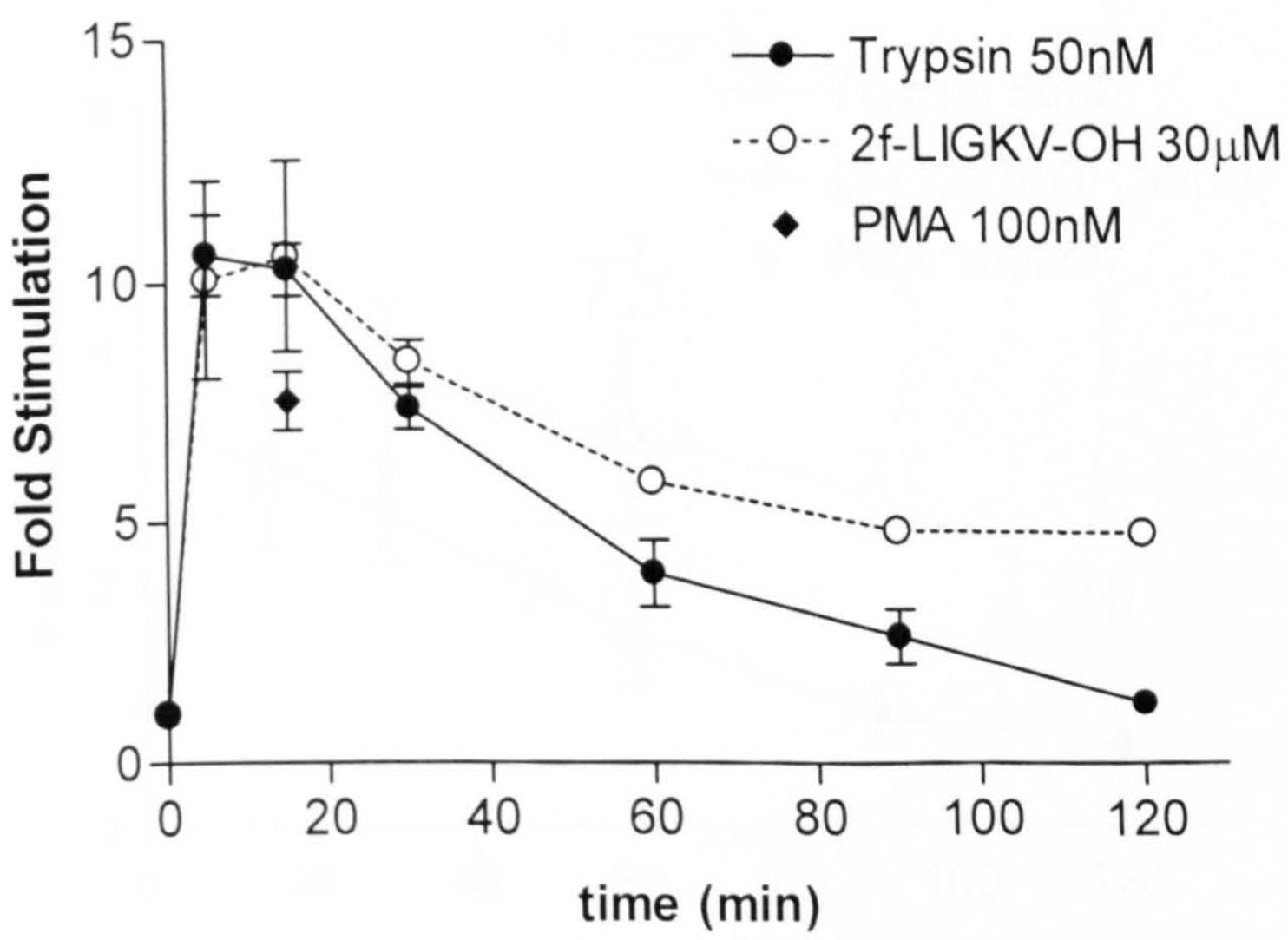
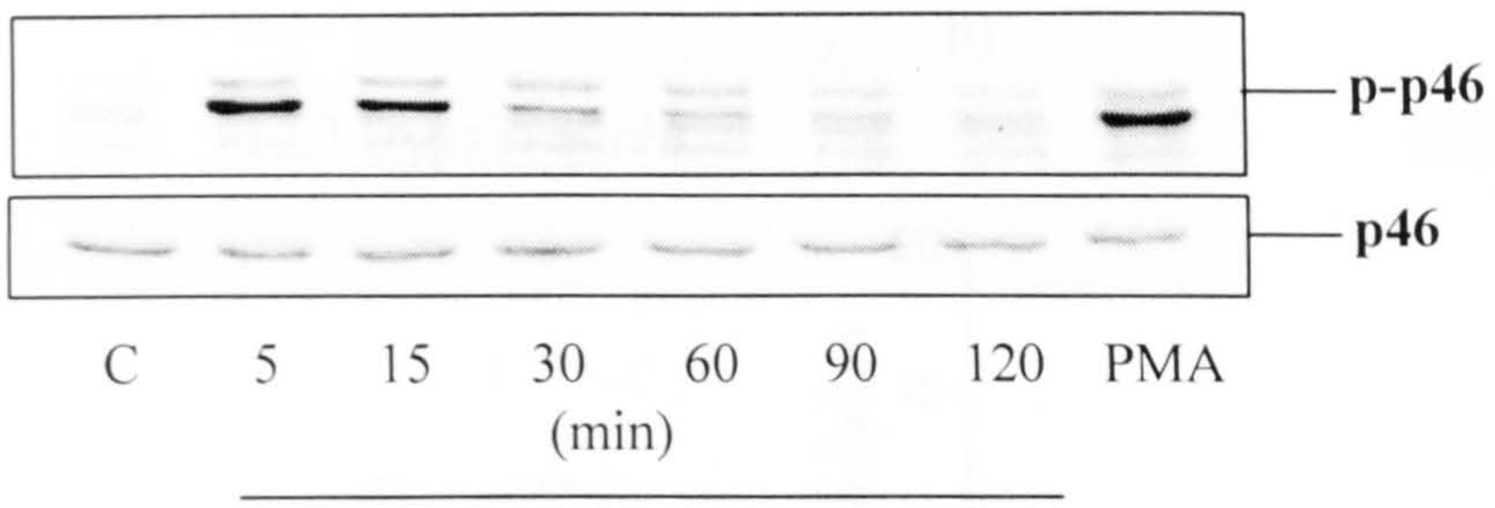


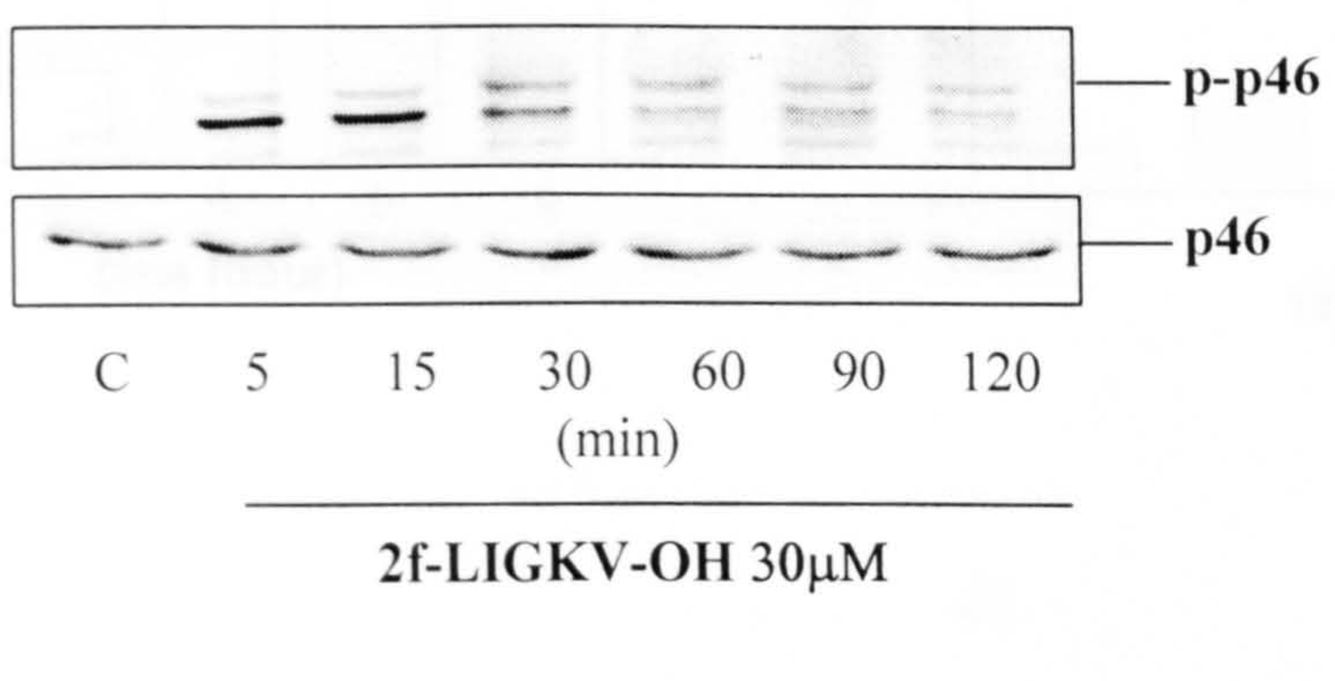
Figure 3.4. PAR-2–mediated phosphorylation of p38 MAPK in clone G cells

Cells were rendered quiescent for 18 hours prior to stimulation with trypsin (50nM) or 2f-LIGKV-OH (30μM) for the times indicated. PMA (100nM) was utilised as a positive control (t = 15 min). Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2). (a) and (b) Blots shown are representative of 3 individual experiments. Equal protein loading was ensured by probing the membrane against total p38 (lower panel) in parallel to p-p38 (top panel). (c) Blots were quantified by densitometry and expressed as mean ± s.e.m. (fold stimulation).

a)



b)



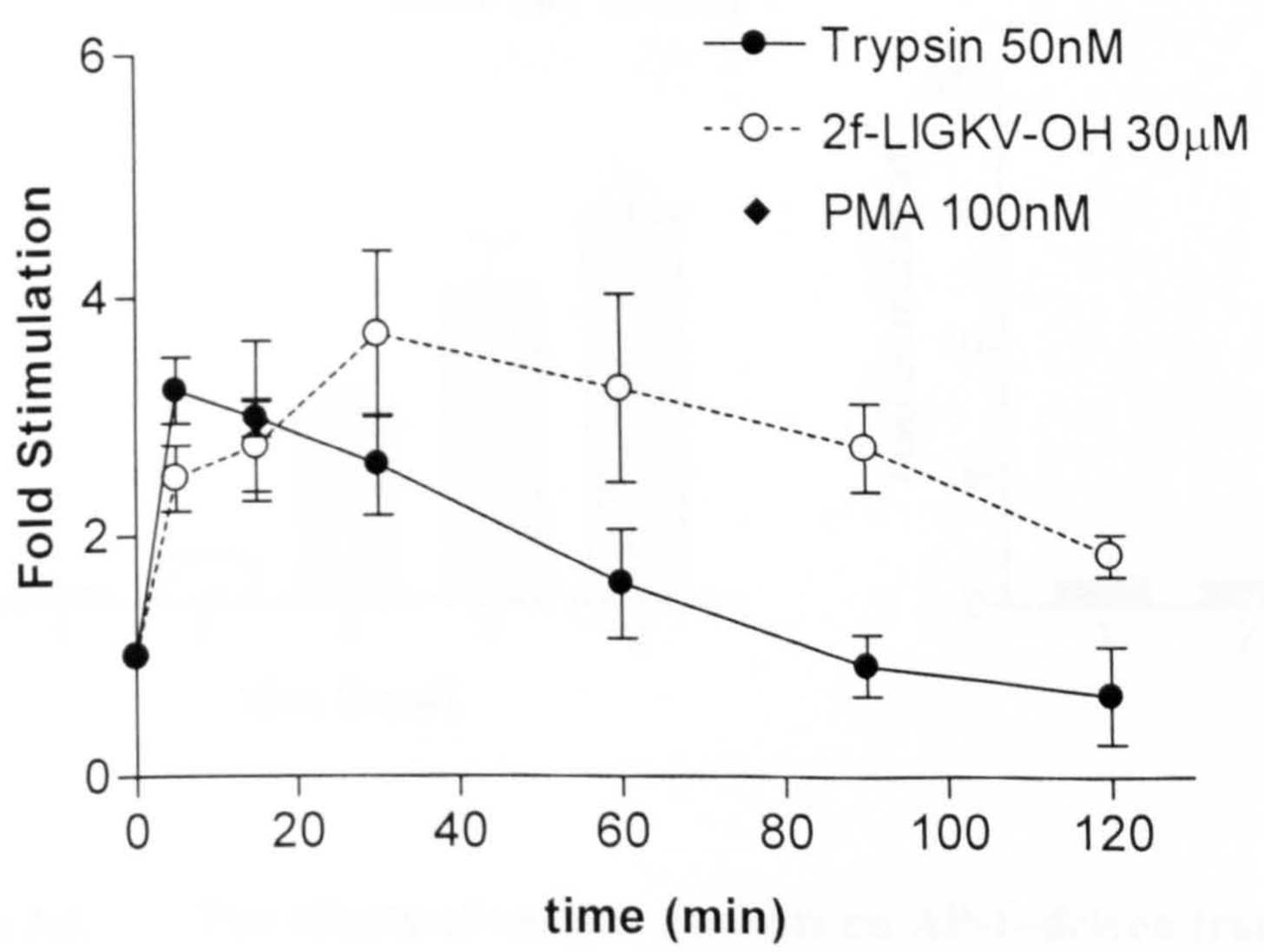


Figure 3.5. PAR-2–mediated phosphorylation of JNK in clone G cells

Cells were rendered quiescent for 18 hours prior to stimulation with 50nM of trypsin or 30μM of 2f-LIGKV-OH for the times indicated with 100nM PMA utilised as a positive control (t = 15 min). Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2). (a) and (b) Blots shown are representative of two others. (c) Blots were quantified by densitometry and expressed as mean ± s.e.m. (fold stimulation).

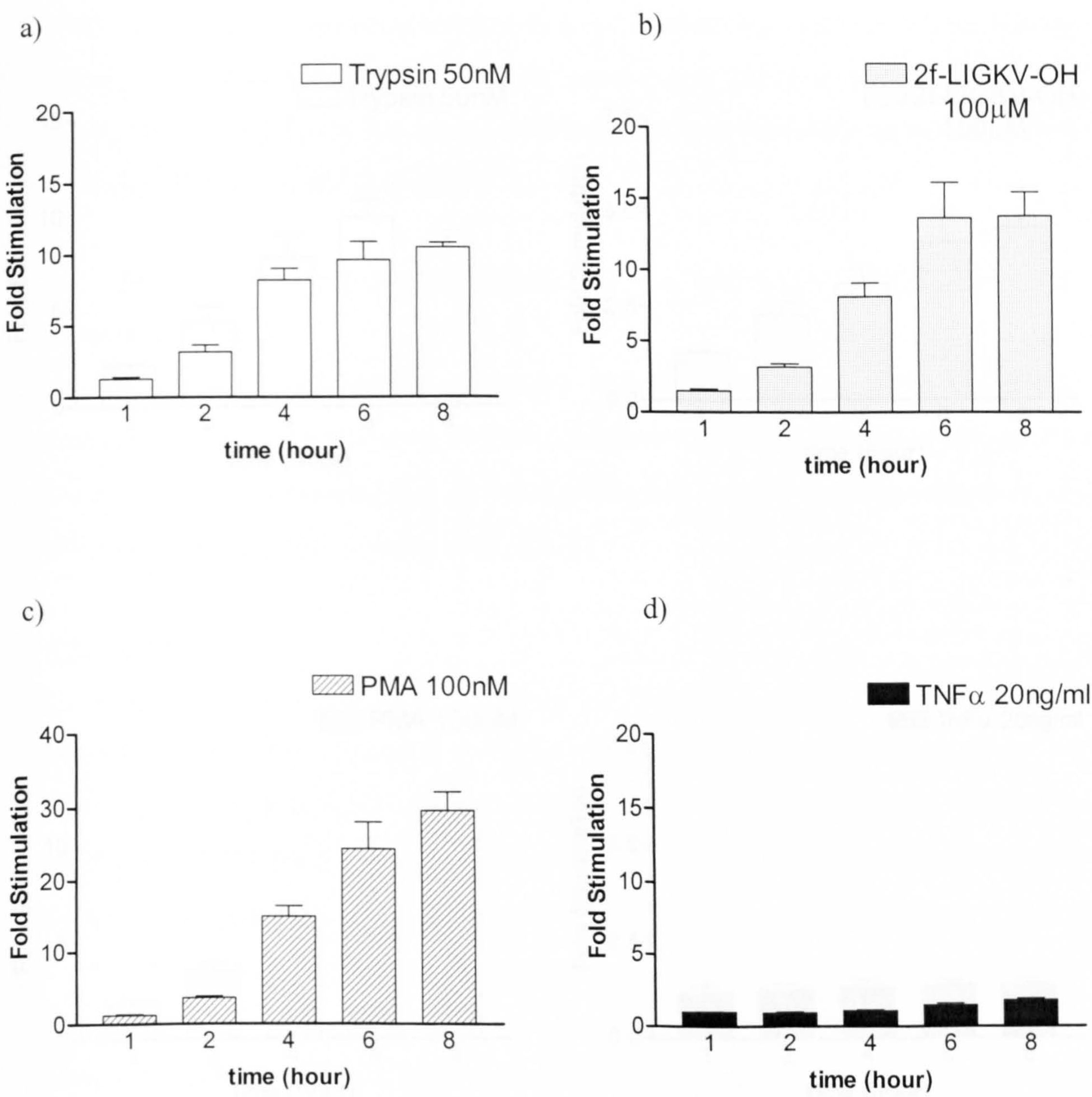


Figure 3.6. The effects of various agonists on AP-1–driven transcriptional activation in clone G cells

Clone G cells having a luciferase gene in the AP-1 reporter region were used in this experiment. Cells were grown to confluency and rendered quiescent for 18 hours before the addition of various agonists for the times indicated. Following termination of stimulation, cells lysates were then assayed for luciferase activity as previously described (Section 2.8). Data shown are expressed as fold over unstimulated basal and each value represents the mean $\pm$ s.e.m. from three separate experiments.

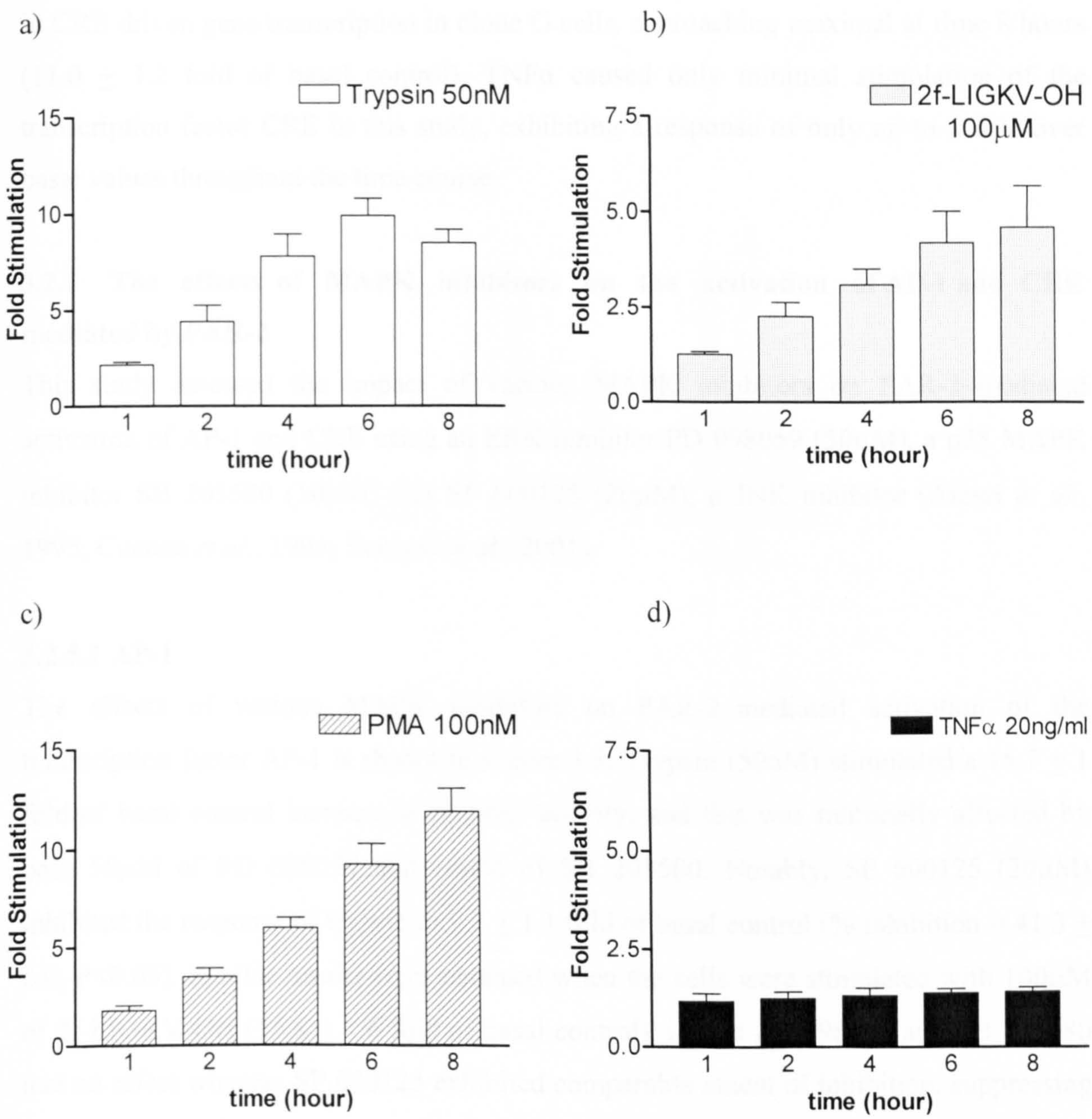


Figure 3.7. The effects of various agonists on CRE–driven transcriptional activation in clone G cells

Clone G cells having a luciferase gene in the CRE reporter region were used in this experiment. Cells were grown to confluency and rendered quiescent for 18 hours before the addition of various agonists for various time points. After termination of stimulation, cells lysates were assessed for luciferase reporter activity as previously described (Section 2.8). Data shown are expressed as fold over unstimulated basal and each value represents the mean $\pm$ s.e.m. from three separate experiments.

in CRE driven gene transcription in clone G cells, approaching maximal at time 8 hours (11.0 ± 1.2 fold of basal control). TNFα caused only minimal stimulation of the transcription factor CRE in this study, exhibiting a response of only up to 2 fold over basal values throughout the time course.

3.2.5 The effects of MAPK inhibitors on the activation of AP-1 and CRE mediated by PAR-2

This study assessed the impact of various MAPK inhibitors on PAR-2–mediated activation of AP-1 and CRE using an ERK inhibitor PD 098059 (50μM), a p38 MAPK inhibitor SB 203580 (10μM) and SP 600125 (20μM), a JNK inhibitor (Alessi *et al.*, 1995; Cuenda *et al.*, 1995; Bennett *et al.*, 2001).

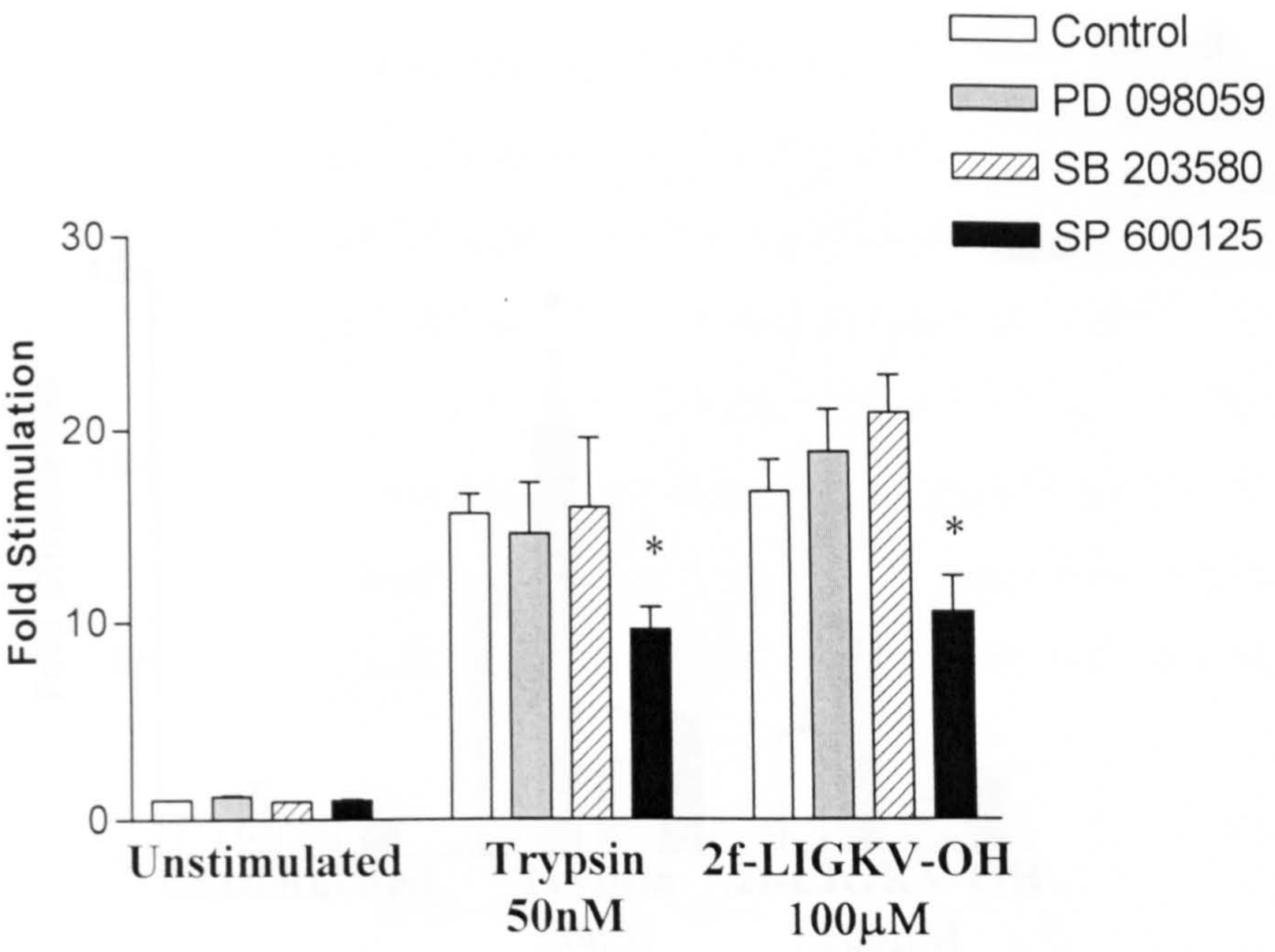
3.2.5.1 AP-1

The effects of various MAPK inhibitors on PAR-2–mediated activation of the transcription factor AP-1 is shown in Figure 3.8. Trypsin (50nM) stimulated a 15.7 ± 1 fold of basal control increase in reporter activity, and this was minimally affected by both 50μM of PD 098059 and 10μM of SB 203580. Notably, SP 600125 (20μM) inhibited the response of trypsin to 9.7 ± 1.1 fold of basal control (% inhibition = 41.3 ± 6.0, $P<0.05$). Similar results were obtained when the cells were stimulated with 100μM of 2f-LIGKV-OH (16.8 ± 1.6 fold of basal control), in that PD 098059 and SB 203580 had no effect whereas SP 600125 exhibited comparable extent of inhibition, suppressing the response to 10.6 ± 1.9 fold of basal control (% inhibition = 40.4 ± 5.7 , $P<0.05$).

3.2.5.2 CRE

Figure 3.9 illustrates the effects of PD 098059, SB 203580 and SP 600125 on trypsin and 2f-LIGKV-OH–stimulated CRE-driven transcriptional activation in clone G cells. Trypsin (50nM) and 2f-LIGKV-OH (100μM) induced the activation of this transcription factor, measured as 6.6 ± 0.7 and 3.0 ± 0.6 fold of basal control respectively at 6 hours post stimulation. These responses were significantly potentiated in the presence of PD

a)



b)

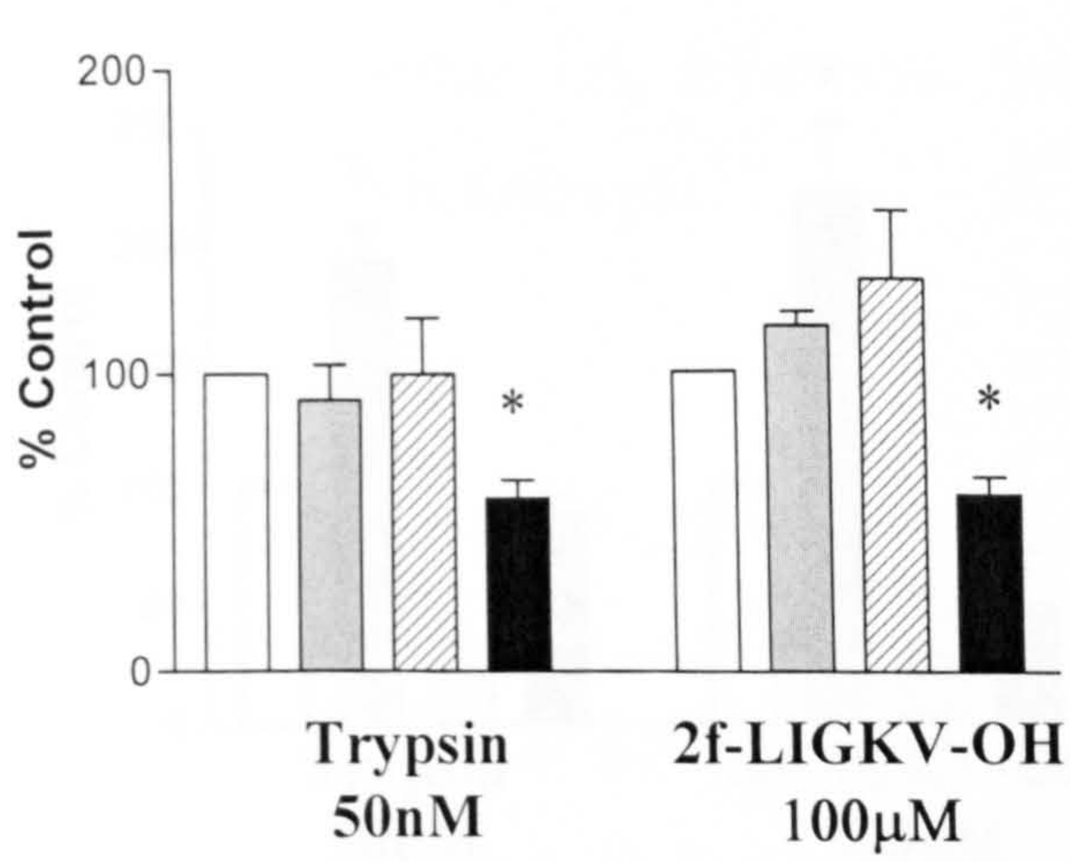
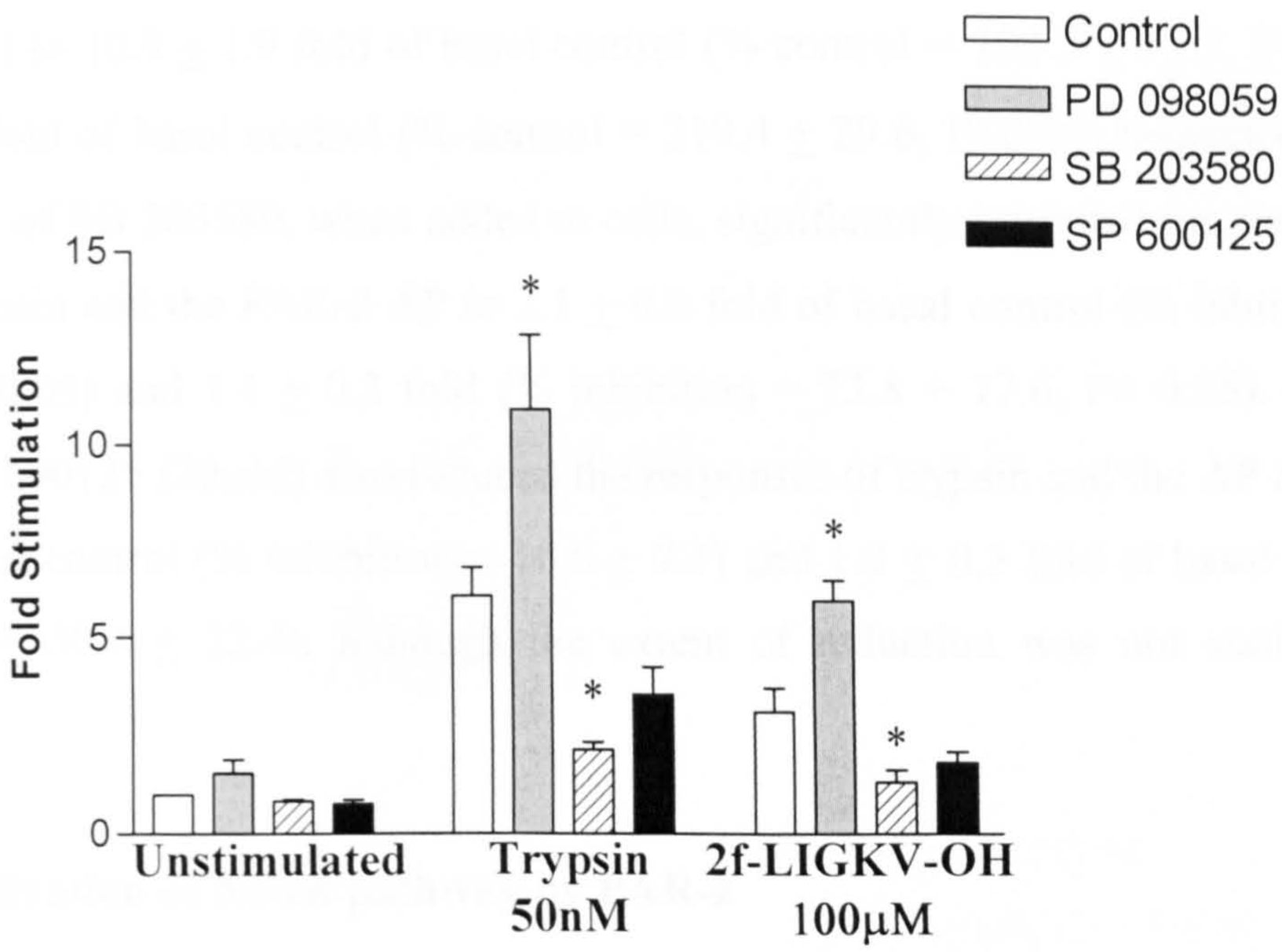


Figure 3.8. The effects of various MAPK inhibitors on PAR-2–stimulated AP-1–induced transcriptional activation in clone G cells

Clone G cells having a luciferase gene in the AP-1 reporter region were used in this experiment. Cells were grown to confluency and rendered quiescent for 18 hours and incubated with PD 098059 (50μM), SB 203580 (10μM) or SP 600125 (20μM) for 30 min before the addition of trypsin (50nM) or 2f-LIGKV-OH (100μM) for 6 hours. Luciferase activity was determined as previously outlined (Section 2.8). Data shown are expressed as (a) fold over unstimulated basal and (b) % of stimulated control with each value representing the mean ± s.e.m. from three separate experiments. * $P < 0.05$ compared with agonist-stimulated control.

a)



b)

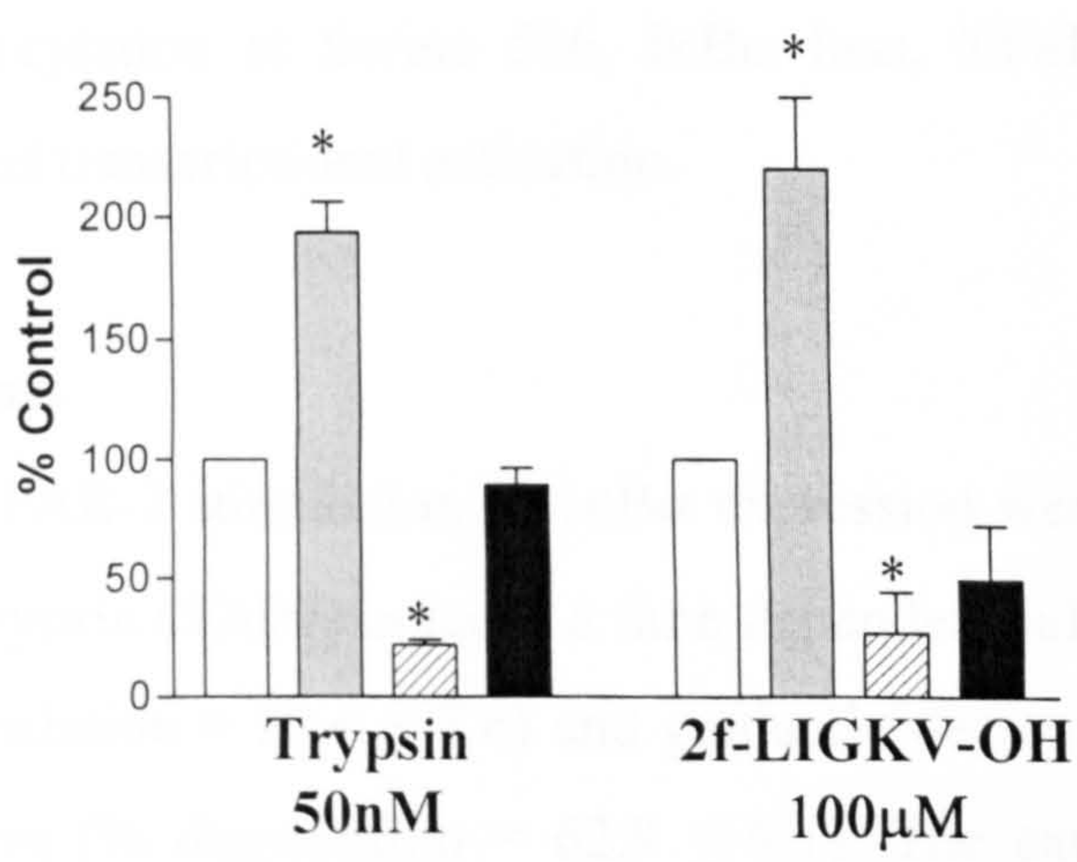


Figure 3.9. The effects of various MAPK inhibitors on PAR-2–stimulated CRE–induced transcriptional activation in clone G cells

Clone G cells having a luciferase gene in the CRE reporter region were used in this experiment. Cells were grown to confluency and rendered quiescent for 18 hours and incubated with PD 098059 (50μM), SB 203580 (10μM) or SP 600125 (20μM) for 30 min before stimulating with trypsin (50nM) or 2f-LIGKV-OH (100μM) for 6 hours. Cells were then assayed for luciferase activity as previously outlined (Section 2.8). Data shown are expressed as (a) fold over unstimulated basal and (b) % of stimulated control with each value representing the mean ± s.e.m. from three separate experiments. * $P < 0.05$ compared with agonist-stimulated control.

098059 (50μM) to 10.9 ± 1.9 fold of basal control (% control = 193.5 ± 12.7, P< 0.05) and 5.3 ± 0.9 fold of basal control (% control = 219.4 ± 29.6, P<0.05) respectively. In contrast, 10μM of SB 203580, when added to cells, significantly inhibited the responses induced by trypsin and the PAR-2 AP to 2.1 ± 0.2 fold of basal control (% inhibition = 78.0 ± 2.1, P<0.05) and 1.4 ± 0.2 fold (% inhibition = 72.8 + 17.6, P< 0.05). On the other hand, SP 600125 (20μM) also reduced the responses of trypsin and the AP to 3.5 ± 0.7 fold of basal control (% inhibition = 10.6 ± 7.1) and 1.8 ± 0.3 fold of basal control (% inhibition = 50.1 ± 22.4), although the extent of reduction was not statistically significant.

3.2.6 The activation of NFκB pathway by PAR-2

This study investigated the activation of NFκB pathway by PAR-2 at the levels of p65 NFκB phosphorylation at Serine 536, IκBα loss, NFκB-DNA binding activity and NFκB–mediated transcriptional activation.

3.2.6.1 IκBα loss

The effects of PAR-2 stimulation on IκBα expression were also assessed as depicted in Figure 3.10. Trypsin (50nM) induced a time-dependent IκBα loss which was apparent at 5 min (% degradation = 20.6 ± 7.6) and gradually decreasing to a minimum by 60 min after stimulation (% degradation = 62.9 ± 9.1). The expression of IκBα was slowly restored thereafter and back to near basal values by 120 min. The activating peptide, 2f-LIGKV-OH (30μM), also induced a time-dependent IκBα loss in clone G cells with similar kinetics, with the maximum protein degradation observed at time 60 min (% degradation = 68.1 ± 9.6). The PKC activator, PMA (100nM), when added to clone G, resulted in IκBα loss detected as % degradation = 74.7 ± 3.3 at 15 min post stimulation.

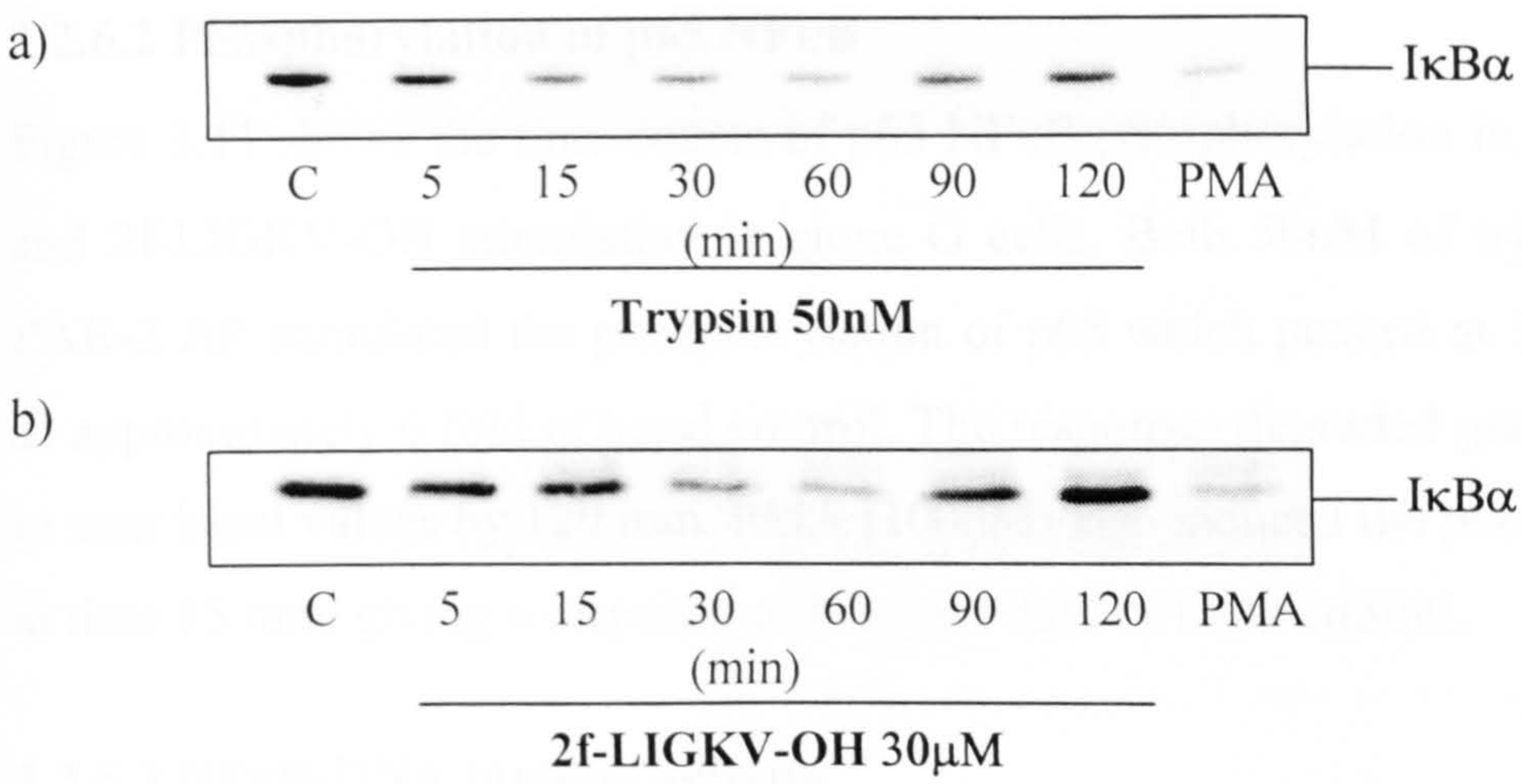


Figure 3.10. PAR-2–stimulated IκBα loss in clone G cells

Cells were rendered quiescent for 18 hours prior to stimulation with 50nM of trypsin or 30μM of 2f-LIGKV-OH for the times indicated with 100nM PMA utilised as a positive control (t=15min). Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and visualised by Western blotting (Section 2.6.2). (a) and (b) Blots shown are representative of two others. (c) Blots were quantified by densitometry and expressed as mean ± s.e.m. (fold stimulation).

3.2.6.2 Phosphorylation of p65 NFκB

Figure 3.11 shows the time-course of p65 NFκB phosphorylation in response to trypsin and 2f-LIGKV-OH stimulation in clone G cells. Both 50nM of trypsin and 30μM of PAR-2 AP stimulated the phosphorylation of p65 which peaked at 15-30 min, detected as approximately 6 fold of basal control. The responses degraded gradually and returned to near basal values by 120 min. PMA (100nM) also induced the phosphorylation of p65 at time 15 min, giving a response of 8.2 $\pm$ 0.6 fold of basal control.

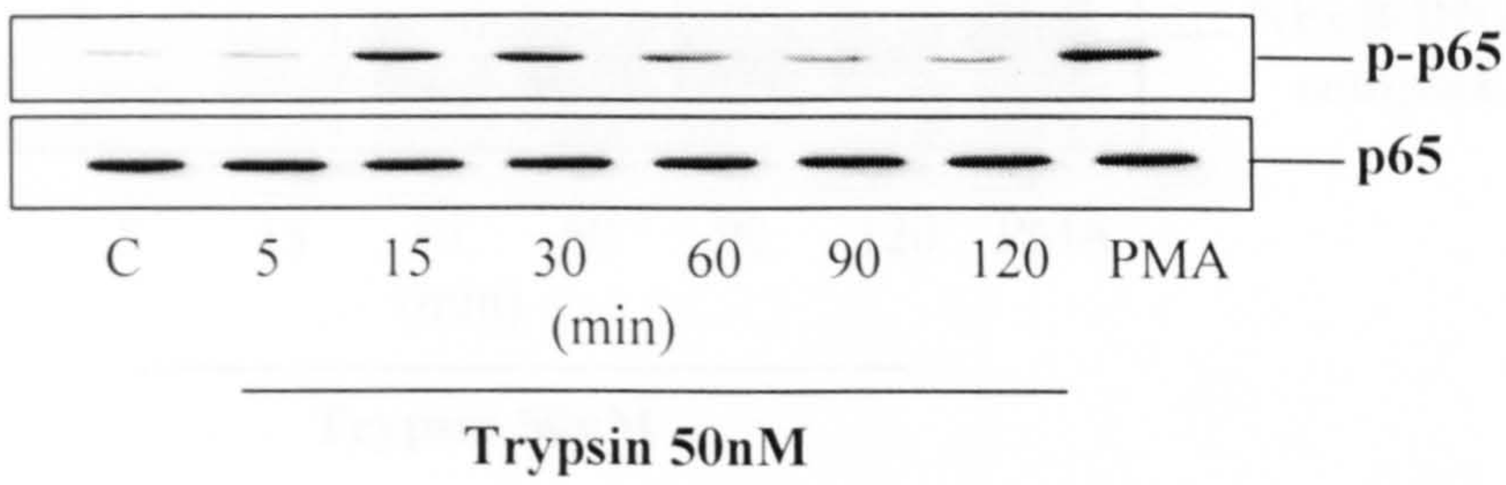
3.2.6.3 NFκB-DNA binding activity

Figure 3.12 illustrates the effects of PAR-2 activation on NFκB-DNA binding activity. Both trypsin (50nM) and 2f-LIGKV-OH (30μM) induced time-dependent increases in the binding of NFκB to DNA, albeit with slightly different kinetics. Trypsin stimulated a response which can be observed at 30 min (1.7 $\pm$ 0.1 fold of basal control), reaching maximum at 60-90 min, with the magnitude of approximately 4 fold above basal (4.4 $\pm$ 0.3 and 4.3 $\pm$ 0.4 fold of basal control for time 60 and 90 min respectively). This response still remained evident by 120 min (2.3 $\pm$ 0.5 fold of basal control). On the other hand, the NFκB-DNA binding activity induced by the PAR-2 AP was apparent at an earlier time, 15 min (2.3 $\pm$ 0.4 fold of basal control); and peaked at 60-90 min, at approximately 5 fold of basal control. This response, however, was observed to decay slowly and still persistent by 120 min (4.3 $\pm$ 0.6 fold of basal control).

3.2.6.4 NFκB-driven transcriptional activity

The activation of NFκB–mediated transcriptional activation was also investigated in response to PAR-2 stimulation over a period of 8 hours as shown in Figure 3.13. Trypsin (50nM) stimulated a time-dependent increase in NFκB–driven gene transcription, reaching a maximum by 6-8 hours with the response observed to be approximately 23 fold of basal control. Similar activation profile was observed with 100μM of the PAR-2 AP, 2f-LIGKV-OH, with the highest response detected as approximately 18 fold of

a)



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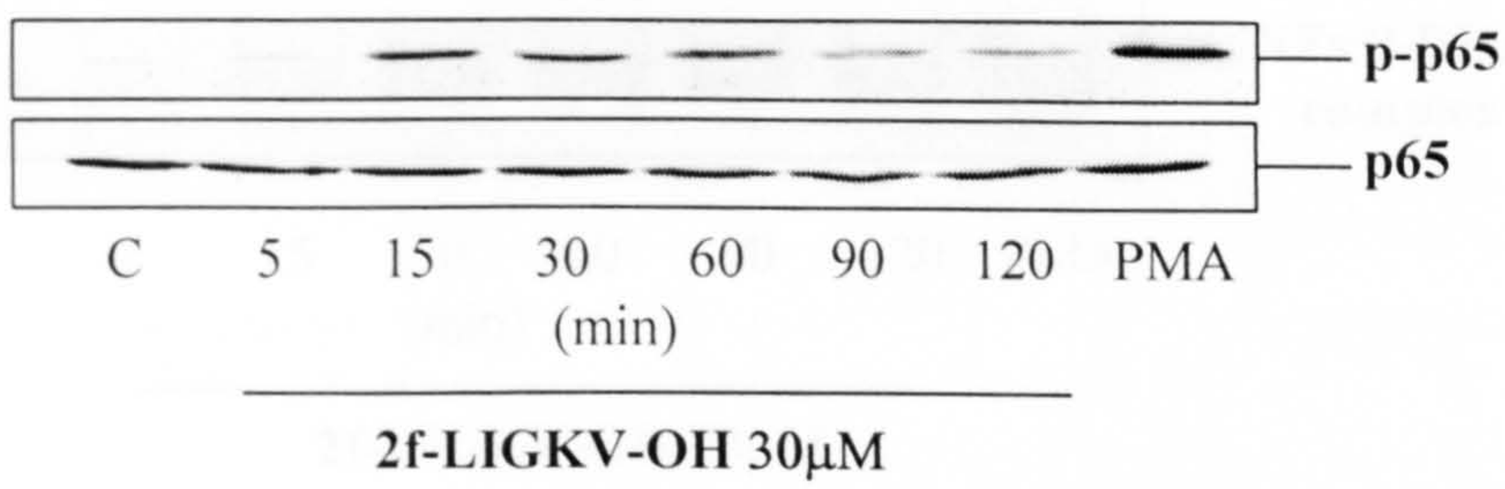

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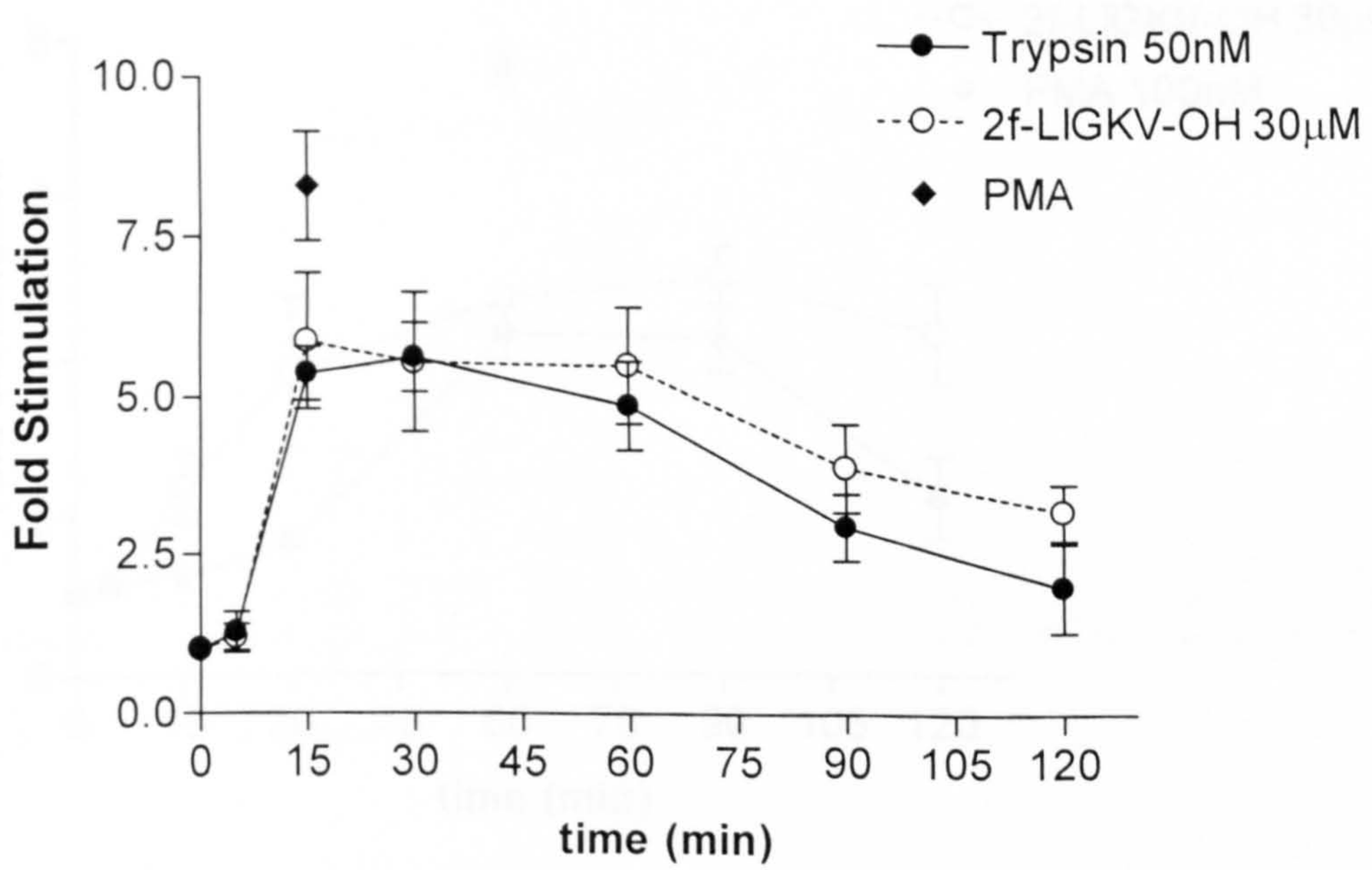


Figure 3.11. PAR-2–mediated phosphorylation of p65 NFκB in clone G cells

Cells were rendered quiescent for 18 hours prior to stimulation with trypsin (50nM) or 2f-LIGKV-OH (30μM) for the times indicated. PMA (100nM) was utilised as a positive control (t = 15 min). Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and were visualised by Western blotting (Section 2.6.2). (a) and (b) Blots shown are representative of 3 individual experiments. (c) Blots were quantified by densitometry and expressed as mean ± s.e.m. (fold stimulation).

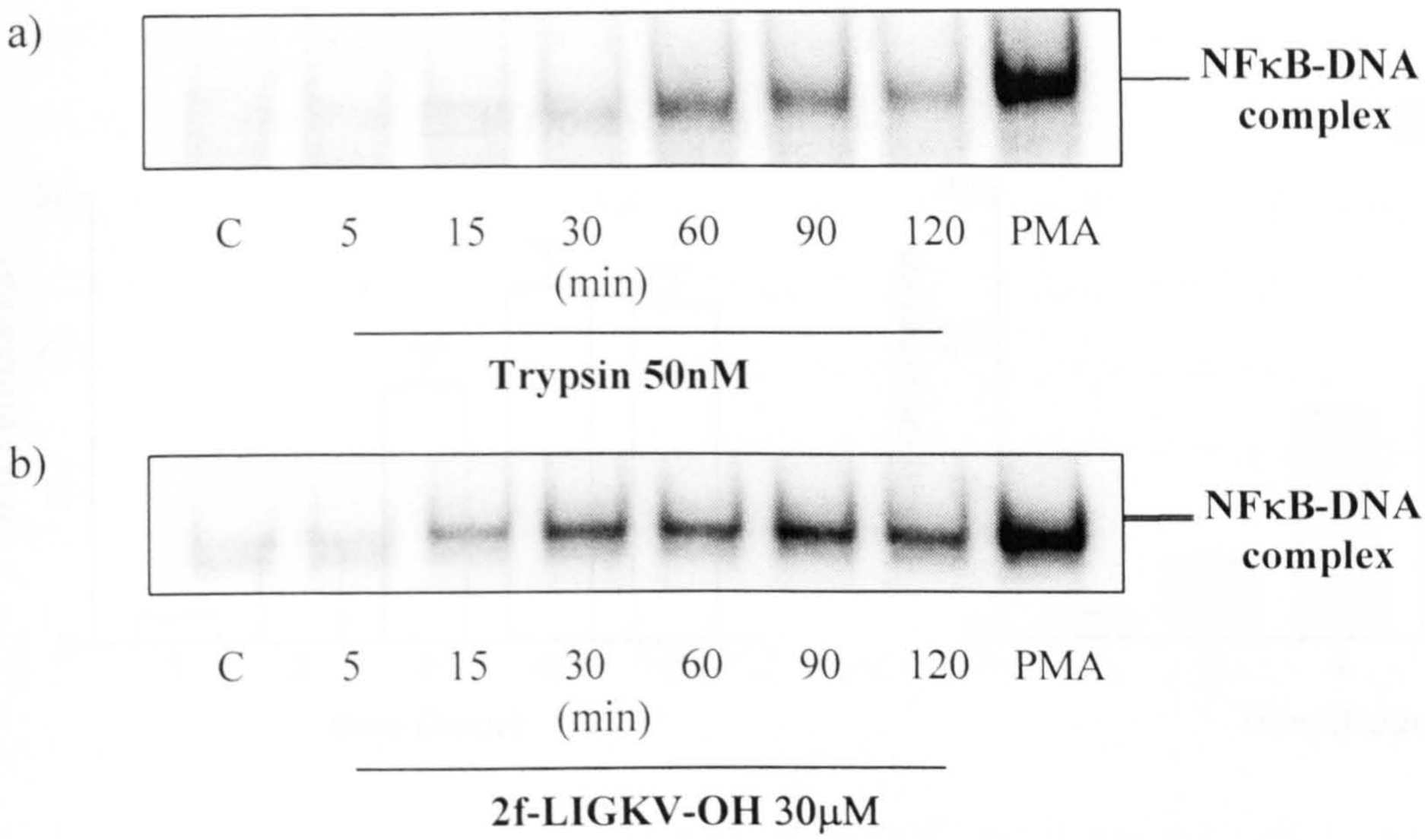


Figure 3.12. PAR-2 stimulated NFκB-DNA binding

Cells were grown to confluent before being rendered queiscent in serum-free medium for 18 hours prior to stimulation with trypsin (50nM) or 2f-LIGKV-OH (30μM) for the indicated time points. Nuclear extracts were prepared and the binding activity was measured by EMSA (Section 2.7). (a) & (b) Autoradiograms shown are representative of two others. (c) Autoradiograms were quantified using densitometry and expressed as mean ± s.e.m. of three separate experiments.

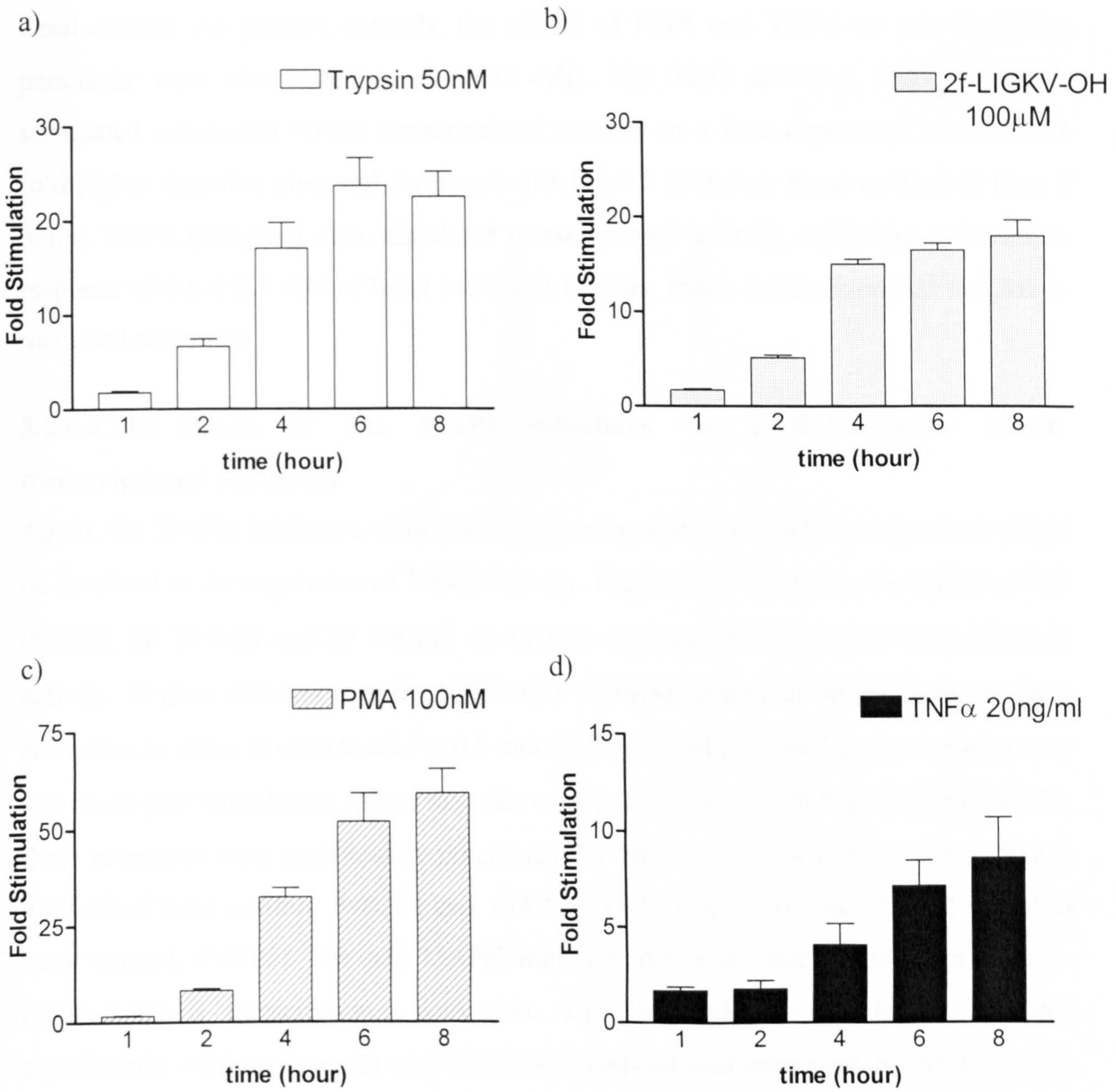


Figure 3.13. The effects of various agonists on NFκB–driven transcriptional activation in clone G cells

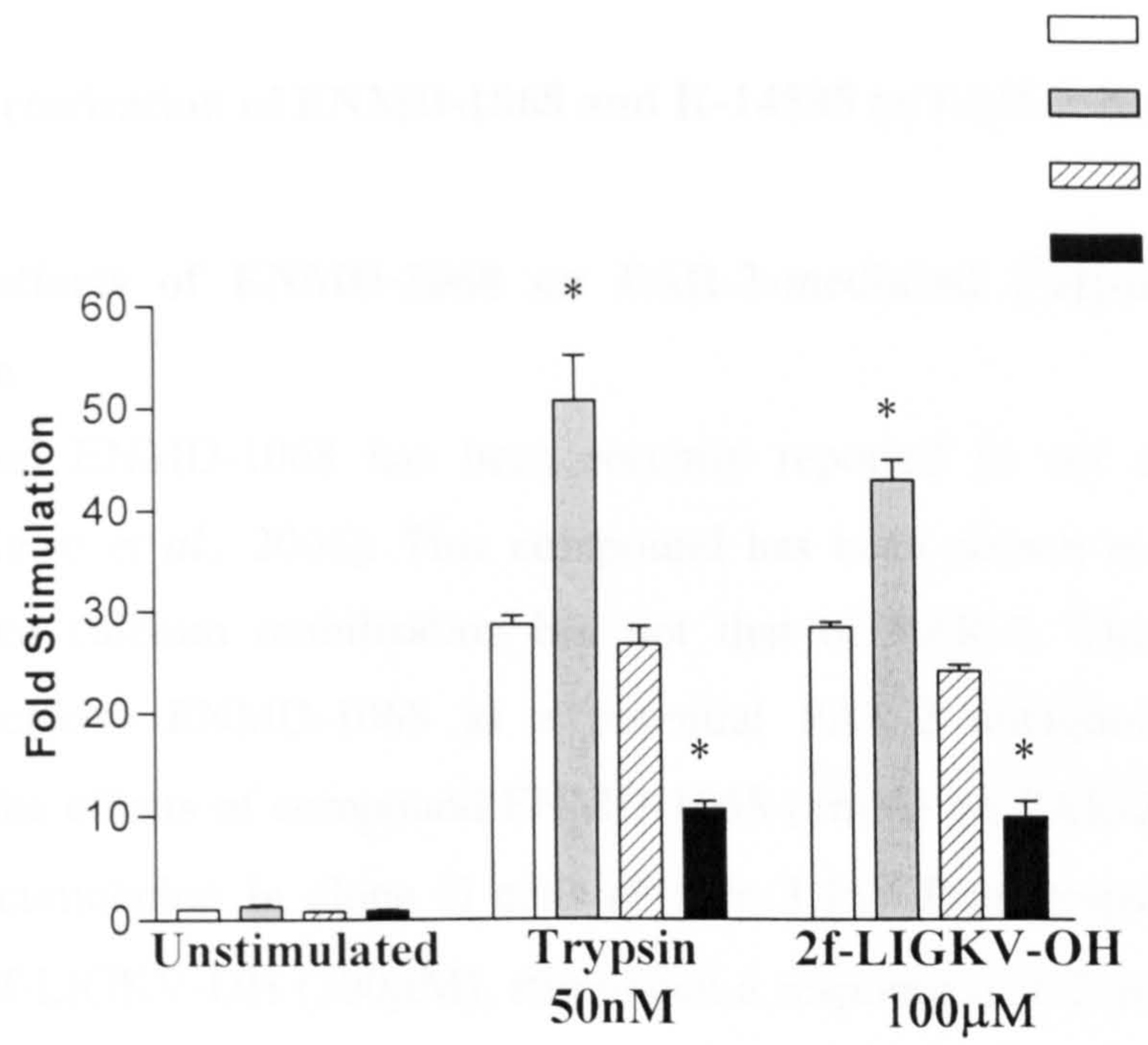
Clone G cells having a luciferase gene in the NFκB reporter region were used in this experiment. Cells were grown to confluency and rendered quiescent for 18 hours before the addition of various agonists for various time points. Cell lysates were then assayed for luciferase activity as previously described (Section 2.8). Data shown are expressed as fold over unstimulated basal and each value represents the mean ± s.e.m. from three separate experiments.

basal control. As positive controls, the effects of PMA and TNFα on this signalling parameter were also tested in clone G cells. The PKC activator, PMA (100nM), stimulated substantial NFκB transcriptional activity in a time-dependent manner, 2-3 fold higher than that observed for trypsin (65.1 ± 1.2 fold over basal control) at time 8 hours. TNFα (20ng/ml) also stimulated transcriptional activity, achieving a maximum response of 8.6 ± 2.1 fold of basal control at 8 hours, much lower compared to PAR-2-mediated responses.

3.2.6.5 The effects of the MAPK inhibitors on PAR-2–induced NFκB transcriptional activation

Again, the MAPK inhibitors were used to investigate if any MAPK components might be involved in the regulation of NFκB activity. Figure 3.14 illustrates the effects of PD 098025, SB 203580 and SP 600125 on PAR-2–mediated NFκB–driven transcriptional activity. Trypsin (50nM) and 2f-LIGKV-OH (100μM) activated NFκB transcriptional activation in clone G cells to 28.7 ± 0.8 and 28.5 ± 0.5 fold of basal control respectively at 6 hours post stimulation. When the cells were preincubated with 50μM of PD 098059, these responses were significantly potentiated by 180.6 ± 22.2 % (28.7 ± 0.8 to 50.8 ± 4.5 fold of basal control, $P<0.05$) and 153.2 ± 10.0% (28.5 ± 0.5 to 42.9 ± 1.9 fold of basal control, $P<0.05$). The p38 MAPK inhibitor, however, had no effect on PAR-2–induced NFκB transcriptional activation. The JNK inhibitor SP 600125 (20μM) significantly inhibited trypsin and 2f-LIGKV-OH-induced responses by 65.4 ± 1.9% (28.7 ± 0.8 to 10.6 ± 0.8 fold of basal control, $P<0.05$) and 67.8 ± 5.3% (28.5 ± 0.5 to 9.9 ± 1.6 fold of basal control, $P<0.05$) respectively.

a)



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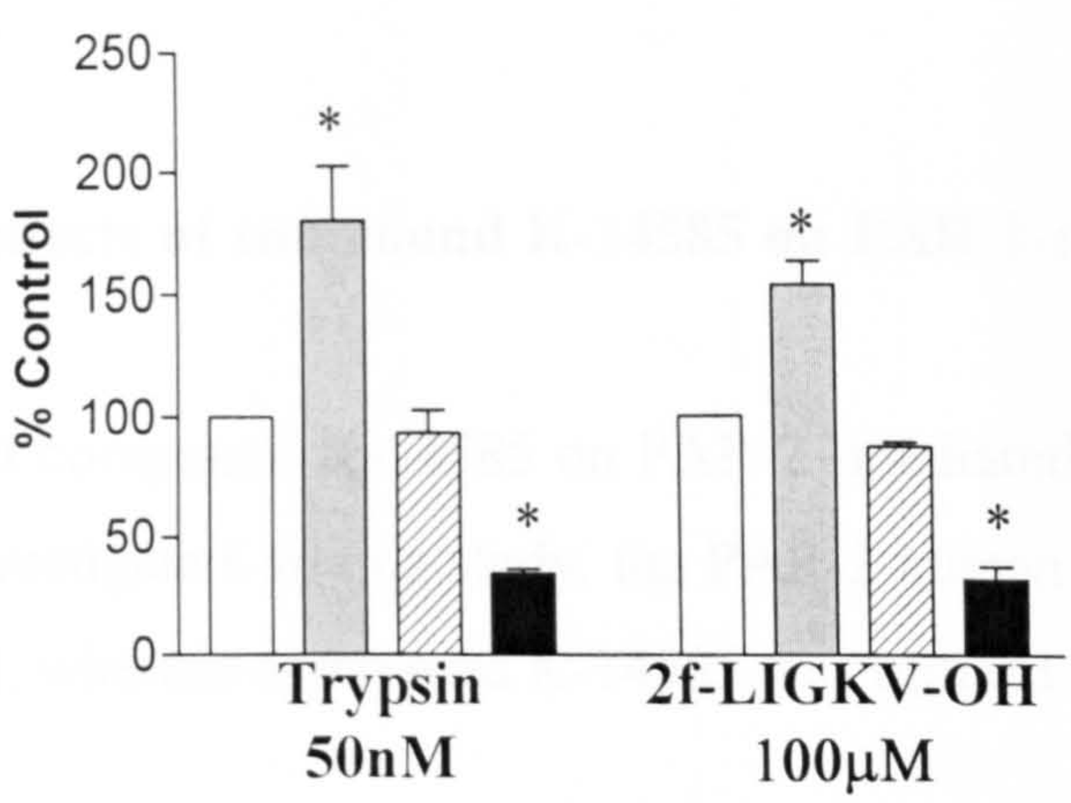


Figure 3.14. The effects of various MAPK inhibitors on PAR-2–stimulated NFκB–induced transcriptional activation in clone G cells

Clone G cells having a luciferase gene in the NFκB reporter region were used in this experiment. Cells were grown to confluency and rendered quiescent for 18 hours before the addition of PD 098059 (50μM), SB 203580 (10μM) or SP 600125 (20μM) for 30 min before stimulating with trypsin (50nM) or 2f-LIGKV-OH (100μM) for 6 hours. Cell lysates were then assayed for luciferase activity as previously described (Section 2.8). Data shown are expressed as (a) fold of unstimulated basal and (b) % of stimulated control with each value representing the mean ± s.e.m. from three separate triplicate experiments. * $P < 0.05$ compared with agonist-stimulated control.

3.3 Characterisation of ENMD-1068 and K-14585 as PAR-2 Antagonists

3.3.1 The effects of ENMD-1068 on PAR-2-mediated [^{3}H]-inositol phosphate accumulation

The compound ENMD-1068 has been recently reported to act as a novel PAR-2 antagonist (Kelso *et al.*, 2006). This compound has been shown to selectively inhibit PAR-2-induced calcium mobilisation but not that of PAR-1. Therefore in order to further characterise ENMD-1068 as a potential PAR-2 antagonist, this study has investigated the effects of compound ENMD-1068 (5mM) on PAR-2–mediated inositol phosphate accumulation in clone G cells (Figure 3.15). Both trypsin (20nM) and the PAR-2 AP, 2f-LIGKV-OH (100μM), stimulated a response of 8.2 and 7.1 fold of basal control respectively. These responses, however, were not affected by the presence of ENMD-1068.

3.3.2 The effects of compound K-14585 on PAR-2-mediated signalling in clone G cells

The effects of compound K-14585 on PAR-2 -mediated signalling cascades in clone G cells were investigated. In this study, the PAR-2 human AP SLIGKV (30μM) was used as the agonist; whereas compound K-14585 was utilised at 30, 5 and 1μM respectively.

3.3.2.1 The effects of K-14585 on PAR-2-stimulated [^{3}H]-inositol phosphate accumulation

SLIGKV (30μM) induced an increase in the accumulation of total [^{3}H]-inositol phosphate in clone G cells at 60 min, with the magnitude of response observed being 6.6 $\pm$1.1 fold of basal control (Figure 3.16). Compound K-14585 at both 30 and 5μM significantly reduced this response to 2.8 $\pm$ 0.6 fold (% inhibition = 68.4 $\pm$ 10.4, $P<0.05$) and 2.9 $\pm$ 0.6 fold (% inhibition = 67.6 $\pm$ 4.6, $P<0.05$) respectively, whereas 1μM of K-14585 inhibited the response to 3.9 $\pm$ 0.6 fold (% inhibition = 43.7 $\pm$ 13.3). Notably, K-14585 at all the concentrations tested did not alter the basal of total [^{3}H]-inositol phosphate accumulation.

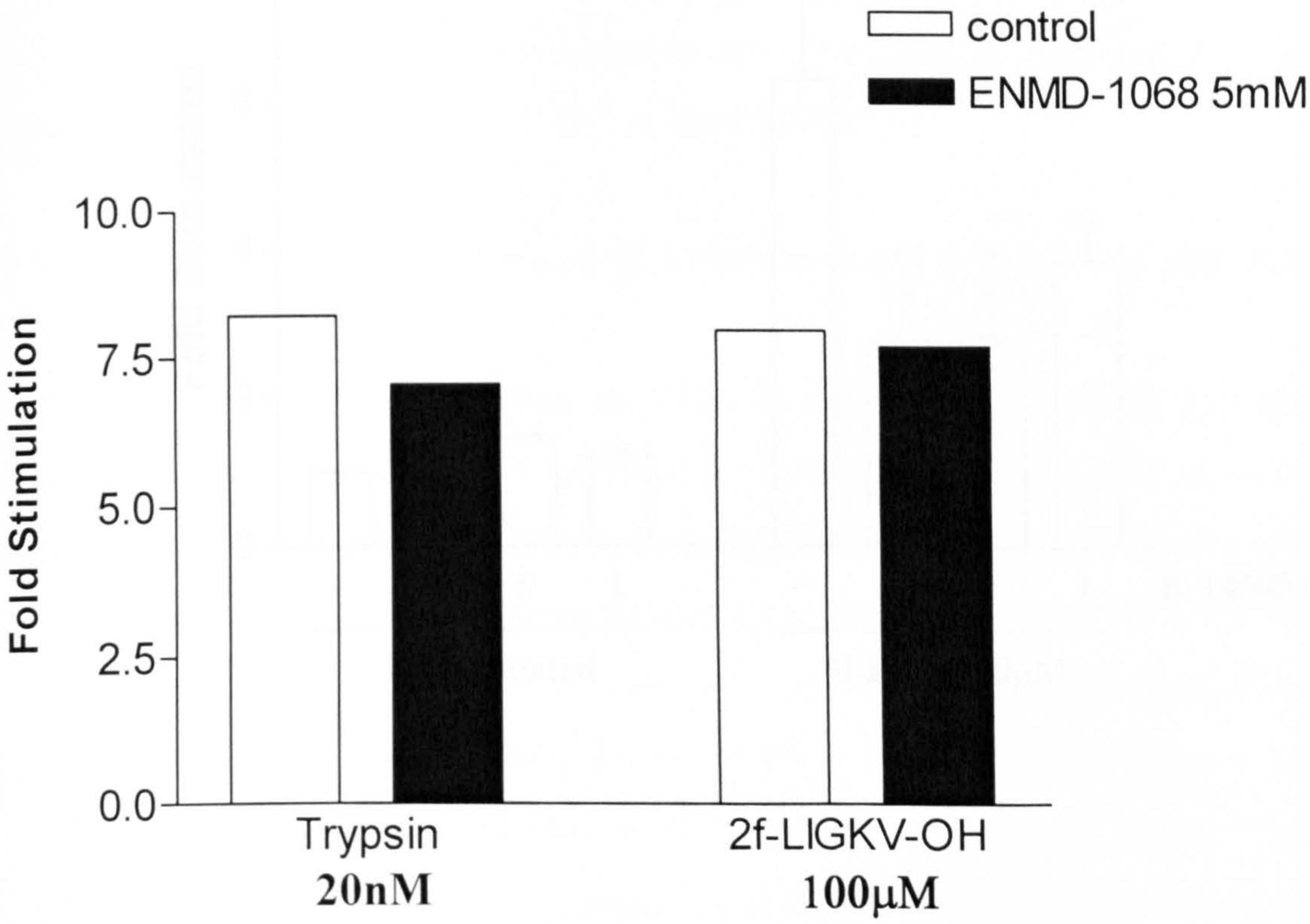


Figure 3.15. The effects of compound ENMD-1068 on PAR-2–mediated [^{3}H]-inositol phosphate accumulation in clone G cells

Cells were prelabelled with [^{3}H]-myo-inositol for 18 hours in serum free medium. LiCl (10mM) was added to the cells prior to incubation with 5mM of ENMD-1068 for 45 min. Subsequently the cells were stimulated with trypsin (20nM) or 2f-LIGKV-OH (100μM) for 45 min. Accumulation of total [^{3}H]-inositol phosphate was measured as outlined (Section 2.5). Each value represents the mean from two separate experiments performed in triplicate.

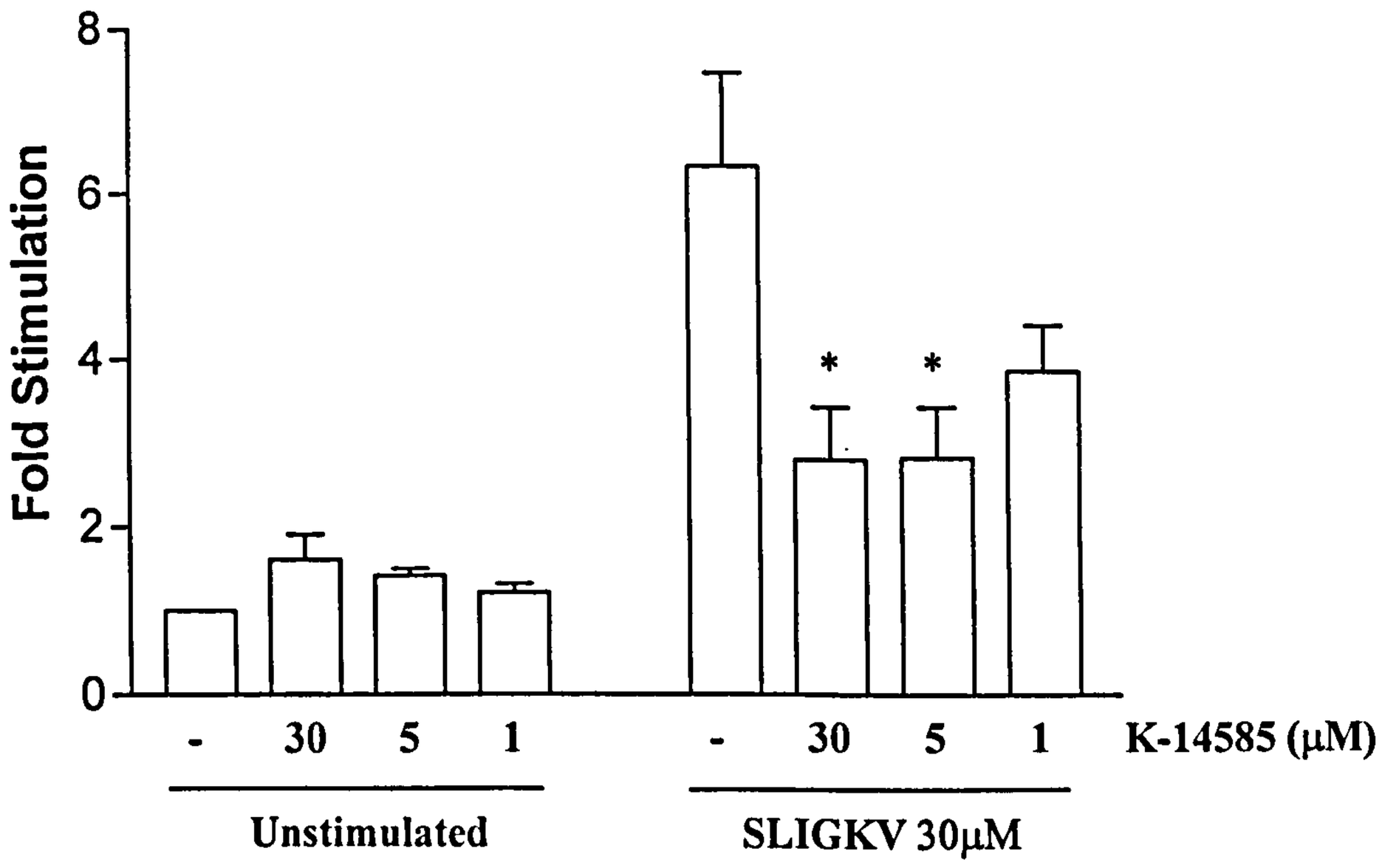


Figure 3.16. The effects of compound K-14585 on SLIGKV-induced [^{3}H]-inositol phosphate accumulation

Cells were prelabelled with [^{3}H]-myo-inositol for 18 hours in serum-free medium and incubated with various concentrations of compound K-14585 for 30 minutes prior to stimulation with 30μM of SLIGKV for 60 min. Accumulation of total [^{3}H]-inositol phosphate was measured as described in Section 2.5. Each value represents the mean ± s.e.m. from three separate experiments performed in triplicate. * $P<0.05$ compared with stimulated control (-).

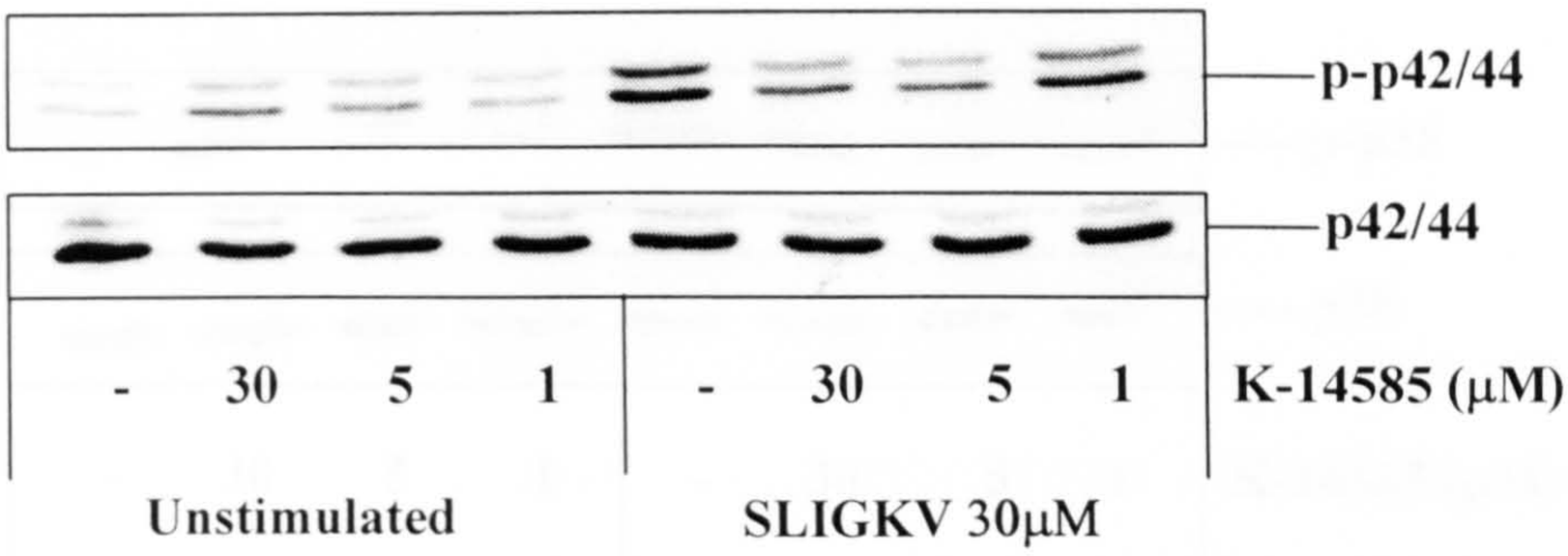
3.3.2.2 The effects of compound K-14585 on PAR-2–mediated p42/44 MAP kinase activation

Figure 3.17 shows the effects of K-14585 on PAR-2-stimulated p42/44 MAP kinase activation. SLIGKV (30μM) increased the phosphorylation of p42/44 MAP kinase to 8.9 ± 0.4 fold of basal control. This response, however, was not significantly altered upon pretreatment of the cells with 30, 5, 1μM of K-14585, with maximal inhibition observed at 30μM (5.9 ± 1.6 fold of basal control, % inhibition = 39.7 ± 18.0). Interestingly, 30μM of K-14585 alone, when added to cells, was able to stimulate the activation of p42/44 MAP kinase to 2.8 ± 1.1 fold of basal control.

3.3.2.3 The effects of compound K-14585 on PAR-2–mediated p38 MAP kinase activation

Figure 3.18 illustrates the effects of K-14585 on SLIGKV–induced p38 MAP kinase activation mediated via PAR-2 stimulation. SLIGKV (30μM) caused an increase in the phosphorylation of p38 MAP kinase. Significant inhibition of the response was observed when cells were preincubated with 5μM of K-14585 (6.3 ± 0.8 to 3.0 ± 0.6 fold of basal control, % inhibition = 63.0 ± 8.5, $P<0.05$). However, at 30 and 1μM, K-14585 only slightly reduced the MAP kinase activity to 4.6 ± 1.1 fold (% inhibition = 32.9 ± 19.0) and 4.6 ± 1.2 fold (% inhibition = 31.3 ± 16.0) respectively. Again, 30μM of the inhibitor alone was observed to enhance p38 MAP kinase activity, with a response of 3.1 ± 1.0 fold of basal control.

a)



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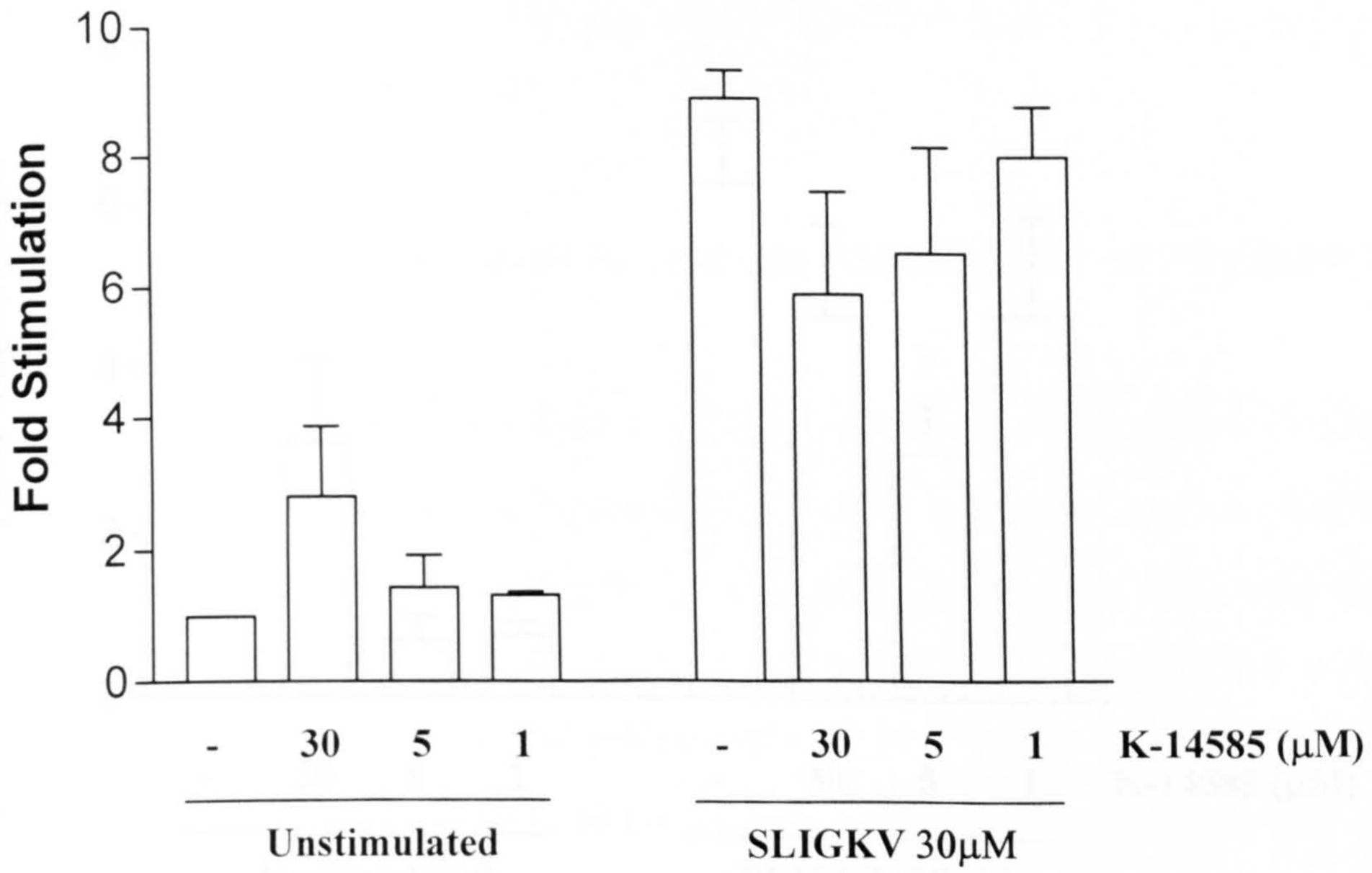
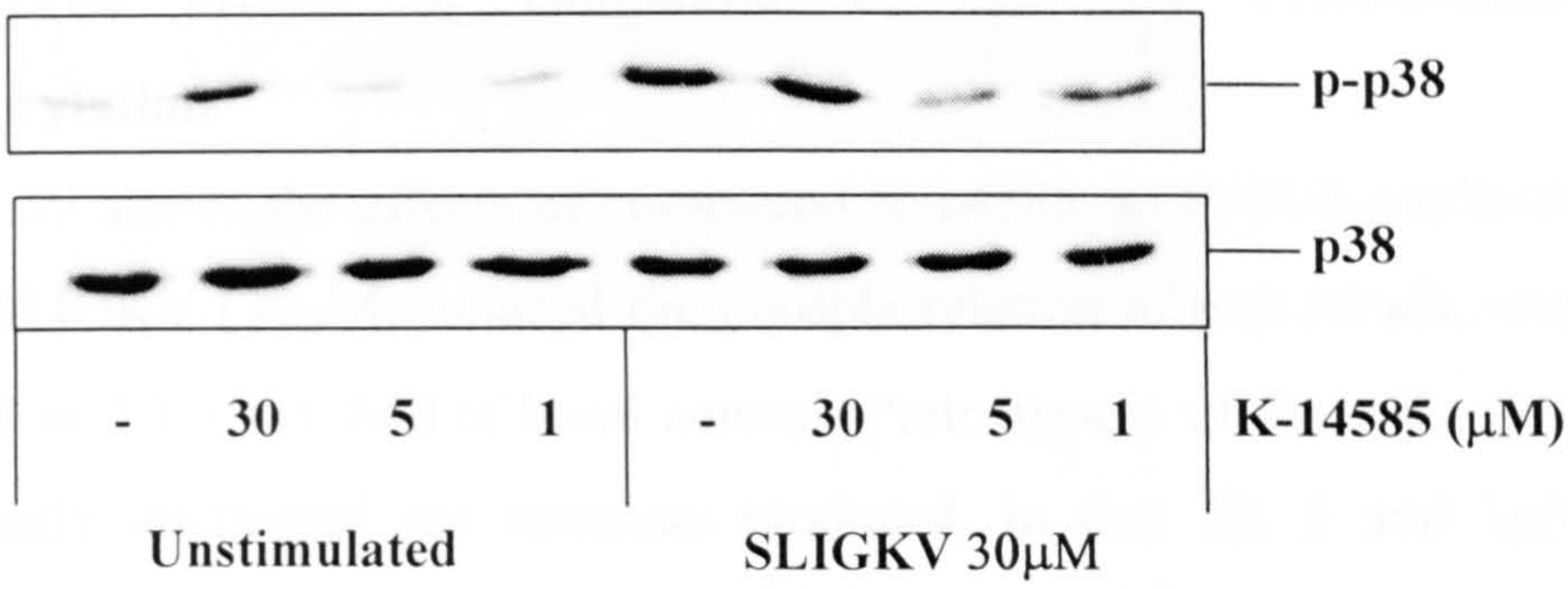


Figure 3.17. The effects of compound K-14585 on SLIGKV-induced p42/44 MAP kinase activation in clone G cells

Cells were grown to confluence and rendered quiescent in serum-free medium for 18 hours and preincubated with increasing concentrations of K-14585 for 30 min prior to stimulation with 30μM SLIGKV for a further 10 min. Whole cell lysates were prepared and processed as described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2).(a) Blots shown are representative of three. (b) Blots were quantified by densitometry and expressed as mean ± s.e.m.

a)



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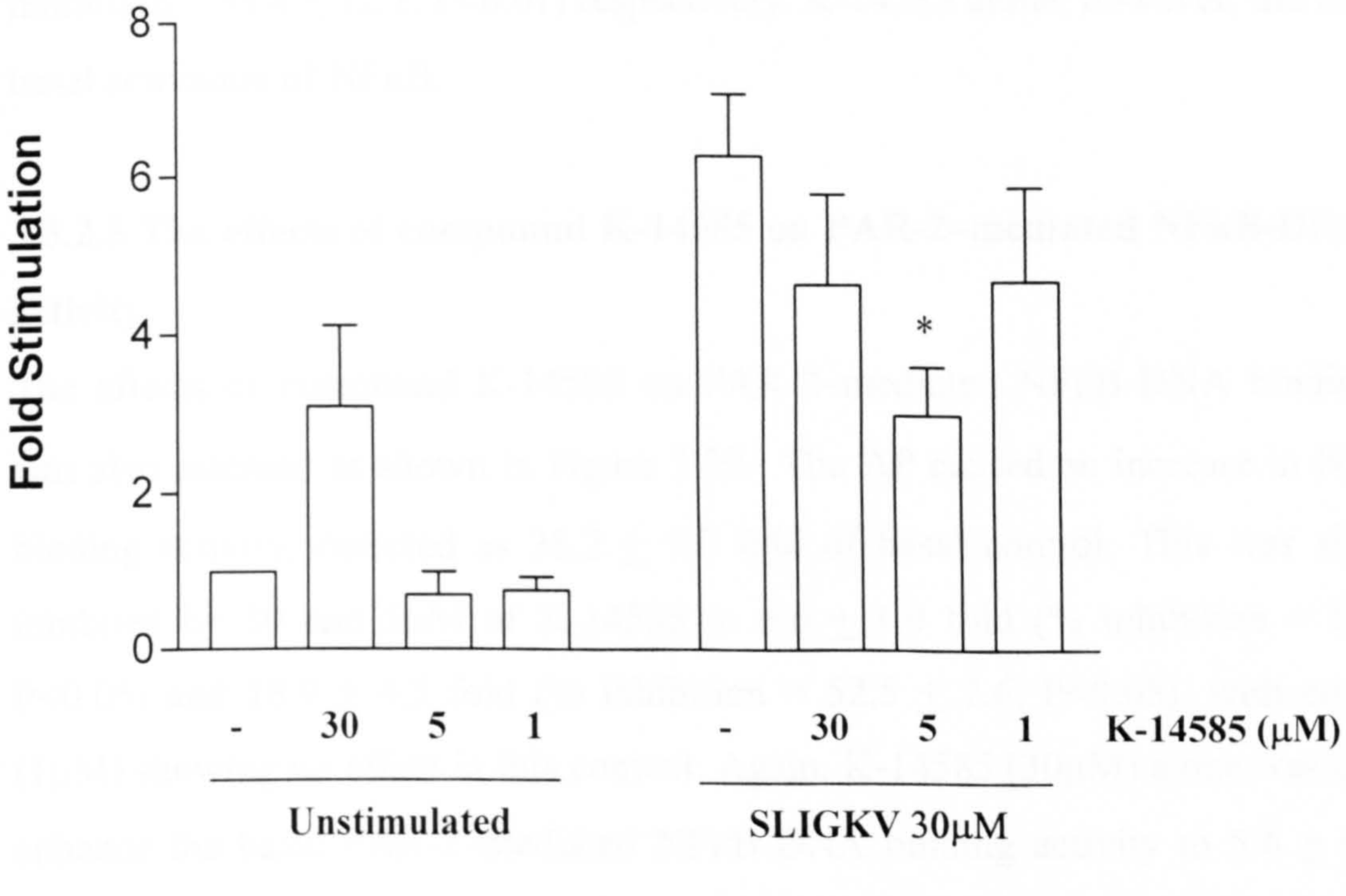


Figure 3.18. The effects of compound K-14585 on SLIGKV-induced p38 MAP kinase activation

Cells were rendered quiescent in serum-free medium for 18 hours and preincubated with increasing concentrations of compound K-14585 for 30 minutes prior to stimulation with 30μM SLIGKV for a further 10 min. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and were resolved by Western blotting (Section 2.6.2). (a) Blots shown are representative of two others. (b) Blots were quantified by densitometry and expressed as mean ± s.e.m. * $P<0.05$ compared with stimulated control (-).

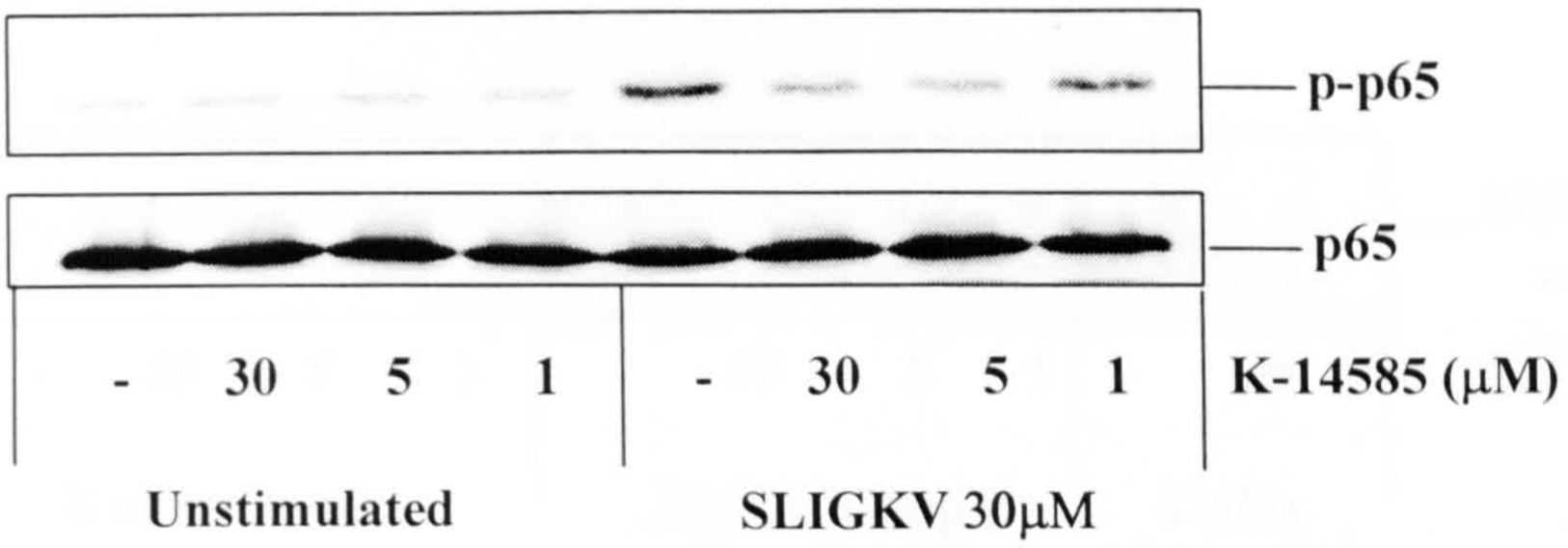
3.3.2.4 The effects of compound K-14585 on PAR-2–mediated NFκB phosphorylation

Figure 3.19 shows the effects of compound K-14585 on PAR-2-mediated activation of NFκB. SLIGKV (30μM) induced the phosphorylation of p65 NFκB, with the response observed as 3.2 ± 0.1 fold of basal control. Pretreatment of the cells with compound K significantly attenuated the response produced, in that 30, 5 and 1μM of K-14585 reduced NFκB activation mediated by PAR-2 to 1.7 ± 0.1 fold (% inhibition = 70.0 ± 2.1, $P<0.01$), 1.3 ± 0.2 fold (% inhibition = 87.5 ± 10.5, $P<0.01$), and 1.9 ± 0.3 fold (% inhibition = 59.4 ± 12.1, $P<0.01$) respectively. K-14585 alone, however, did not alter the basal activation of NFκB.

3.3.2.5 The effects of compound K-14585 on PAR-2–mediated NFκB-DNA binding activity

The effects of compound K-14585 on PAR-2–mediated NFκB-DNA binding activity was also assessed as shown in Figure 3.20. The AP caused an increase in NFκB-DNA binding activity, detected as 38.2 ± 5.5 fold of basal control. This was significantly inhibited by 30 and 5μM of K-14585 to 6.6 ± 1.0 fold (% inhibition = 83.4 ± 5.7, $P<0.05$) and 18.9 ± 4.5 fold (% inhibition = 52.5 ± 7.6, $P<0.05$), with compound K (1μM) showing no effect in this context. Again, K-14585 (30μM) alone was observed to enhance the basal PAR-2–mediated NFκB-DNA binding activity to 5.6 ± 0.8 fold of basal control. TNFα (20ng/ml) also enhanced the binding of NFκB to the nucleus, and this response remained unaffected in the presence of 30μM K-14585.

a)



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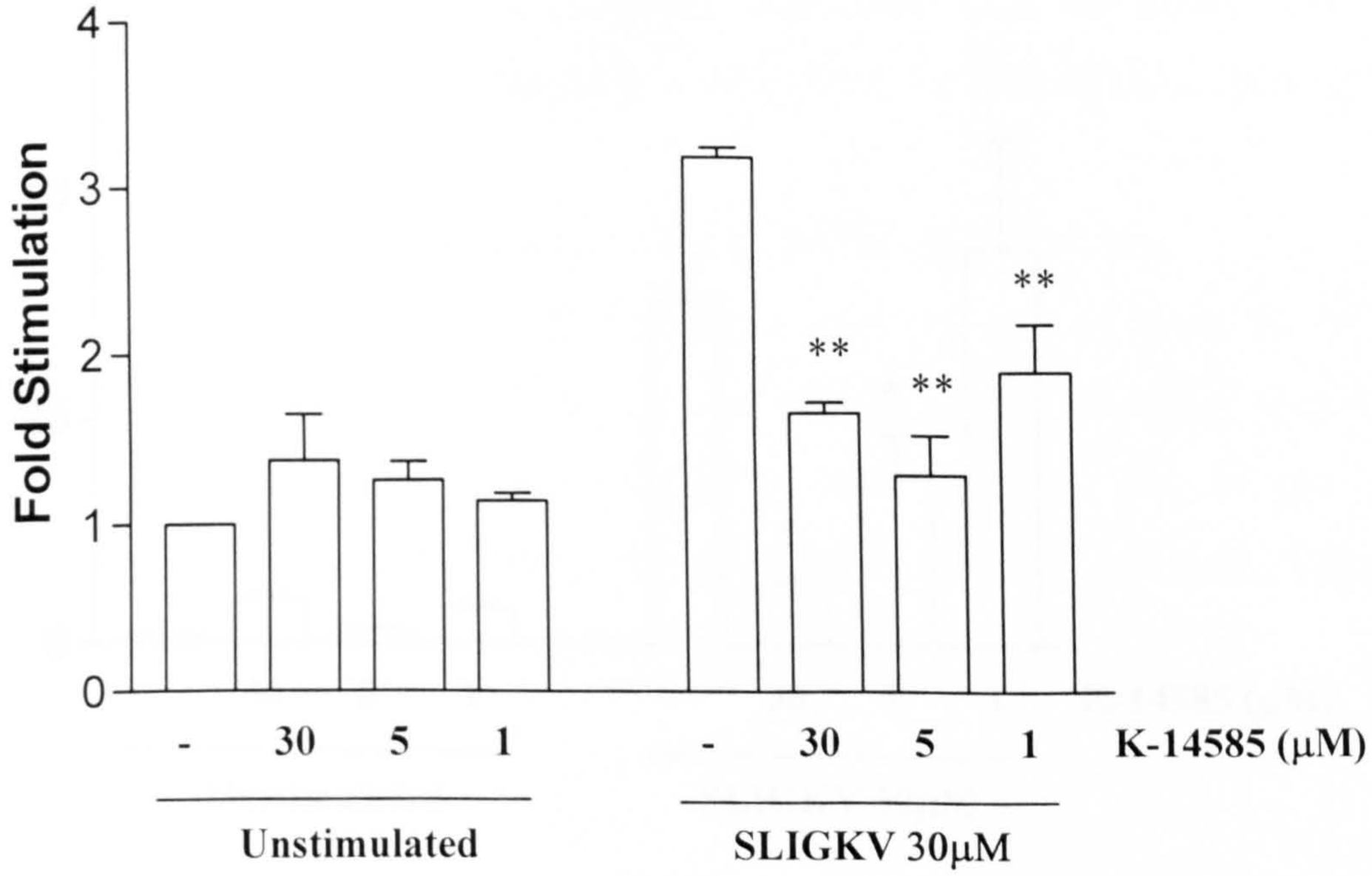
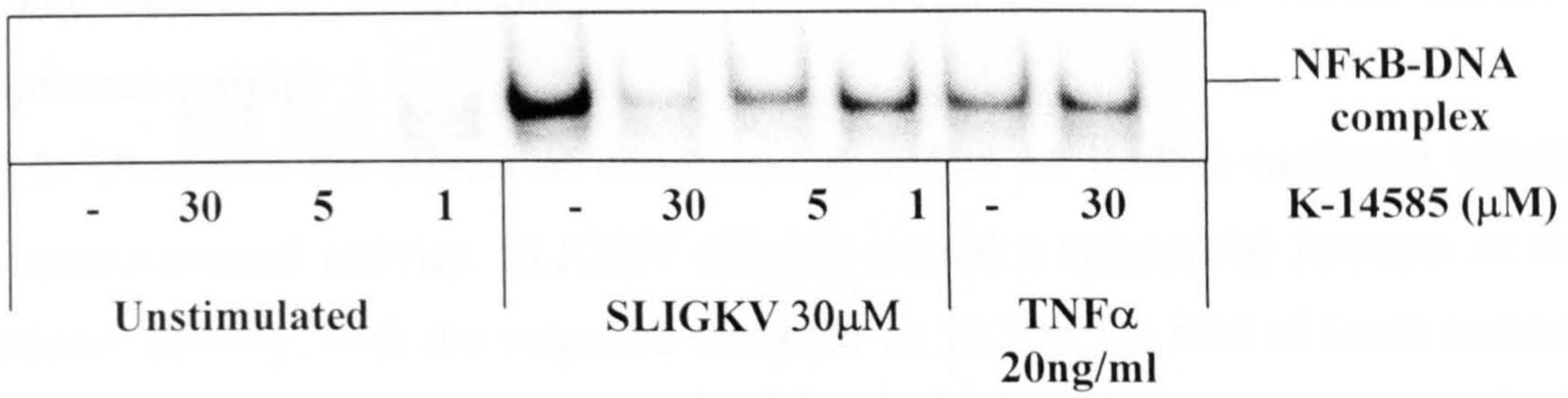


Figure 3.19. The effects of K-14585 on SLIGKV-mediated p65 NFκB phosphorylation in clone G cells

Cells were rendered quiescent in serum-free medium for 18 hours prior to stimulation with 30μM of SLIGKV for a further 30 min following incubation of the cells with various concentrations of K-14585 for 30 min. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2). (a) Blots shown are representative of two others. (b) Blots were quantified by densitometry and expressed as mean ± s.e.m. **P<0.01 compared with stimulated control (-).

a)



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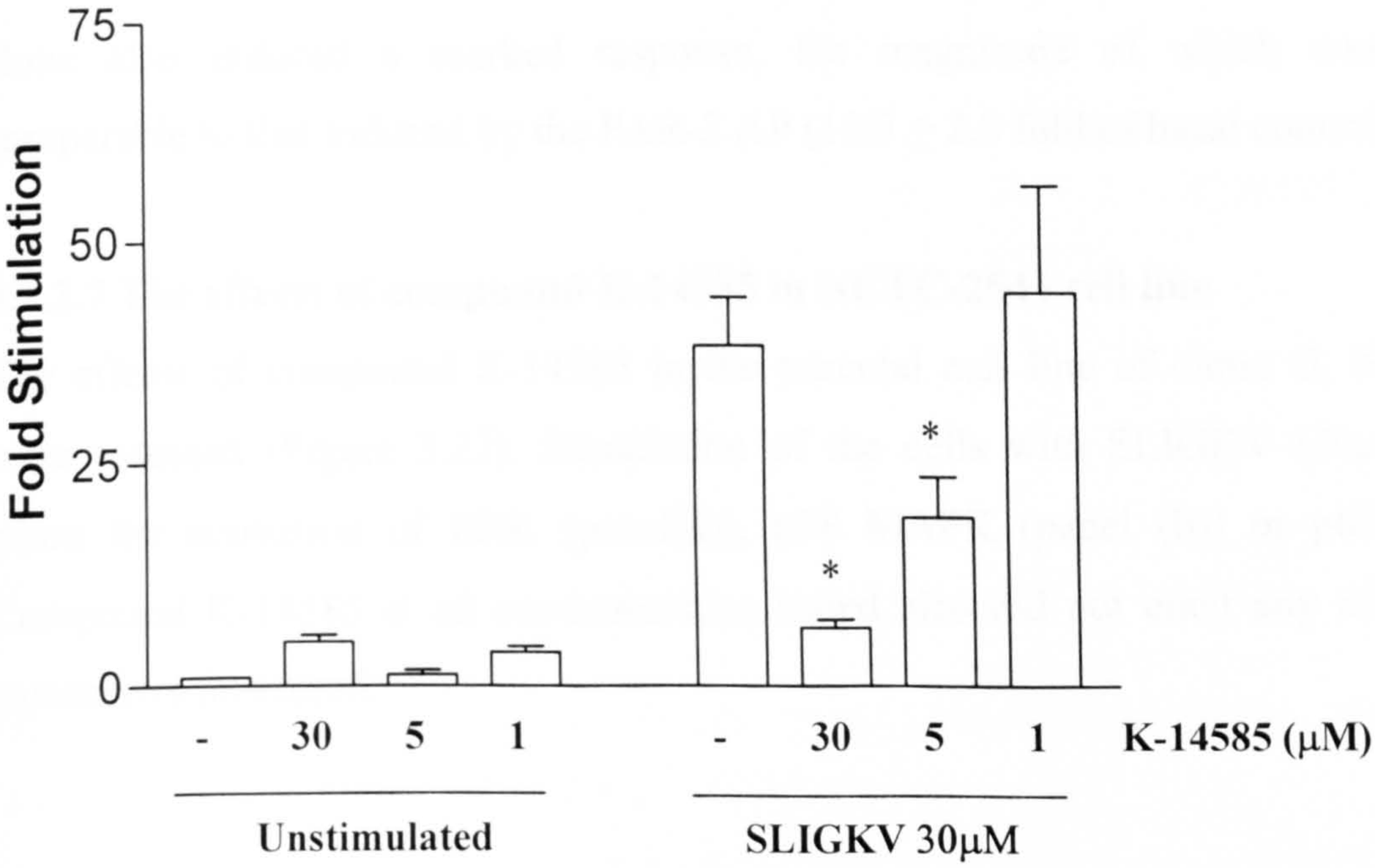


Figure 3.20. The effects of K-14585 on SLIGKV-mediated NFκB-DNA binding activity in clone G cells

Cells were grown to confluence before being rendered quiescent in serum-free medium for 18 hours prior to 30 min incubation with increasing concentrations of K-14585 . Cells were then stimulated with the appropriate agonists for 60 min. Nuclear extracts were prepared and binding activity was measured by EMSA (Section 2.7). (a) The autoradiogram shown is the representative of three. (b) Blots were quantified using densitometry and expressed as mean ± s.e.m. *P<0.05 compared with stimulated control (-).

3.3.2.6 The effects of compound K-14585 on PAR-2–mediated NFκB-induced transcriptional activity

Figure 3.21 illustrates the effects of compound K-14585 on PAR-2–mediated NFκB-induced transcriptional activity. SLIGKV (30μM) caused a substantial increase in the transcriptional activity, with the response observed as 10.7 ± 1.2 fold of basal control. This response was significantly attenuated by 5μM K-14585 to 3.6 ± 1.1 fold (% inhibition = 70.0 ± 14.3, $P<0.05$), with no significant alteration observed following pretreatment of the cells with 30 and 1μM K-14585. Strikingly, compound K (30μM) alone also induced a marked response, the magnitude of which was relatively comparable to that induced by the PAR-2 AP (11.7 ± 2.8 fold of basal control).

3.3.2.7 The effects of compound K-14585 in NCTC-2544 cell line

The effects of compound K-14585 in the parental cell line of clone G, NCTC-2544, were assessed (Figure 3.22). Stimulation of the cells with SLIGKV (30μM) did not cause the activation of ERK (panel(a)), p38 MAPK (panel (b)) or p65 (panel(c)). Compound K-14585 at all concentrations tested also did not elicit any effects on the parameters measured.

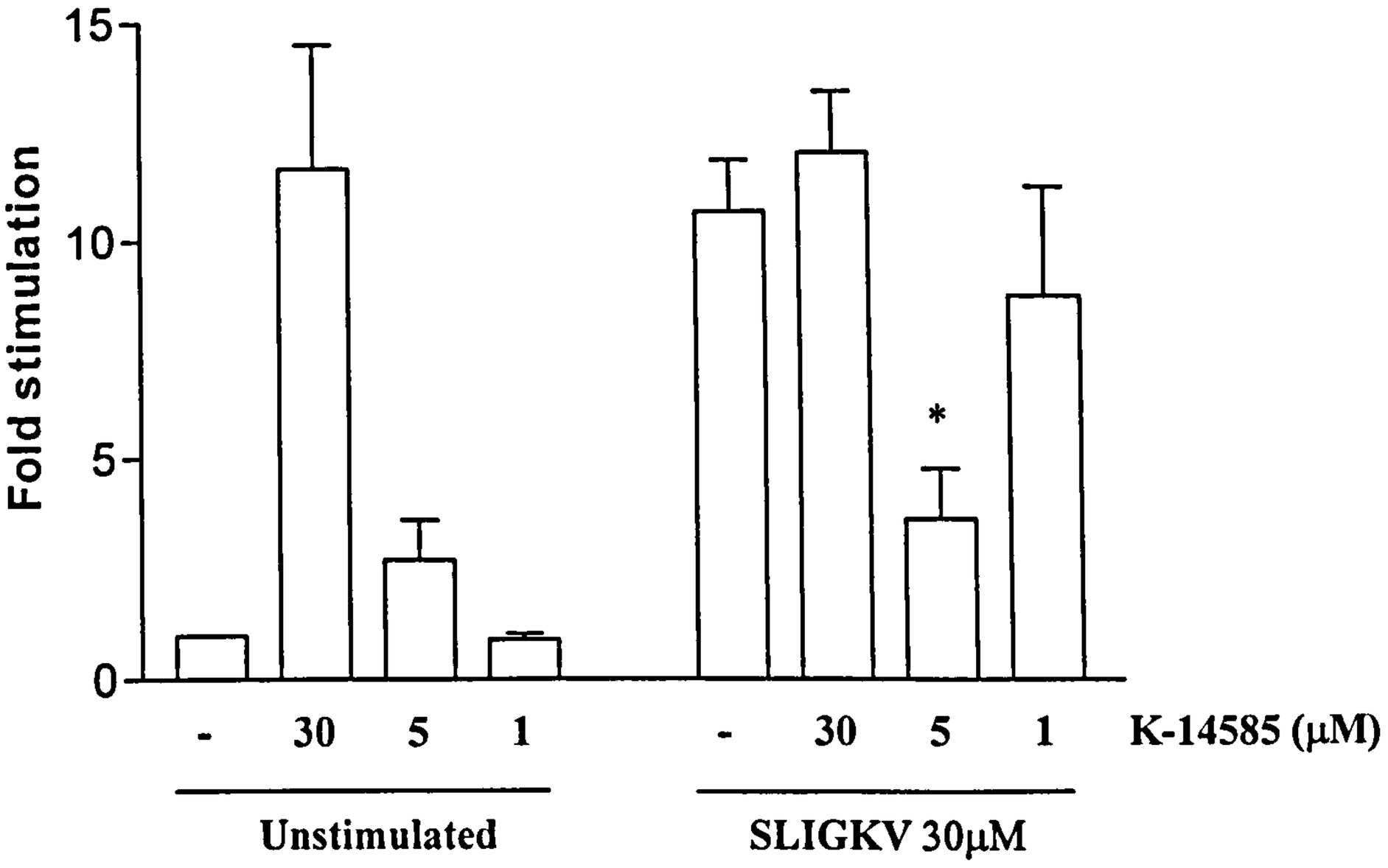


Figure 3.21. The effects of K-14585 on SLIGKV-mediated NFκB-induced transcriptional activation in clone G

Clone G cells having a luciferase gene in the NFκB reporter region were used in this experiment. Cells were grown to confluency and rendered quiescent for 18 hours before being pretreated with various concentrations of K-14585 for 30 min. Cells were then stimulated with 30μM of SLIGKV for 6 hours and luciferase activity was determined as previously described (Section 2.8). Data shown are expressed as fold over unstimulated basal and each value represents the mean ± s.e.m. from three separate experiments. *P<0.05 compared with stimulated control (-).

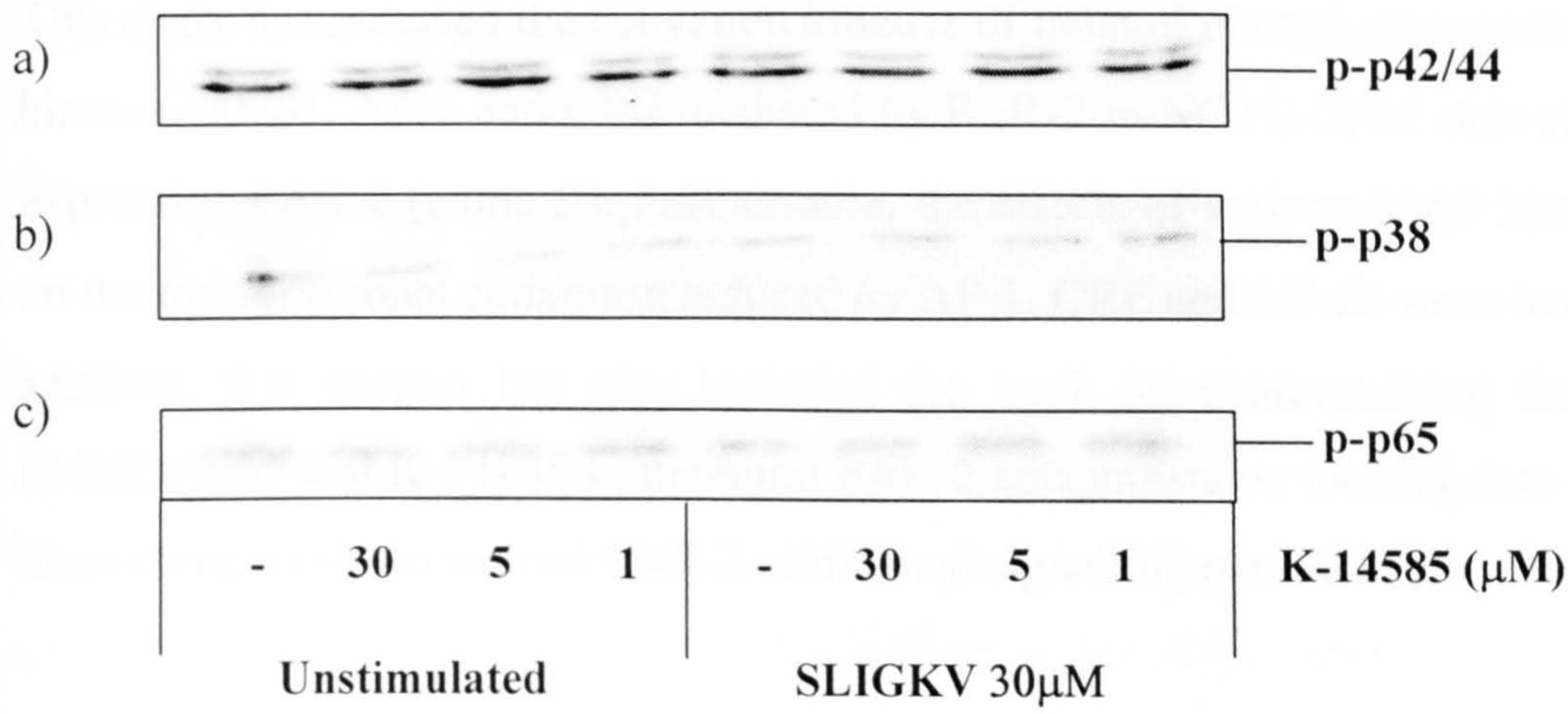


Figure 3.22. The effects of compound K-14585 in NCTC-2544 cells

Cells were grown to confluence before being rendered quiescent with serum-free medium for 18 hours before 30 min incubation with various concentrations of K-14585. Cells were then stimulated with 30μM SLIGKV for 10 min (a & b) or 30 min (c). Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2) using specific antibodies for (a) p-p42/44 MAP kinase, (b) p-p38 MAP kinase and (c) p-p65. Blots shown are the representative of two others.

3.3 Discussion

This study has assessed the activation kinetics of inositol phosphate accumulation, MAP kinases, NFκB, AP-1 and CRE mediated by PAR-2 in NCTC-2544 skin cell line stably expressing PAR-2 (clone G). Furthermore, the effects of various MAP kinase inhibitors on the transcriptional activation induced by AP-1, CRE and NFκB were investigated. In addition, this chapter has also included the work on characterising the compounds ENMD-1068 and K-14585 as potential PAR-2 antagonists by looking into the effects of these compounds on several PAR-2-stimulated signalling parameters in clone G cells.

Measurement of total inositol phosphate accumulation provides an index of GPCR–induced PLC activity. In this project, PAR-2 activation in clone G cells stimulated both a time and concentration-dependent accumulation of inositol phosphate, indicating the activation of PLC activity modulated by this receptor. This is consistent with previous studies in that PAR-2 agonists have been shown to be able to induce labelled inositol phosphate accumulation in several cell lines including KNRK, COS-1 and keratinocytes (Kong *et al.*, 1997; Molino *et al.*, 1997a; Santulli *et al.*, 1995).

Classically, activation of PLC is also associated with an increase in intracellular calcium level as a result of inositol phosphate formation (discussed in Section 1.1). In fact, calcium mobilisation has been detected in multiple cell types such as CHO, KNRK and colonic myocytes following PAR-2 stimulation, suggesting that PAR-2 couples to IP_3/Ca^{2+} axis (Nystedt *et al.*, 1995a; Böhm *et al.*, 1996b; Corvera *et al.*, 1997). Although it was not assessed in this project, NCTC-2544 cells transfected with PAR-2 have been shown to result in calcium mobilisation in response to PAR-2 agonists (Seatter *et al.*, 2004). Thus as with previous studies, these results suggest the coupling of PAR-2 to $G\alpha_{q/11}$ to result in IP_3/Ca^{2+} responses (Nystedt *et al.*, 1994, Santulli *et al.*, 1995, Böhm *et al.*, 1996b) .

The data presented here clearly illustrates the activation of ERK, p38 MAP kinase and JNK by PAR-2. The stimulation of the classical MAP kinase, ERK, through GPCRs

such as PAR-1, β-adrenergic receptor and muscarinic receptors has been well documented (Vouret-Craviari *et al.*, 1993; Maudsley *et al.*, 2000; Mochizuki *et al.*, 1999). PAR-2–mediated phoshorylation of ERK, on the other hand, has been reported in several cell types including rat aortic smooth muscle cells (RASMCs), human umbilical vein endothelial cells (HUVECs), astrocytes, intestinal epithelial cells and human colon cancer cells (Belham *et al.*, 1996; Woolkalis *et al.*, 1996; Ge *et al.*, 2003; Fyfe *et al.*, 2005; Nishibori *et al.*, 2005). In clone G cells, PAR-2 activation induced by trypsin led to rapid and transient activation of ERK. This is consistent with previous findings (Belham *et al.*, 1996; Jin *et al.*, 2005; Kawao *et al.*, 2005; Koo *et al.*, 2002) but not others which have implied either sustained or biphasic kinetics (Ge *et al.*, 2003; Chokki *et al.*, 2005). Similarly, PAR-1-induced phosphorylation of p42/44 MAP kinase has also been shown to exhibit distinct kinetics in various studies (Kahan *et al.*, 1992; Wang *et al.*, 2002). Such phenomenon points to the fact that as with other GPCRs, PAR-1 and PAR-2–induced ERK activation might involve distinct mechanisms in different cell models (Luttrell, 2005). Notably, the phosphorylation of ERK induced by the PAR-2 AP, 2f-LIGKV-OH, exhibited a more sustained profile compared to that of trypsin.

Various studies have identified the roles of ERK in linking PAR-2 activation to several downstream effects. For example, in human airway epithelial cells, PAR-2 is found to mediate prostaglandin E_2 formation, IL-8 secretion and the release of an autocrine factor, amphiregulin; through the p42/44 MAPK pathway (Kawao *et al.*, 2005; Page *et al.*, 2003; Page *et al.*, 2005; Chokki *et al.*, 2005). Also, PAR-2–induced TNFα and pepsinogen secretions are thought to be modulated via this MAPK pathway in human leukemic mast cell line and gastric chief cells respectively (Kang *et al.*, 2003; Fiorucci *et al.*, 2003). In addition, PAR-2–stimulated cell mitogenesis has been well described in several instances including smooth muscle and endothelial cells as well as human lung fibroblasts (Bono *et al.*, 1997; Mirza *et al.*, 1996; Akers *et al.*, 2000). As ERK is a well-known signalling pathway that mediates mitogenic signals induced by GPCRs, it is very likely PAR-2 utilises this axis to exert mitogenic effects.

As with ERK, PAR-2 activation has been shown to result in p38 MAP kinase stimulation in a large number of cells such as smooth muscle cells, epithelial cells, keratinocytes, endothelial cells, pancreatic stellate cells and cultured human pulp cells (Belham *et al.*, 1996; Kawao *et al.*, 2005; Kanke *et al.*, 2001; Ma *et al.*, 2006; Masamune *et al.*, 2005; Tancharoen *et al.*, 2005). The kinetics of p38 MAP kinase phosphorylation stimulated by trypsin in this study is slightly more sustained than that of p42/44 MAP kinase, and is consistent with Kawao *et al.* (2005) and Tancharoen *et al.* (2005) but not other studies which reported only transient activation of this MAP kinase (Masamune *et al.*, 2005; Ma *et al.*, 2006). Again, the PAR-2 AP has been observed to induce a more persistent signal than the enzyme in this context.

The ability of PAR-2 to signal to p38 MAPK might have important implications in mediating cellular stress responses. A recent study has proposed the role of this MAPK along with other signalling components such as NFκB in mediating the release of COX-2 and 6-keto-PGF (1 alpha), a stable product of prostacyclin; in human endothelial cells (Syeda *et al.*, 2006). The roles of p38 MAPK in modulating prostaglandin and neuropeptide release have also been implicated in lung epithelial and human pulp cells (Kawao *et al.*, 2005; Tancharoen *et al.*, 2005). In keratinocytes, p38 MAPK has been identified as the important mediator for ultraviolet and reactive oxygen–induced inflammation and apoptosis (Maytin *et al.*, 2001; Chouinard *et al.*, 2002; Hildesheim *et al.*, 2004). Therefore, although the significance of p38 MAPK activation by PAR-2 in keratinocytes has yet to be elucidated, it is possible that this signalling component might underlie stress-induced cellular responses which could be mediated through PAR-2.

The stress-activated protein kinase, JNK, on the other hand, appeared to be weakly stimulated by PAR-2 in clone G cells compared to the two aforementioned MAP kinases. Studies using smooth muscle cells and culture human pulp cells have provided evidence of PAR-2 coupling to p42/44 and p38 MAPK more efficiently than that of JNK in these models (Belham *et al.*, 1996; Tancharoen *et al.*, 2005). In contrast, by using another methodological approach to assess the kinase activity of this signalling

component, Kanke *et al.* (2001) reported a more substantial magnitude of JNK activation in the same cell line as reported herein. The contradicting results might reflect the difference in the sensitivity of the experimental techniques involved. As with the case of ERK and p38 MAP kinase, stimulation of PAR-2 by 2f-LIGKV-OH resulted in a more sustained JNK phosphorylation compared to that of trypsin.

Unlike p42/44 and p38 MAPK, the significance of JNK activation by PAR-2 remains largely unexplored, except for a recent study which described the role of this MAPK in pancreatic fibrosis (Masamune *et al.*, 2005). The authors concluded that JNK together with p38 MAPK serve as the predominant players in mediating trypsin, tryptase and SLIGRL–induced pancreatic stellate cell proliferation and collagen synthesis. Nevertheless, as JNK activation can lead to stimulation of transcription factors such as AP-1, as well as inhibition of cell growth and apoptosis, the role of PAR-2 in this context is anticipated (Xia *et al.*, 1995).

Thus far this report has shown that in clone G cells, PAR-2 stimulation leads to activation of the MAP kinases. This project has then extended to look at the potential roles of PAR-2 in the stimulation of ERK and SAPK downstream components, namely AP-1 and CRE–driven transcriptional activation. Transcription factors that preferentially bind to AP-1 reporter site are homodimers of Jun (v-Jun, c-Jun, Jun B and Jun D) or heterodimers of Jun and fos (v-Fos, c-Fos, Fos B, Fra-1 and Fra-2) (Karin *et al.*, 1997). Alternatively, heterodimers formed from Jun and ATF family members (ATF-2, ATF-3/LRF-1 and B-ATF) bind to CRE site which has the base sequence of TGACCGTCA (Karin *et al.*, 1997).

The results presented here indicate that PAR-2 couples to both AP-1 and CRE–mediated transcription. The response obtained for AP-1–modulated protein expression appeared to be slightly higher than that of CRE, suggesting that PAR-2 links to AP-1 more efficiently than CRE. The direct PKC activator, PMA, activates both AP-1 and CRE–dependent transcription in clone G cells with much higher magnitude of responses

compared to PAR-2 agonists and TNFα. In contrast, TNFα stimulates minimal activation of AP-1 and CRE–driven protein expression, indicating that, amongst the agonists tested, this cytokine couples least effectively to these pathways in clone G cells. Stimulation of AP-1–induced transcriptional activation by PAR-2 has been reported by other studies (Tancharoen *et al.*, 2005; Luo *et al.*, 2006). Tancharoen *et al.* (2005) reported the role of PAR-2/AP-1 transcription activation in mediating neuropeptide release in dental pulp tissue, whereas Luo *et al.* (2006) provided the novel evidence of the involvement of Jab-1 protein in modulating the PAR-2/AP-1 pathway. PAR-2–mediated CRE–driven transcription activation, however, has yet to be reported.

The roles of MAPK in the signalling axis linking PAR-2 to the AP-1/CRE pathway were then investigated using PD 098059, a MEK/ERK inhibitor (Alessi *et al.*, 1995); SB 203580, a p38 MAPK inhibitor (Cuenda *et al.*, 1995); and SP 600125, a JNK inhibitor (Bennett *et al.*, 2001).

Of all the inhibitors tested, only the JNK inhibitor, SP 600125, significantly inhibited PAR-2–induced AP-1 transcription, whereas both PD 098059 and SB 203580 had no effect. This indicates that both ERK and p38 MAPK are not involved in the axis of PAR-2/AP-1 and JNK might play a partial role in this context. This is because the extent of inhibition imposed by SP 600125 was only approximately 40%, and it is likely the residual response could be modulated by other as yet unidentified signalling components.

These results are in line with other studies which supported the notion that JNK is a well known activator of AP-1 transcription (Schwabe *et al.*, 2003; Zhang *et al.*, 2004). In contrast, the data obtained here is inconsistent with a recent report which described the coupling of PAR-2 to AP-1 through p38 MAP kinase pathway and not JNK (Tancharoen *et al.*, 2005). This implies that PAR-2 might utilise distinct mechanisms to regulate gene expression in different models.

CRE–driven transcriptional activation mediated by PAR-2, on the other hand, was significantly affected by PD 098059 and SB 203580, in which potentiation and inhibition of response were observed respectively. This implies that the ERK pathway may exert a negative regulatory role in PAR-2/CRE axis. This is in concert with the findings in the literature suggesting that CRE appears to be regulated by ERK (reviewed in Shaywitz & Greenberg, 1999). In fact a recent study supported such a notion by providing evidence for ERK-mediated inhibition of CRE–modulated transcription through its action on the ribosomal kinase, $pp90^{RSK}$ in a human hepatoma cell line (Wang *et al.*, 2003). However the exact mechanism underlying PAR-2/ERK/CRE axis awaits further investigation. Both p38 MAPK and JNK have been reported to activate Jun and ATF proteins, thus serve as stimulant for inducing CRE activity (reviewed in Treisman, 1996; Whitmarsh & Davis, 1996). However in this study it appears that p38 MAPK might have a more prominent role than JNK to mediate the signalling of PAR-2 to CRE transcriptional activation, although inhibition of JNK activity did reduce the response to a certain extent but it was not statistically significant.

NFκB represents the major transcription factor in inflammation, however, its activation paradigm is less well characterised in GPCRs compared to cytokine receptors (discussed in Section 1.4.6). This study has clearly demonstrated the activation of NFκB by PAR-2 as exemplified at the levels of p65 phosphorylation, IκBα loss, NFκB-DNA binding and NFκB–induced transcriptional activation. Other studies have reported the activation of NFκB by PAR-2 in several cells lines such as keratinocytes, endothelial cells and smooth muscle cells (Buddenkotte *et al.*, 2005; Macfarlane *et al.*, 2005; Syeda *et al.*, 2006; Shpacovitch *et al.*, 2002; Bretschneider *et al.*, 1999).

The loss in IκBα expression is often used as an initial indicator for the activation of NFκB. The maximum extent of IκBα loss in clone G cells modulated by PAR-2 was far less than that observed for PMA. Perhaps the population of NFκB being activated by

PAR-2 is not as abundant as in the case of PMA, as PMA–induced NFκB-DNA binding activity and transcriptional activation exhibited a higher magnitude than that of PAR-2.

Phosphorylation of NFκB is a mode of post-translational modification that is imposed on this signalling component to secondarily regulate its activity (reviewed in Viatour *et al.*, 2005). That are at least nine inducible phosphorylation sites in p65, most of which have been shown to be important in regulating transcriptional activity. This study focused on the phosphorylation of p65 at Ser-536, which is known to be one of the most important phosphorylation sites of this component (Viatour *et al.*, 2005). The data presented here indicates that in clone G cells, PAR-2 is able to phosphorylate p65 at Ser-536, with similar kinetics observed in both trypsin and 2f-LIGKV-OH–induced responses. Relatively little information has been reported on PAR-2-induced NFκB phosphorylation in other experimental models, until a recent study provided evidence of NFκB phosphorylation induced by PAR-2 in endothelial cells (Syeda *et al.*, 2006).

Interestingly, the results obtained herein indicate that phosphorylation of p65 precedes IκBα loss, suggesting that the phosphorylation process might have taken place while p65 is still physically associated with its inhibitory molecules. Several studies have provided evidence that phosphorylation of p65 in fact occurs while NFκB is still intact with IκBα (Mattioli *et al.*, 2004; Schwabe & Sakurai, 2005); whereas another study proposed the prior degradation of the inhibitory molecule (Wang *et al.*, 2000).

Removal of IκBα enables the translocation of NFκB into the nucleus to bind to DNA. The kinetics of NFκB-DNA binding induced by PAR-2 reported in this study is similar to that reported in primary keratinocytes in that the maximal binding occurs at 1 hour (Buddenkotte *et al.*, 2005). Notably, TNFα when added to clone G cells also stimulated NFκB–driven transcriptional activation, albeit with a much lower magnitude of response. This observation indicates that TNFα might not couple to NFκB pathway as effectively as PMA or PAR-2 does in clone G cells.

The roles of PAR-2–induced NFκB activation have yet to be elucidated except in mediating ICAM-1 and COX-2 secretion in primary keratinocytes and endothelial cells respectively (Buddenkotte *et al.*, 2005; Syeda *et al.*, 2006). Given the fact that NFκB is a major determinant of the switch between proliferation and differentiation of keratinocytes as well as an inducer of various inflammatory mediators, coupling of PAR-2 to this signalling component indicates the possible roles of PAR-2 in this context and more investigation is needed.

PAR-2–stimulated NFκB–driven protein expression was also investigated using the aforementioned MAP kinase inhibitors to examine if there is any potential cross talk between this pathway and the MAPK cascades. Interestingly, the results showed that in the presence of the MEK inhibitor, PD 098059, both trypsin and PAR-2 AP-stimulated responses were significantly potentiated. Again, this implies that ERK pathway might negatively regulate NFκB activation, although this is not consistent with previous literature which supported the positive role of ERK in this context (Dhawan & Richmond, 2002; Jiang *et al.*, 2001; Chen *et al.*, 2004; Chang *et al.*, 2005). In contrast, JNK seems to mediate about 60% of PAR-2/NFκB pathway, suggesting a crosstalk between these two cascades, highlighting an interesting phenomenon thus urging more studies.

The concentrations of trypsin and PAR-2 AP used in this study lies in the range of nanomolar and micromolar respectively, indicating that the enzyme is near 1000 times more potent than the synthetic ligand. The same phenomenon has also been reported in PAR-1 (discussed in Sections 1.2.1.2 and 1.2.2.2). There could be a few explanations for this. One postulation is that a single molecule of trypsin is able to cleave multiple receptors; whereas one molecule of AP is only able to bind and activate one receptor. Also, there might be conformational differences between the endogenous ligand and the synthetic peptide, which could possibly lead to a lower binding affinity of the AP for the receptor compared to trypsin. Another plausible explanation is that trypsin might activate receptor(s) other than PAR-2 in clone G cells. However, this remains unlikely as

stimulation of clone G parental cell line, NCTC-2544, with trypsin did not exhibit any detectable responses (Kanke *et al.*, 2001).

The PAR-2 AP utilised in this project, 2f-LIGKV-OH, is derived from the native human PAR-2 tethered ligand, SLIGKV, and having a furoyl group in place of serine residue (Kawabata *et al.*, 2004). Incorporation of the furoyl group has been proposed to enhance the potency of the AP by preventing aminopeptidase degradation. This peptide has been utilised in various studies to confirm PAR-2–mediated responses (Ferrell *et al.*, 2003; Johansson *et al.*, 2005; Ikawa *et al.*, 2005). Interestingly, on various occasions, the kinetics of signalling components activation were observed to differ in the case of trypsin and 2f-LIGKV-OH–induced responses. For example, the PAR-2 AP induced a more persistent phosphorylation of ERK, p38 MAPK and JNK compared to that of trypsin. In addition, the PAR-2 AP stimulated NFκB-DNA binding appeared to be stronger than that of trypsin at earlier time points. All these phenomenon point to the fact that there might be a difference between endogenous ligand and synthetic peptide-induced PAR-2 activation.

The underlying mechanism for the observed differential responses induced by trypsin and PAR-2 AP, however, has yet to be elucidated. It is possible that the enzyme and the synthetic ligand might interact with the receptor differentially, thus giving rise to distinct kinetics of responses. For example, previous literature has reported that the ligand-binding sites of a tethered ligand and a soluble ligand could be different on PAR-2 (Al-Ani *et al.*, 1999b; Al-Ani *et al.*, 2002a). The same phenomenon has also been described for PAR-1 (Blackhart *et al.*, 2000; McLaughlin *et al.*, 2005). Such discrepancy between the putative tethered ligand and synthetic APs for PARs might also fit into the model of signalling selective-agonism, which is also referred to as 'agonist-specific trafficking of receptor signalling' (Kenakin *et al.*, 1995a & b). The concept of this model is that structural modifications of peptide agonists might result in differential activation of the signalling components mediated by the respective GPCRs. This concept has been demonstrated in several GPCRs, including adrenergic, dopamine, serotonin and

tachykinin receptors (reviewed in Hermans, 2003). For example, Palanche *et al.* (2001) has shown that activation of the tachykinin receptor by its natural agonist resulted in only calcium mobilisation; whereas the synthetic ligand stimulated both calcium mobilisation and cAMP responses. The authors concluded that the receptor can adopt several active states, as opposed to the classical idea of having only either active or inactive state. Distinct receptor conformations would then lead to differential activation of the signalling cascades.

Similarly, such a paradigm has also been demonstrated in the antagonism of certain GPCRs such as cholecytokinin B and tachykinin receptor (Pommier *et al.*, 1999; Sagan *et al.*, 1996). This possibility for PAR-2 can be exemplified by K-14585, which is a novel peptide synthesized based on the PAR-2 tethered ligand sequence as a potential receptor antagonist (see Figure 3.23). The effects of compound K-14585 on PAR-2–mediated inositol phosphate production, MAP kinases activation and NFκB signalling were examined in clone G cells, and the data presented here show that different concentrations of compound K-14585 were required to inhibit different signalling targets. Not only that, K-14585 alone was able to induce the activation of certain signalling mediators within different assays, indicating that it might possess some agonistic activity on PAR-2. Notably, studies using the parental cell line of clone G, NCTC-2544, confirmed that the effects of K-14585 in clone G cells were specific to PAR-2. Thus, it is possible that compound K-14585 at a given concentration might selectively antagonise and/or modulate distinct signalling pathways affected by PAR-2 agonists. Due to limited availability of K-14585, the effects of this compound on trypsin–induced responses in clone G were not pursued. More investigations such as ligand-binding assay and competition studies as well as stability profile of the compound in the solution might provide useful information on the characteristics of K-14585.

Another potential PAR-2 antagonist that has been examined in this project is the compound ENMD-1068. ENMD-1068 is a small molecule of N^1-3-methylbutyryl-N^4-6-aminohexanoyl-piperazine which has been shown to act specifically on PAR-2 to

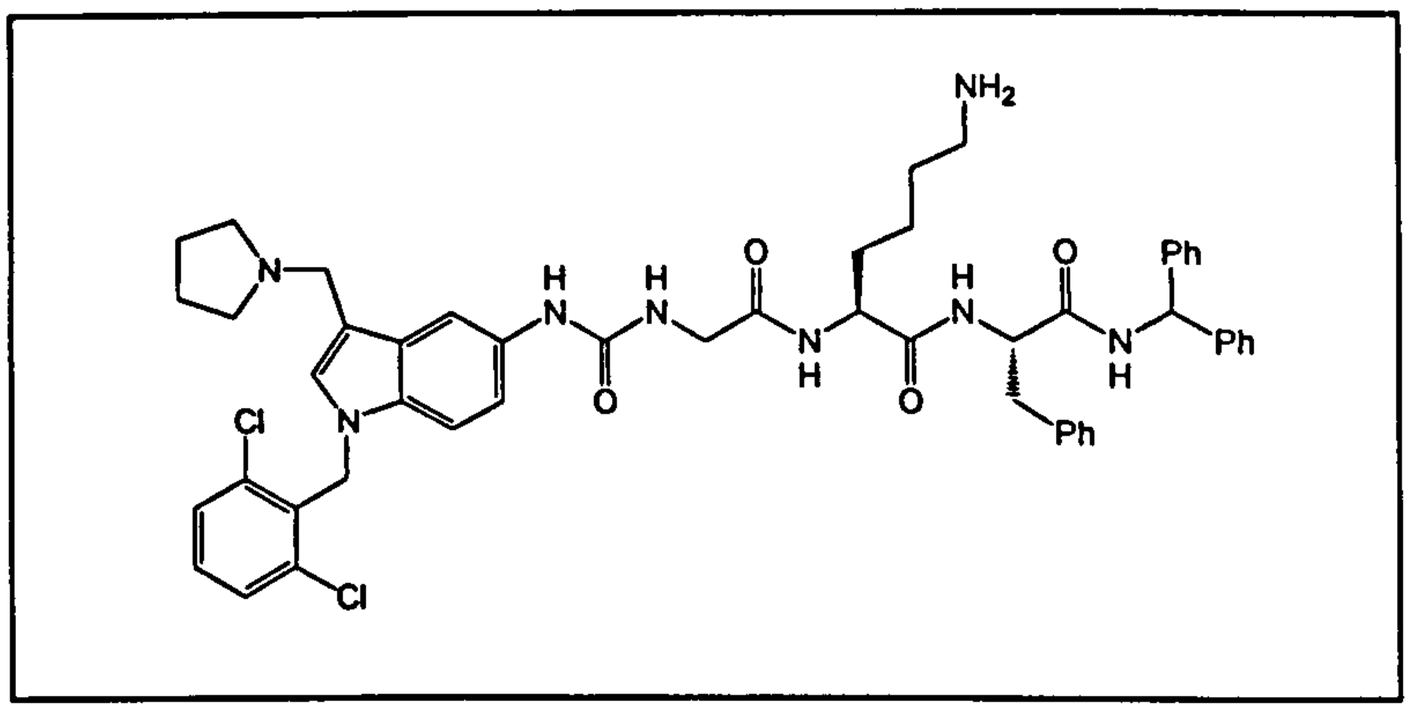


Figure 3.23. The structure of K-14585.

antagonise the pro-arthritic effects of this receptor, thus represents a novel therapeutic agent (Kelso *et al.*, 2006). However, ENMD-1068 at 5mM had no effect on both trypsin and AP–induced responses as reported herein. One possible explanation to account for the observed difference is that the level of PAR-2 expression in clone G cells was much higher than that of cells employed in the reported study and thus 5mM of ENMD-1068 was not sufficient to antagonise PAR-2–induced activity. Thus, the potential role of ENMD-1068 as a PAR-2 antagonist was not justified in this study.

Collectively, the results presented here indicate that PAR-2 is able to couple to IP_3/Ca^{2+}, ERK, p38 MAP kinase, JNK, the distal transcription factors AP-1 and CRE as well as NFκB activation in PAR-2 transfected NCTC-2544 skin cell line. PAR-2 might utilise all these signalling components to exert various cellular effects such as proliferation, differentiation and inflammation in keratinocytes. Nevertheless, the mechanisms of activation and the biological roles of these signalling parameters remain largely undefined, in addition to the discrepancy observed between endogenous ligand and synthetic AP for PAR-2. Thus, more investigation is needed in order to provide a clearer picture in these contexts and it remains to be determined if ENMD-1068 and K-14585 would serve as useful therapeutic agents targeting PAR-2.

CHAPTER 4

THE REGULATION OF PAR-2–MEDIATED NFκB ACTIVATION

4.1 Introduction

Nuclear factor kappa B (NFκB) is a ubiquitously expressed transcription factor and has been implied as the principal mediator of immunity and inflammation (reviewed in Baldwin, 2001). The activation of NFκB involves a series of sequential events and several signalling components which would eventually lead to the translocation of this transcription factor from the cytoplasm into the nucleus to modulate target genes expression (discussed in Section 1.4).

However, the signalling paradigm of NFκB pathway modulated by GPCRs, unlike cytokine receptors, still remains largely undefined. As with other GPCRs such as bradykinin and PAF receptor, PAR-2 has been shown to associate with NFκB activation in various studies (Ye, 2001; Bretschneider *et al.*, 1999; Kanke *et al.*, 2001; Shpacovitch *et al.*, 2002). In skin, PAR-2 is involved in mediating synthesis and release of several inflammatory mediators such as GM-CSF, IL-6 and IL-8 (Wakita *et al.*, 1997; Hou *et al.*, 1998). Such effects exerted by PAR-2 could be mediated via the NFκB pathway.

Nevertheless, little is known regarding the mechanisms involved in linking PAR-2 to the stimulation of NFκB. A good understanding of PAR-2/NFκB axis is vital for identifying potential new therapies. Therefore this study sought to address the mechanisms and the intermediary components involved in PAR-2–stimulated NFκB activation in PAR-2 stable cell line (clone G) which has been previously characterised in Chapter 3. Several approaches including transgene delivery using adenoviral constructs, small interfering RNA (siRNA) technology and pharmacological tools were employed to look at the possible roles of IKKα, IKKβ, PKC, intracellular calcium and $G\alpha_{q/11}$ in this signalling paradigm. In addition, the roles of these signalling components in PAR-2/NFκB were also confirmed using normal human epidermal keratinocytes (NHEK), the primary cells that endogenously express PAR-2.

4.2 The Roles of IKKα and IKKβ

The ability of PAR-2 agonists to induce cellular degradation of IκBα indicates that PAR-2 may utilise the IKK complex as an upstream mediator of the NFκB pathway (see Chapter 3, discussed in Section 1.4). In order to investigate the roles of IKKα and IKKβ which serve as catalytic subunits in the regulation of PAR-2/NFκB activation, adenoviral constructs encoding dominant negative IKKα (Adv. IKKα$^{+/-}$) or IKKβ$^{+/-}$ were employed as experimental tools in this study. These dominant negative constructs have an alanine residue in place of Lysine-44 of the kinase domain thus rendering them catalytically inactive (Regnier *et al.*, 1997; Oitzinger *et al.*, 2001). The effects of these adenoviral constructs in clone G cells and NHEK on PAR-2–mediated NFκB activation were then assessed at the levels of IκBα expression, phosphorylation of p65 NFκB, NFκB-DNA binding and transcriptional activation.

4.2.1 The effects of Adv. IKKα$^{+/-}$ and IKKβ$^{+/-}$ on cellular degradation of IκBα in clone G cells

Figure 4.1 shows the effects of Adv. IKKα$^{+/-}$ and Adv. IKKβ$^{+/-}$ on PAR-2–induced IκBα loss in clone G cells. Infection of cells with 300 pfu/cell of either construct for 48 hours had no effect on the basal expression of IκBα. When the cells were exposed to 50nM of trypsin for 30 min, a substantial loss of IκBα expression was detected (% inhibition = 52.0 $\pm$ 1.3). The extent of IκBα degradation, however, was not altered in the presence of Adv. IKKα$^{+/-}$ at this time point. In contrast, infection of cells with Adv. IKKβ$^{+/-}$ significantly reversed the degradation of IκBα induced by trypsin to a level which was comparable to that of unstimulated control ($P<0.05$ compared to agonist-stimulated control). In this experiment, TNFα which was used as a positive control, also induced significant IκBα loss at this time point; the extent of degradation being much greater than that observed for trypsin (% degradation = 87.2 $\pm$ 5.4). Again, Adv. IKKα$^{+/-}$ appeared to have no impact on the response produced. On the contrary, Adv. IKKβ$^{+/-}$

significantly restored cellular degradation of IκBα induced by TNFα ($P<0.05$ compared to agonist- stimulated control).

4.2.2 The effects of Adv. IKKα$^{+/-}$ and IKKβ$^{+/-}$ on phosphorylation of p65 in clone G cells

The effects of the dominant negative IKK proteins were then investigated at the level of p65 NFκB phosphorylation in clone G cells, as shown in Figure 4.2. Again, infection of the cells with the adenoviral constructs did not change the basal level of p65 phosphorylation. Upon stimulation by trypsin (50nM) for 30 min, a response of 5.8 ± 0.3 fold of basal control was detected. This response, however, was not modified with the infection of cells with Adv. IKKα$^{+/-}$. In contrast, Adv. IKKβ$^{+/-}$ significantly inhibited trypsin–stimulated p65 phosphorylation, a residual response of 1.7 ± 0.4 fold of basal control was recorded (% inhibition = 76.5 ± 10.7, $P<0.05$). TNFα also induced the phosphorylation of p65 (5.0 ± 0.2 fold of basal control). Similarly, this response was not altered by Adv. IKKα$^{+/-}$ but Adv. IKKβ$^{+/-}$ significantly reduced the magnitude of p65 phosphorylation to 1.7 ± 0.5 fold of basal control (% inhibition = 74.7 ± 13.3, $P<0.05$).

4.2.3 The effects of Adv. IKKα$^{+/-}$ and IKKβ$^{+/-}$ on NFκB-DNA binding activity in clone G cells

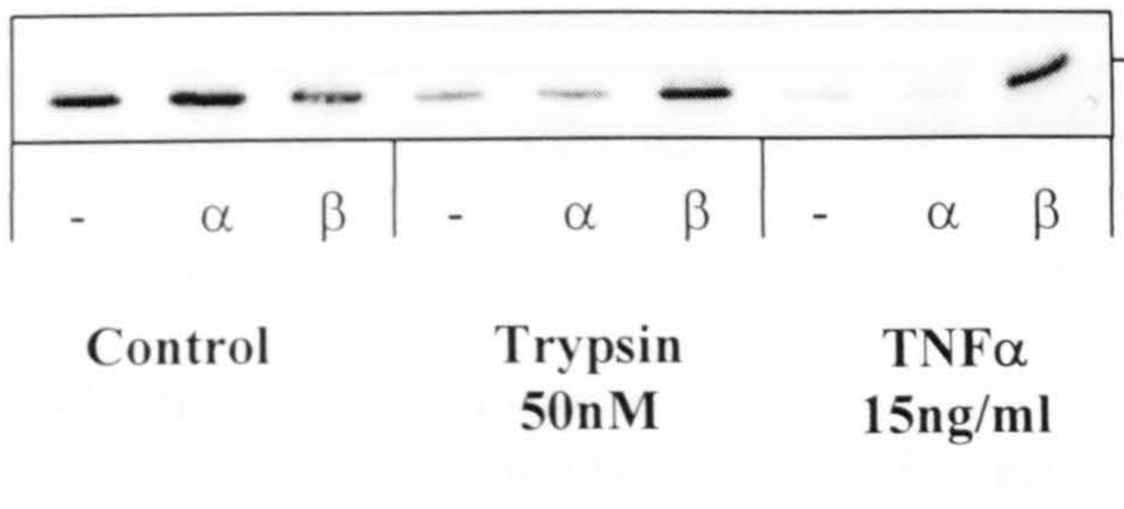
As Adv. IKKβ$^{+/-}$ has been shown to inhibit PAR-2–induced IκBα loss and p65 phosphorylation, this adenoviral construct was then employed to look at the role of IKKβ on NFκB-DNA binding activity. Figure 4.3 shows the effects of Adv. IKKβ$^{+/-}$ on NFκB-DNA binding induced by trypsin and TNFα. Infection of clone G cells with the adenoviral construct did not alter the basal activity of this signalling parameter. Trypsin (50nM) stimulated an increase in NFκB-DNA binding at 60 min (4.8 ± 0.2 fold of basal control). This activity was significantly reduced by Adv. IKKβ$^{+/-}$ to 1.3 ± 0.3 fold of basal control, giving an inhibition of $91.9 \pm 8.9\%$ ($P<0.05$). A similar inhibition profile was also observed when the cells were treated with 15ng/ml of TNFα. A response of 6.2 $\pm$ 0.9 fold of basal control was detected with TNFα stimulation, the magnitude of which

was significantly inhibited in the presence of Adv. IKKβ$^{+/-}$ to 1.5 ± 0.4 fold of basal control (% inhibition = 91.8 ± 8.0, P<0.05).

4.2.4 The effects of Adv. I KKα$^{+/-}$ and IKKβ$^{+/-}$ on NFκB-driven transcriptional activation in clone G cells

The adenoviral dominant negative IKK constructs were then used to investigate a possible role for the kinases on NFκB–induced luciferase expression via PAR-2 stimulation as illustrated in Figure 4.4. Post-infected clone G cells were treated with trypsin, 2f-LIGKV-OH or TNFα for up to 6 hours. Trypsin (50nM) induced a response of 17.1 ± 2.7 fold of basal control which remained unaffected in the presence of Adv. IKKα$^{+/-}$. In contrast, Adv. IKKβ$^{+/-}$ significantly reduced this activity to only 3.1 ± 0.7 fold of basal control (% inhibition = 86.7 ± 4.7, P<0.05). A similar profile was also obtained in the case of 2f-LIGKV-OH (30μM), in that the magnitude of response stimulated by this PAR-2 AP was significantly inhibited by Adv. IKKβ$^{+/-}$ (12.5 ± 2.5 to 2.4 ± 0.5 fold of basal control, % inhibition = 88.0 ± 2.7, P <0.05) but not affected by Adv. IKKα$^{+/-}$. TNFα (15ng/ml) also induced NFκB–driven transcriptional activation in clone G cells (10.0 ± 2.1 fold of basal control). Similarly, Adv. IKKβ$^{+/-}$ but not Adv. IKKα$^{+/-}$ appeared to significantly suppress this response to 1.2 ± 0.2 fold of basal control (% inhibition = 98.9 ± 2.2, P<0.05).

a)



b)

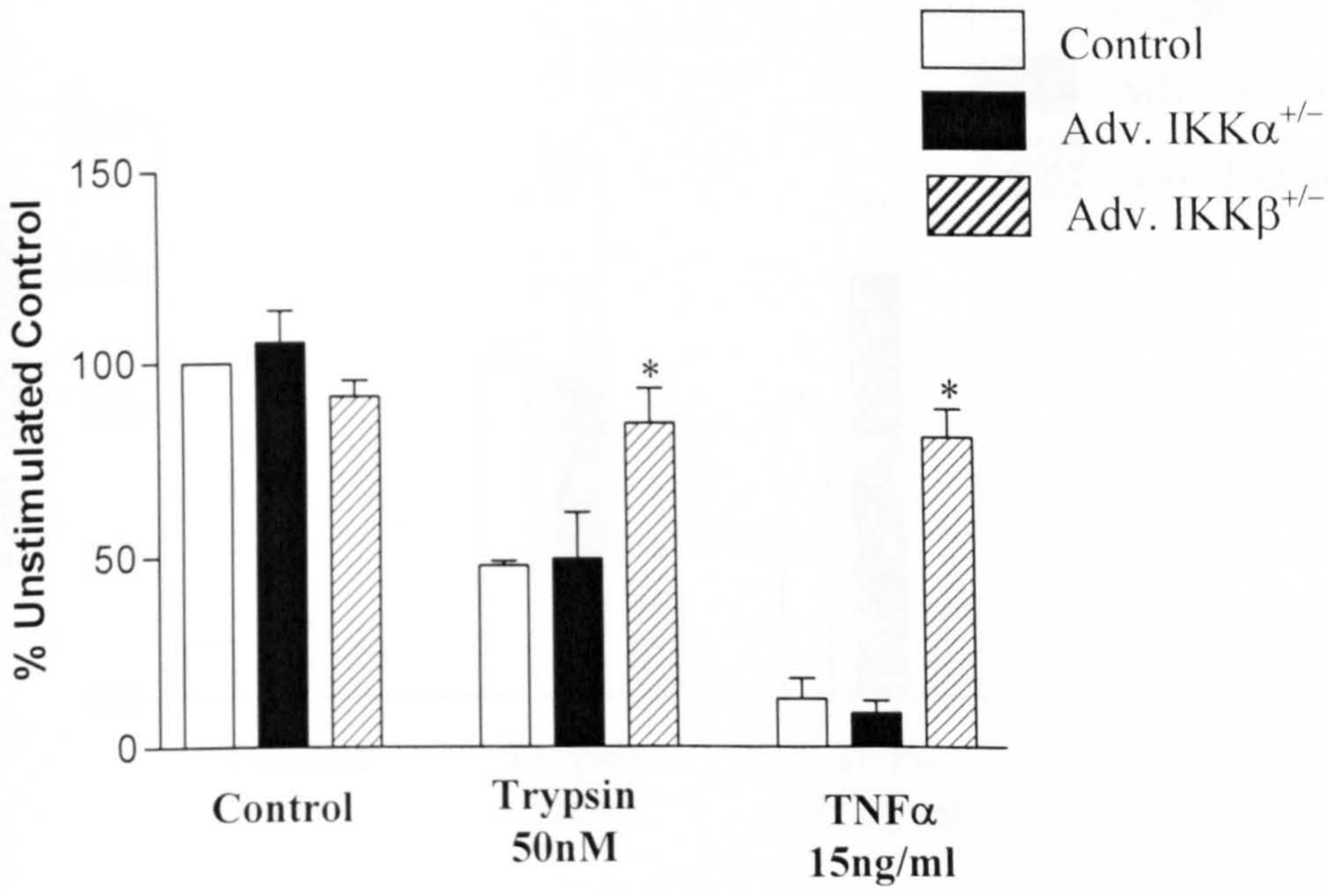
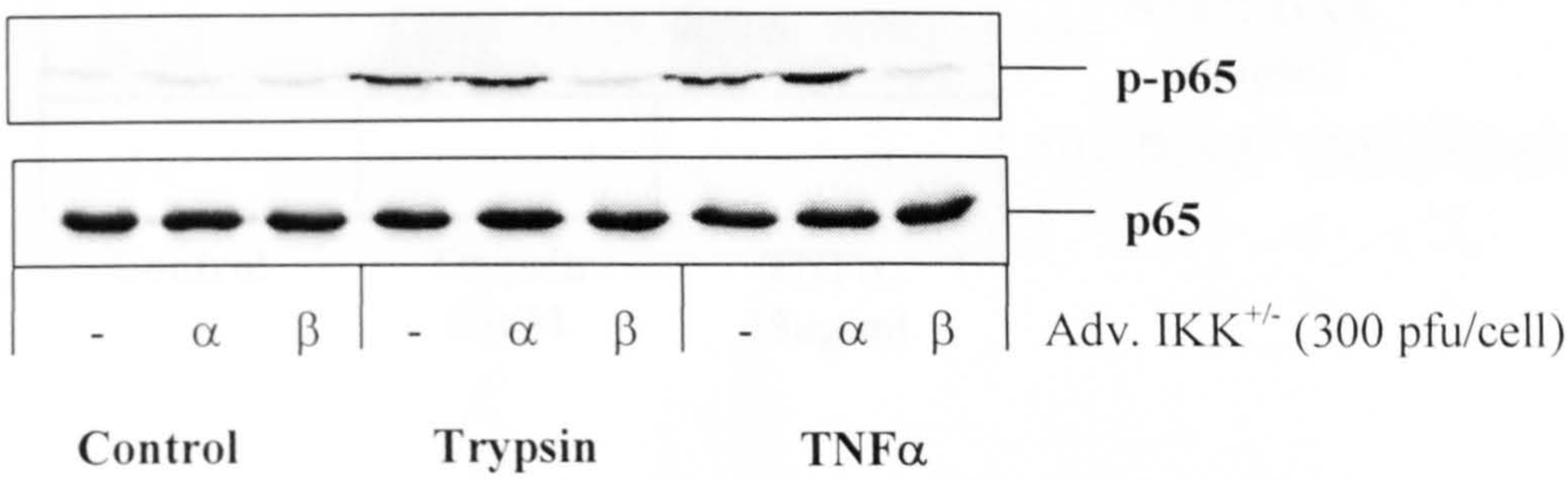


Figure 4.1. The effects of Adv. IKK$\alpha^{+/-}$ and IKK$\beta^{+/-}$ on trypsin and TNFα–stimulated IκBα loss in clone G cells

Cells were infected with the respective adenoviral constructs for 48 hours and rendered quiescent for 18 hours prior to stimulation with 50nM of trypsin or 15ng/ml of TNFα for 30 min. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and were resolved by Western blotting (Section 2.6.2). (a) The blot shown is the representative of two others. (b) Blots were quantified by densitometry and expressed as mean ± s.e.m. (% unstimulated control). *$P<0.05$ compared with agonist-stimulated control.

a)



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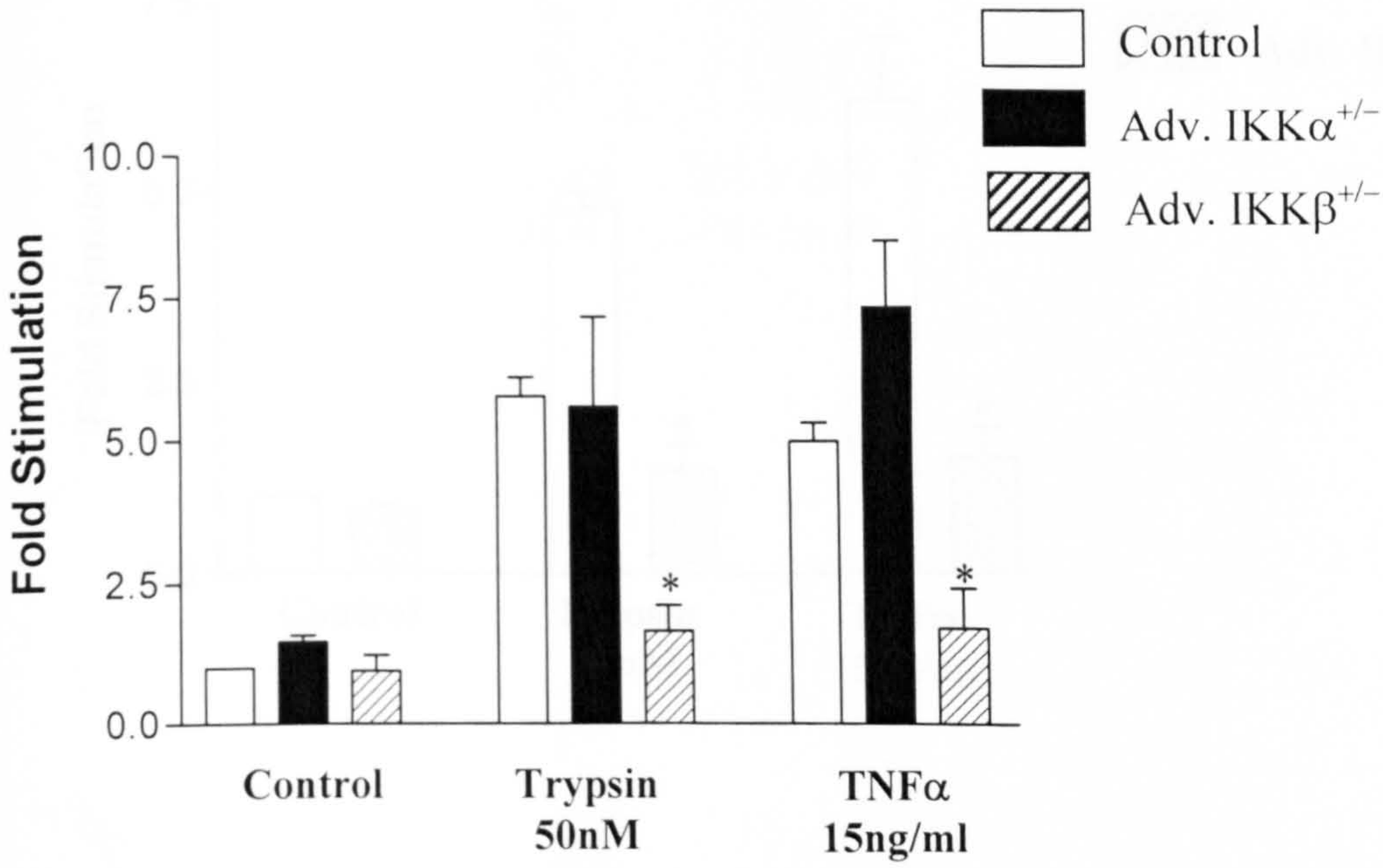
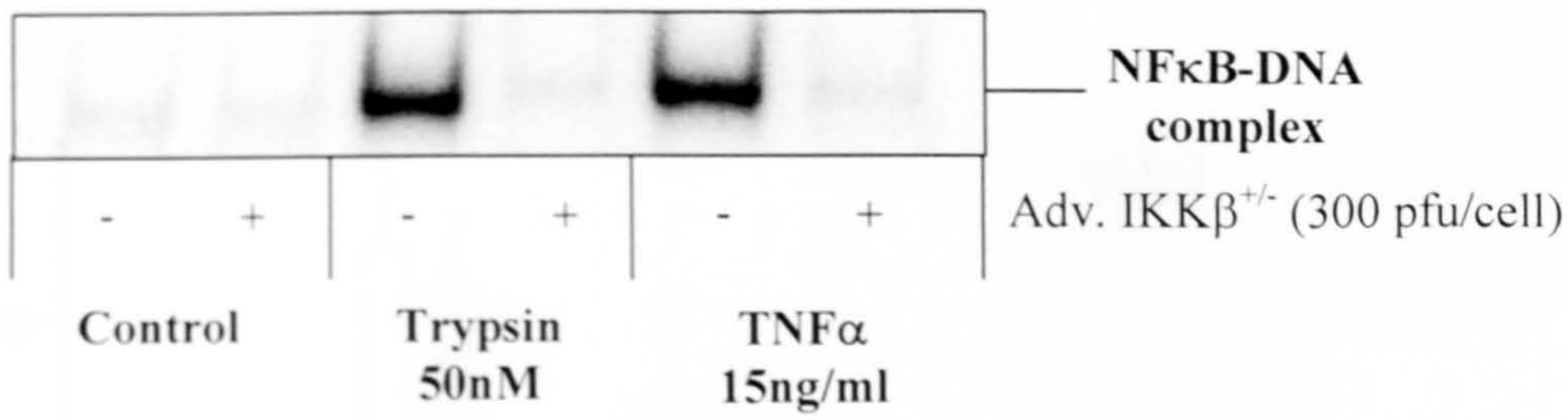


Figure 4.2. The effects of Adv. IKK$\alpha^{+/-}$ and IKK$\beta^{+/-}$ on trypsin and TNFα–stimulated phosphorylation of p65 in clone G cells

Cells were infected with the respective adenoviral constructs for 48 hours and rendered quiescent for 18 hours prior to stimulation with trypsin (50nM) or TNFα (15ng/ml) for 30 min. Whole cell lysates were prepared and processed as previously outlined (Section 2.6.1), and were resolved by Western blotting (Section 2.6.2). (a) The blot shown is the representative of three, equal protein loading was ensured by probing the membrane against total p65 (lower panel) in parallel to p-p65 (top panel). (b) Blots were quantified by densitometry and expressed as mean $\pm$ s.e.m. (fold stimulation). *$P<0.05$ compared with agonist-stimulated control.

a)



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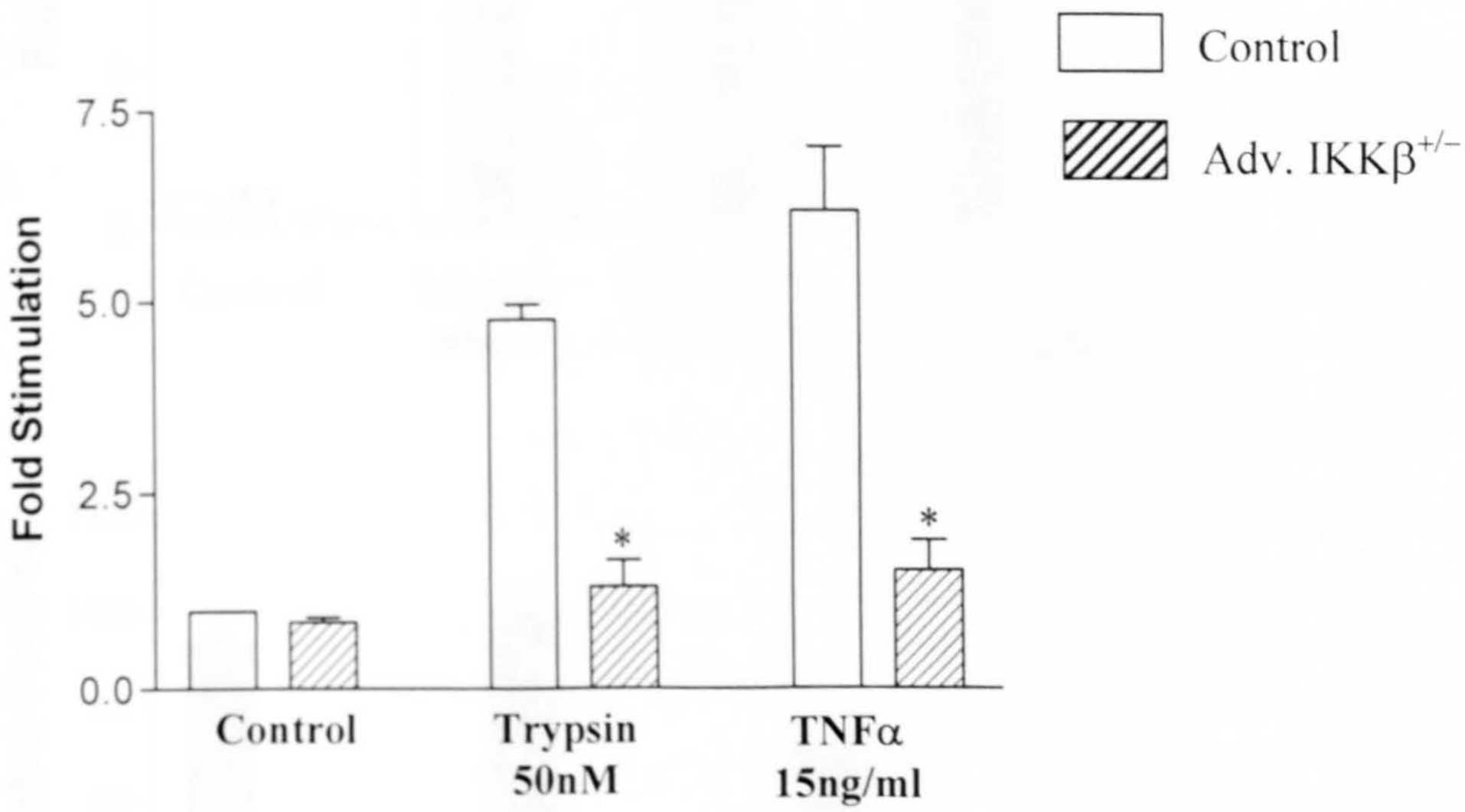


Figure 4.3. The effects of Adv. IKKβ$^{+/-}$ on trypsin and TNFα–stimulated NFκB-DNA binding in clone G cells.

Cells were infected with the adenoviral construct encoding Adv. IKKβ$^{+/-}$ for 48 hours and rendered quiescent for 18 hours. Cells were then exposed to either trypsin (50nM) or TNFα (15ng/ml) for 60 min. Crude nuclear lysates were prepared and processed as previously outlined (Sections 2.7.1 and 2.7.2), and the binding was measured by EMSA (Section 2.7.4). (a) The autoradiogram shown is the representative of three. (b) Autoradiograms were quantified by densitometry and expressed as mean ± s.e.m. (fold stimulation). *P<0.05 compared with agonist-stimulated control.

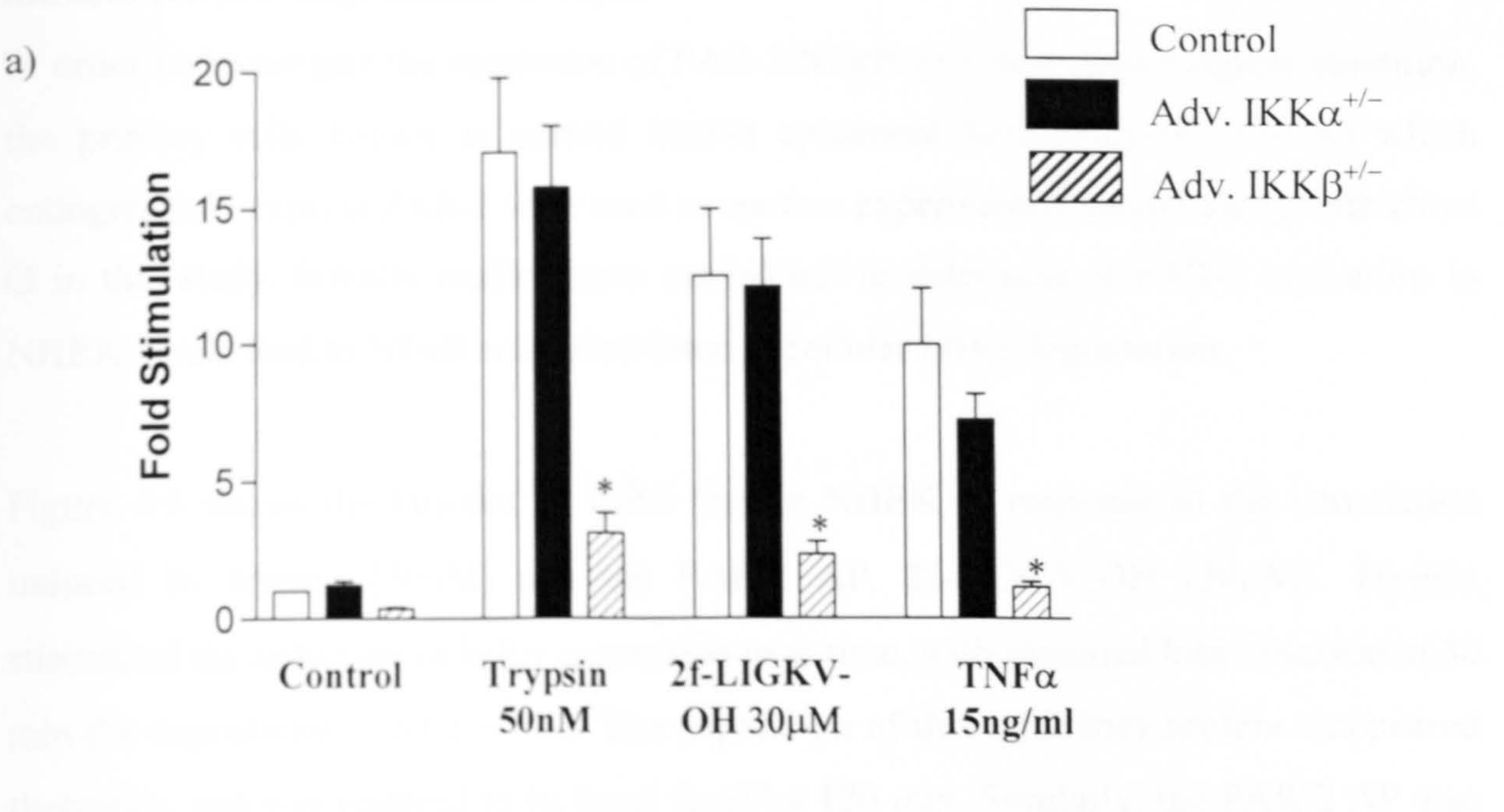


Figure 4.4. The effects of Adv. IKKα$^{+/-}$ and IKKβ$^{+/-}$ on PAR-2 and TNFα–induced NFκB-driven transcription in clone G cells

Cells were infected with the respective adenoviral constructs for 48 hours and rendered quiescent for 18 hours prior to stimulation with trypsin (50nM), 2f-LIGKV-OH (30μM) or TNFα (15ng/ml) for 6 hours. Transcriptional activation was measured as previously described (Section 2.8). Data shown are expressed as mean ± s.e.m. (a) fold stimulation and (b) % stimulated control. *P<0.05 compared with agonist-stimulated control.

4.2.5 PAR-2 activation in normal human epidermal keratinocytes (NHEK) induces cellular degradation of IκBα

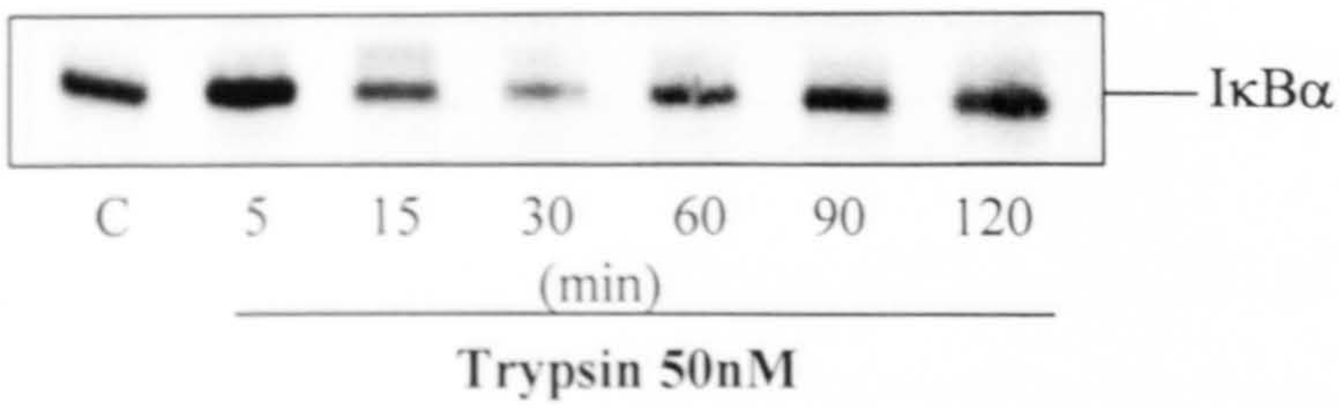
In order to investigate the regulation of PAR-2/NFκB axis in a physiological condition, the primary cells known as normal human epidermal keratinocytes (NHEK) which endogenously express PAR-2 were used as another experimental model along with clone G in this study. Initially studies were carried out to determine if PAR-2 activation in NHEK would lead to NFκB activation through cellular IκBα degradation,

Figure 4.5 shows the kinetics of IκBα loss in NHEK in response to the stimulation induced by trypsin (50nM) and the PAR-2 AP, 2f-LIGKV-OH (30μM). Trypsin stimulated the reduction in IκBα expression over time, with maximal loss observed at 30 min (% degradation = 53.2 ± 0.5). The expression of this inhibitory protein reappeared thereafter, and was restored to its basal level by 120 min. Similarly, the PAR-2 AP also induced a similar pattern of IκBα loss, albeit with a slightly different profile. 2f-LIGKV-OH induced a time-dependent decrease in IκBα expression, which was detected to be the greatest at 60 min, a delayed time point compared to that of trypsin with % degradation = 69.7± 7.8. Again, the degradation of the protein was restored to a level which was comparable to a basal value by 120 min.

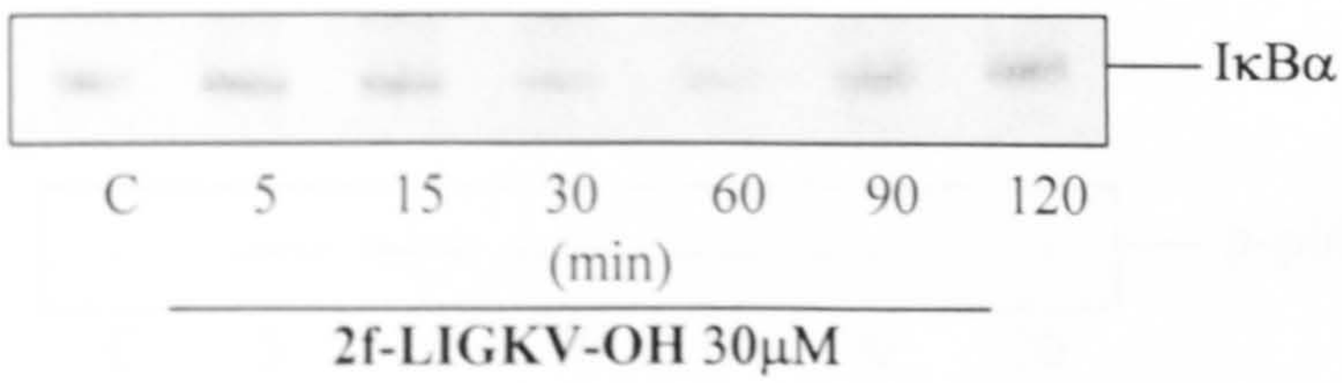
4.2.6 PAR-2 activation in NHEK leads to phosphorylation of p65 NFκB

Another level of NFκB activation assessed in NHEK in response to PAR-2 agonists was phosphorylation of p65 NFκB on Ser-536 as illustrated in Figure 4.6. Trypsin (50nM) stimulated a response which was evident and maximal at 15-30 min (5-6 fold of basal control). This response slowly degraded thereafter and remained slightly above basal by 120 min. Again, 2f-LIGKV-OH was used to confirm the response of PAR-2 activation in this experiment. Cells treated with the AP at 30μM also produced the response of p65 NFκB phosphorylation with kinetics similar to that of trypsin; peaking at 15-30 min (~5 fold above basal). This response decayed gradually and returned near to basal values at the end of experiment.

a)



b)



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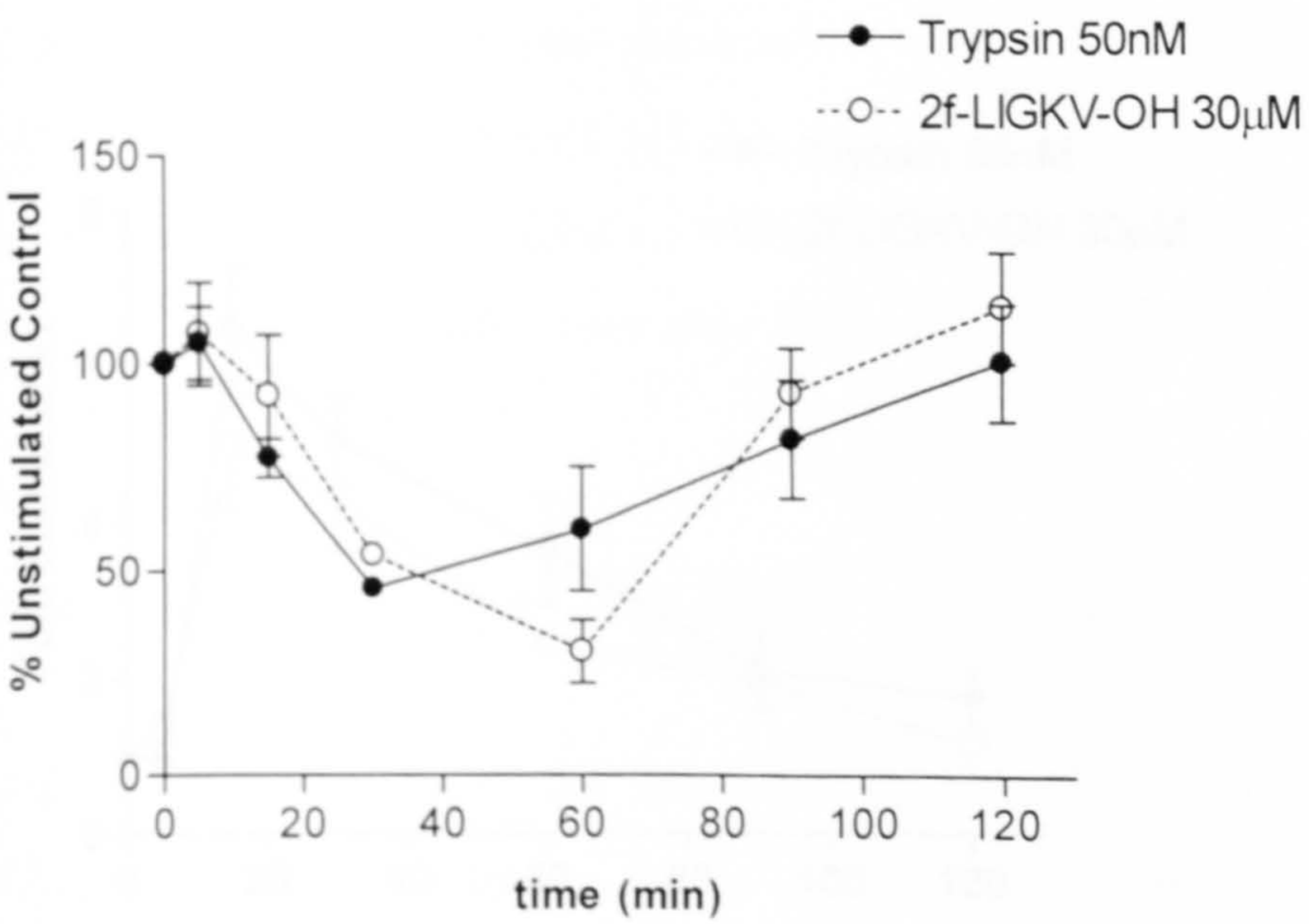
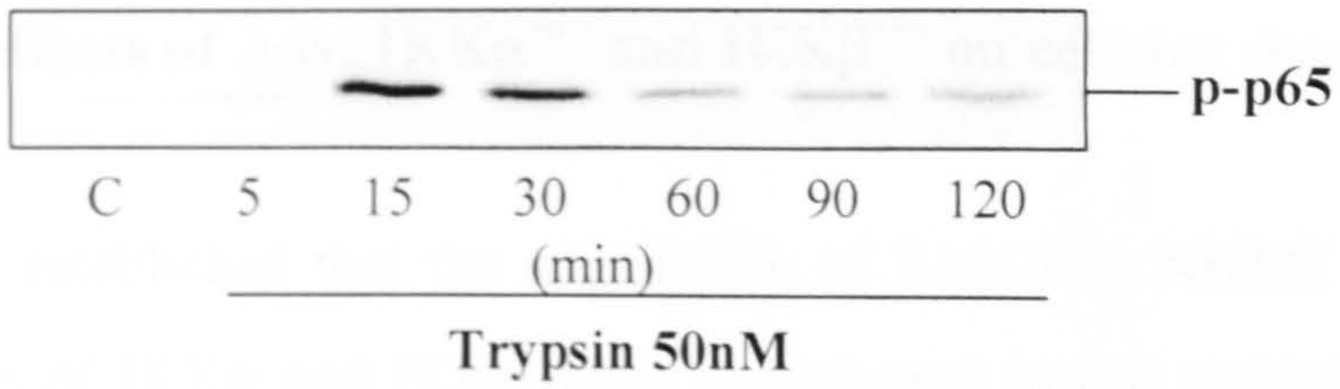


Figure 4.5. PAR-2–stimulated IκBα loss in NHEK

Cells were rendered quiescent for 18 hours prior to stimulation with 50nM of trypsin or 30μM of 2f-LIGKV-OH for the times indicated. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and visualised by Western blotting (Section 2.6.2). (a) and (b) Blots shown are representative of two others. (c) Blots were quantified using densitometry and expressed as mean ± s.e.m. (% unstimulated control).

a)



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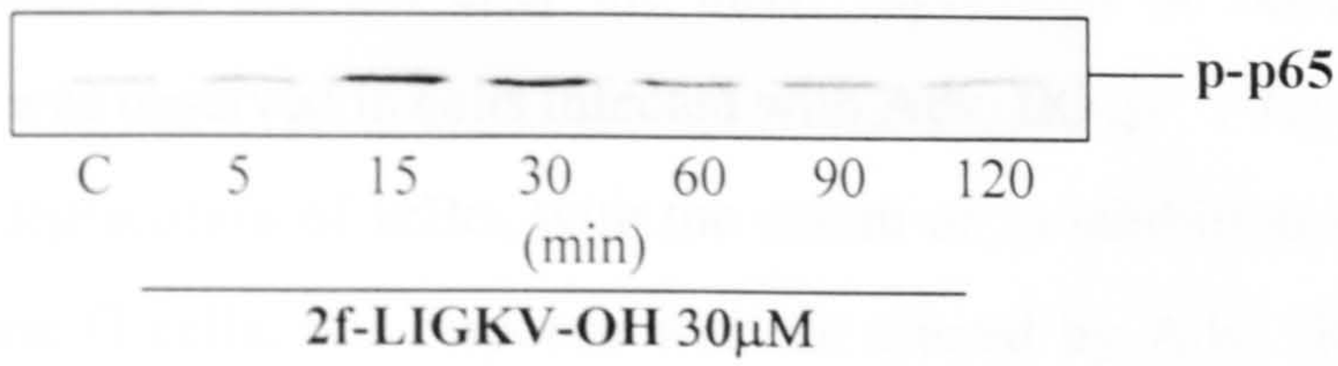

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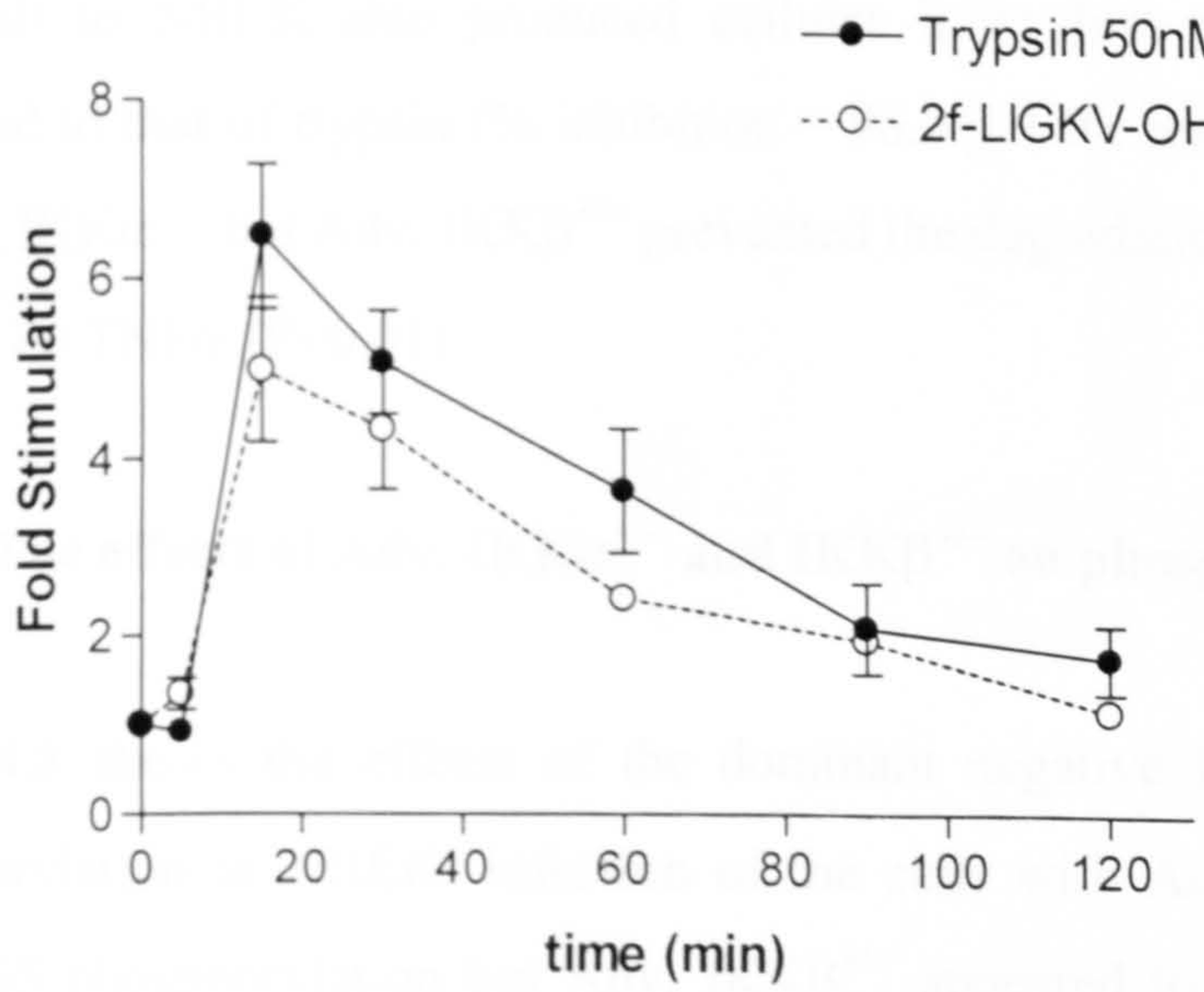


Figure 4.6. PAR-2–stimulated phosphorylation of p65 NFκB in NHEK

Cells were rendered quiescent for 18 hours prior to stimulation with trypsin (50nM) or 2f-LIGKV-OH (30μM) for the times indicated. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and were visualised by Western blotting (Section 2.6.2). (a) and (b) Blots shown are representative of 3 individual experiments. (c) Blots were quantified by densitometry and expressed as mean ± s.e.m. (fold stimulation).

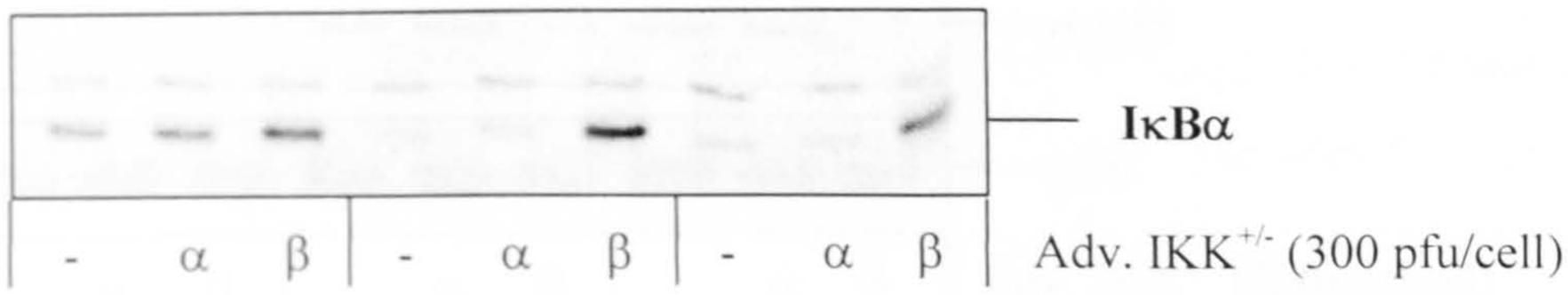
4.2.7 The effects of Adv. IKKα$^{+/-}$ and IKKβ$^{+/-}$ on cellular degradation of IκBα in NHEK

After having established that the stimulation of PAR-2 in NHEK would lead to IκBα loss, the roles of IKKα and IKKβ were investigated in this model using the respective dominant negative adenoviral constructs. Figure 4.7 shows the effects of Adv. IKKα$^{+/-}$ and IKKβ$^{+/-}$ on PAR-2 induced IκBα expression in NHEK. Infection of cells with the adenoviral constructs did not alter the basal expression of IκBα, although a slight enhancement was observed in cells infected with Adv. IKKβ$^{+/-}$. Trypsin (50nM) induced a significant degradation of IκBα, with the extent of % inhibition = 77.9 $\pm$ 3.2. Similar to that of clone G cells, this response was not altered by Adv. IKKα$^{+/-}$ whereas Adv. IKKβ$^{+/-}$ significantly reversed this phenomenon ($P<0.05$). The addition of TNFα (15ng/ml) to NHEK also produced cellular IκBα loss albeit with a greater extent compared to that of trypsin (% inhibition = 96.3 $\pm$ 1.0). Similarly, this was not affected by Adv. IKKα$^{+/-}$ but Adv. IKKβ$^{+/-}$ prevented the degradation of this inhibitory molecule induced by TNFα ($P<0.01$).

4.2.8 The effects of Adv. IKKα$^{+/-}$ and IKKβ$^{+/-}$ on phosphorylation of p65 NFκB in NHEK

Figure 4.8 shows the effects of the dominant negative IKK proteins on p65 NFκB phosphorylation in NHEK. Infection of the cells with Adv. IKKα$^{+/-}$ did not alter the basal p65 phosphorylation but Adv. IKKβ$^{+/-}$ appeared to decrease this basal activity. Addition of trypsin (50nM) induced a response of 6.9 $\pm$ 0.4 fold of basal control in NHEK. Similar to that of clone G cells, this response was not affected by the presence of Adv. IKKα$^{+/-}$ whereas Adv. IKKβ$^{+/-}$, on the contrary, reduced the magnitude of activation significantly to 1.5 $\pm$ 0.3 fold of basal control (% inhibition = 91.3 $\pm$ 4.5, $P< 0.01$). Similarly, TNFα (15ng/ml)-induced p65 phosphorylation in NHEK was significantly reduced by Adv. IKKβ$^{+/-}$ (8.3 $\pm$ 0.2 to 1.2 $\pm$ 0.2 fold of basal control, % inhibition = 99.0 $\pm$ 3.3, $P< 0.01$) but remained unaffected in the presence of Adv. IKKα$^{+/-}$.

a)



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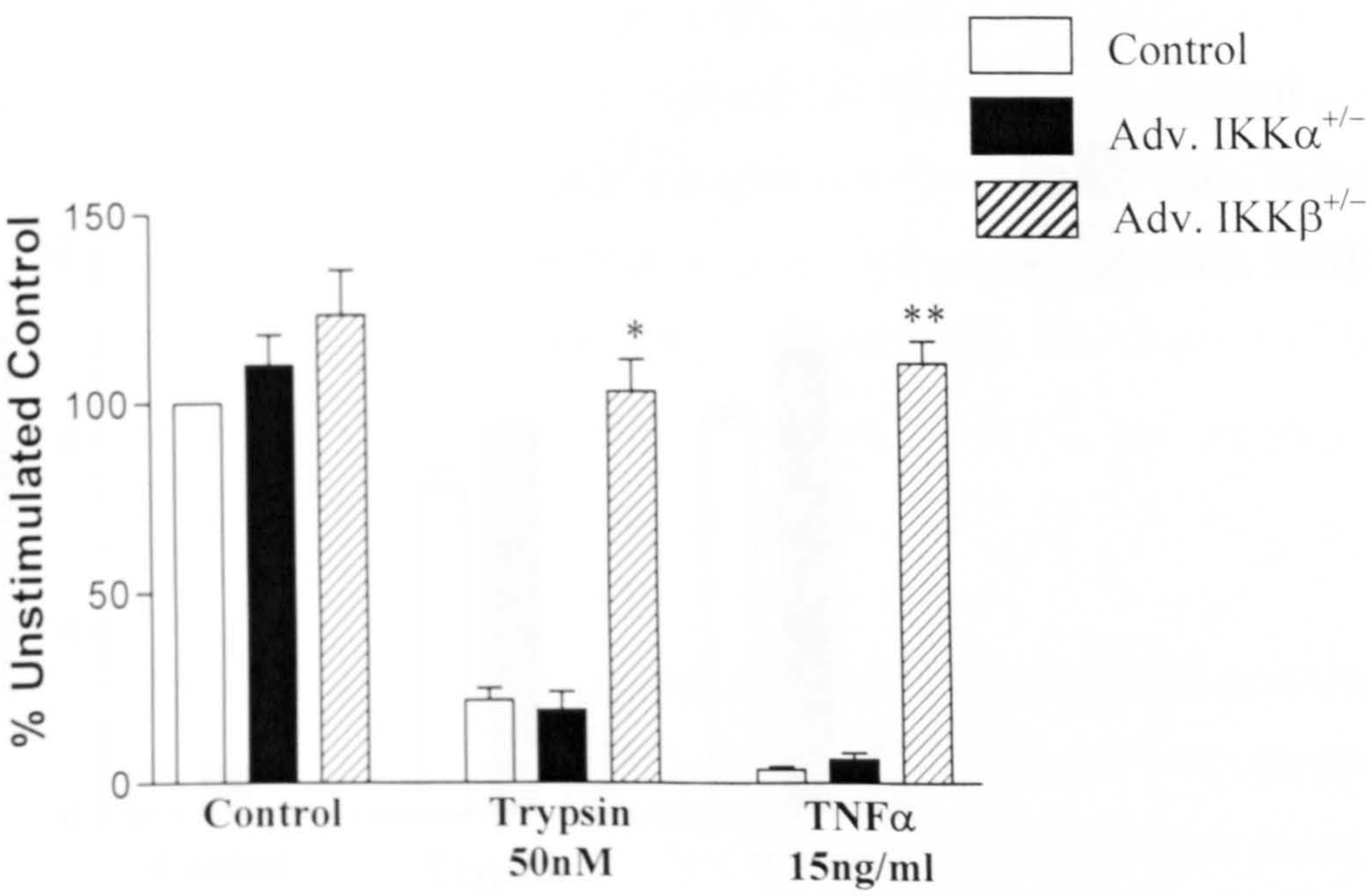
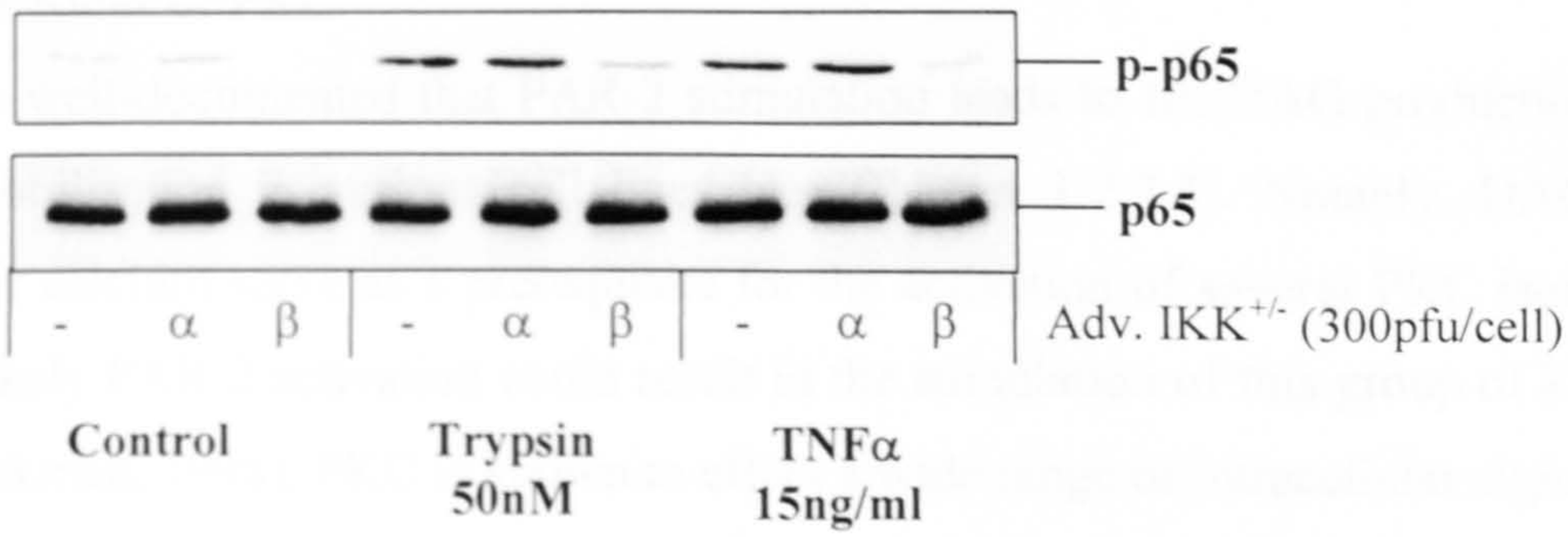


Figure 4.7. The effects of Adv. IKKα$^{+/-}$ and IKKβ$^{+/-}$ on trypsin and TNFα–stimulated IκBα loss in NHEK

Cells were infected with the respective adenoviral constructs for 48 hours and rendered quiescent for 18 hours prior to stimulation with 50nM of trypsin or 15ng/ml of TNFα for 30 min. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and were resolved by Western blotting (Section 2.6.2). (a) The blot shown is the representative of two others. (b) Blots were quantified using densitometry and expressed as mean ± s.e.m. (% unstimulated control). *P<0.05, **P<0.01 compared with agonist-stimulated control.

a)



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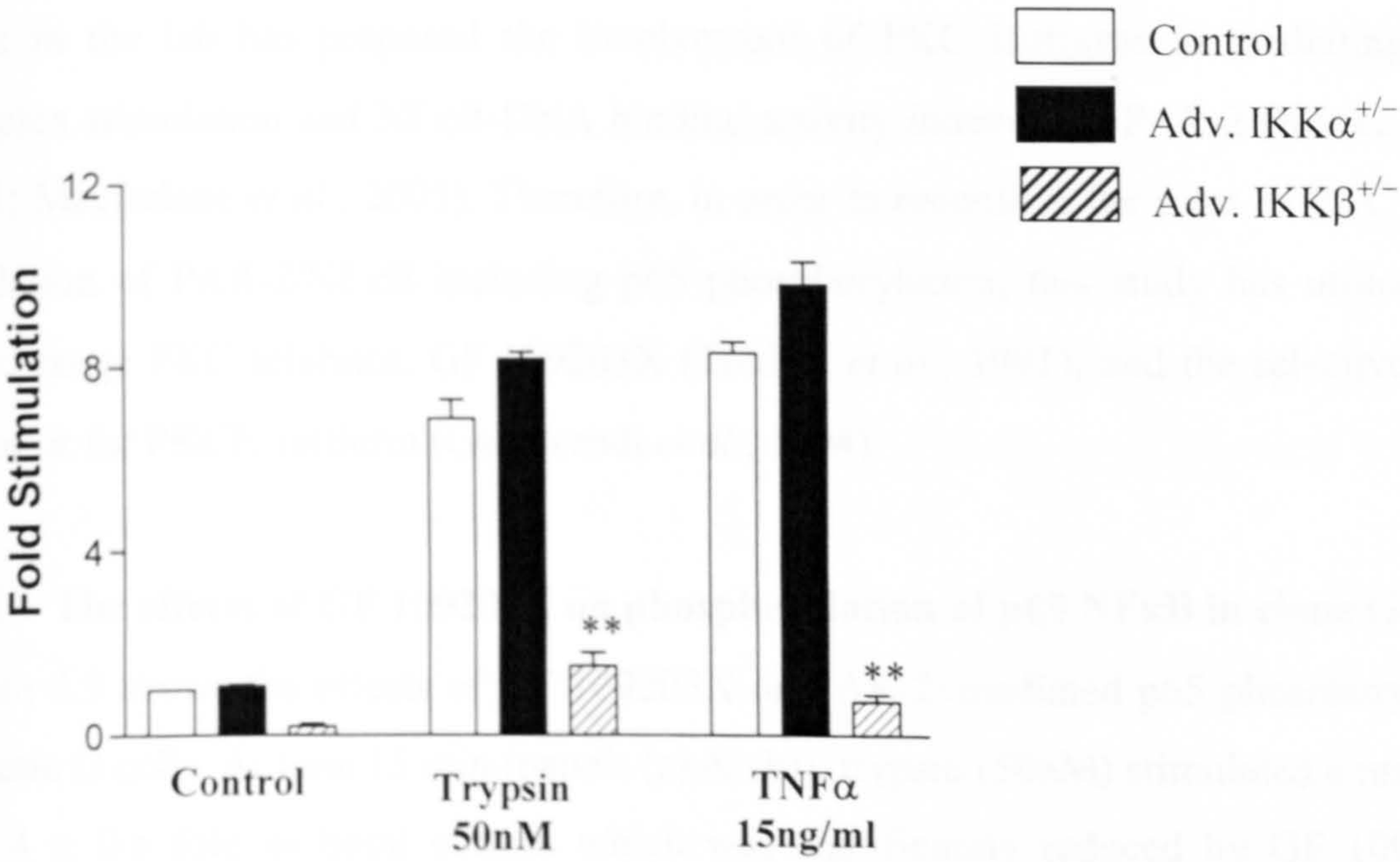


Figure 4.8. The effects of Adv. IKKα$^{+/-}$ and IKKβ$^{+/-}$ on trypsin and TNFα–stimulated phosphorylation of p65 in NHEK

Cells were infected with the respective adenoviral constructs for 48 hours and rendered quiescent for 18 hours prior to stimulation with trypsin (50nM) or TNFα (15ng/ml) for 30 min. Whole cell lysates were prepared and processed as previously outlined (Section 2.6.1), and were resolved by Western blotting (Section 2.6.2). (a) The blot shown is the representative of three. (b) Blots were quantified using densitometry and expressed as mean ± s.e.m. (fold stimulation). **P<0.01 compared with agonist-stimulated control.

4.3 The Roles of PKC

It has been well-documented that PAR-2 stimulation leads to IP_3/DAG production and calcium mobilisation in various cell lines (see Chapter 1.2.2.4). Notably, DAG and intracellular calcium serve as a prerequisite for the activation of several PKC isoforms, thus it is likely PAR-2 activation could result in the stimulation of this group of kinases (Liu & Heckman, 1998). PKC is known to effect a wide range of intracellular signalling, amongst these include its ability to induce IKK activity in response to a wide range of stimuli (Sanz *et al.*, 1999; Lallena *et al.*, 1999; Tojima *et al.*, 2000). In fact previous work in the lab has proposed the involvement of PKC isoforms in mediating IKK complex stimulation and NFκB-DNA binding activity induced by PAR-2 (Kanke *et al.*, 2001; Macfarlane *et al.*, 2005). Therefore, in order to reconfirm the roles of PKC in the regulation of PAR-2/NFκB including p65 phosphorylation, this study has utilised the broad range PKC inhibitor, GF 109203X (Toullec *et al.*, 1991); and the selective PKC inhibitor for PKCδ, rottlerin (Gschwendt *et al.*, 1994).

4.3.1 The effects of GF 109203X on phosphorylation of p65 NFκB in clone G cells

Figure 4.9 shows the effects of GF 109203X on PAR-2–mediated p65 phosphorylation in clone G cells. At time 15 min (panels (a) & (b)), trypsin (50nM) stimulated a response of 5.4 $\pm$ 0.6 fold of basal control which was significantly reduced by GF 109203X (10μM) to 2.1 $\pm$ 0.2 fold of basal control (% inhibition = 73.2 $\pm$ 3.9, $P<0.05$). The PAR-2 AP, 2f-LIGKV-OH at 30μM also induced an activity of similar magnitude (6.5 $\pm$ 0.6 fold of basal control) and GF 109203X (10μM) again significantly inhibited this response to 2.4 $\pm$ 0.1 fold of basal control (% inhibition = 64.6 $\pm$ 2.3, $P<0.05$). The PKC activator, PMA, has been utilised in this experiment as a control. Treatment of cells with 100nM of PMA resulted in a response of 6.4 $\pm$ 0.8 fold of basal control which was reduced significantly by GF 109203X to 1.4 $\pm$ 0.1 fold of basal control (% inhibition = 91.4 $\pm$ 1.5, $P<0.05$).

Interestingly, when the experiment was performed for a longer period of time, it was found that the inhibitory effect of GF 109203X (1-10μM) on PAR-2–induced phosphorylation of p65 was only observed at the earlier (15 min) but not the later time (30 min) (panel (c)).

4.3.2 The effects of GF 109203X on phosphorylation of p65 NFκB in NHEK

The role of PKC on phosphorylation of p65 NFκB mediated by PAR-2 in NHEK was also investigated in parallel to clone G cells using GF 109203X (10μM) as shown in Figure 4.10. GF 109203X significantly reduced the responses induced by trypsin (3.6 ± 0.5 to 1.6 ± 0.4 fold of basal control, % inhibition = 80.1 ± 13.9, $P<0.05$) and 2f-LIGKV-OH (3.5 ± 0.4 to 1.5 ± 0.3 fold of basal control, % inhibition = 80.1 ± 12.2, $P<0.05$) at time 15 min. Similarly, p65 phosphorylation stimulated by the agonists at time 30 min were also significantly suppressed by GF 109203X (trypsin: 3.7 ± 0.6 to 2.1 ± 0.4 fold of basal control, % inhibition = 60.8 ± 16.0, $P<0.05$; AP: 4.5 ± 0.9 to 1.2 ± 0.6 fold of basal control, % inhibition = 78.9 ± 12.1, $P<0.05$).

4.3.3 The effects of rottlerin on phosphorylation of p65 NFκB in clone G cells

The effects of rottlerin, a selective PKC inhibitor for the novel PKC isoform, PKCδ; was employed to investigate the possible role of this PKC isoform in this signalling paradigm for two time points, 15 and 30 min post stimulation as illustrated in Figure 4.11. In the presence of rottlerin (10μM), the basal activity of p65 phosphorylation remained unchanged compared to the unstimulated control. The addition of trypsin (50nM) stimulated responses of 5.7 and 6.7 fold of basal control at 15 and 30 min, both of which were markedly reduced by rottlerin to 1.1 and 1.5 fold of basal control respectively. Similarly, the response stimulated by 100nM of PMA (6.4 fold of basal control) was also largely abolished by rottlerin. However, in this experiment the statistical significance of the extent of reduction by rottlerin could not be justified due to insufficient n numbers (n=2).

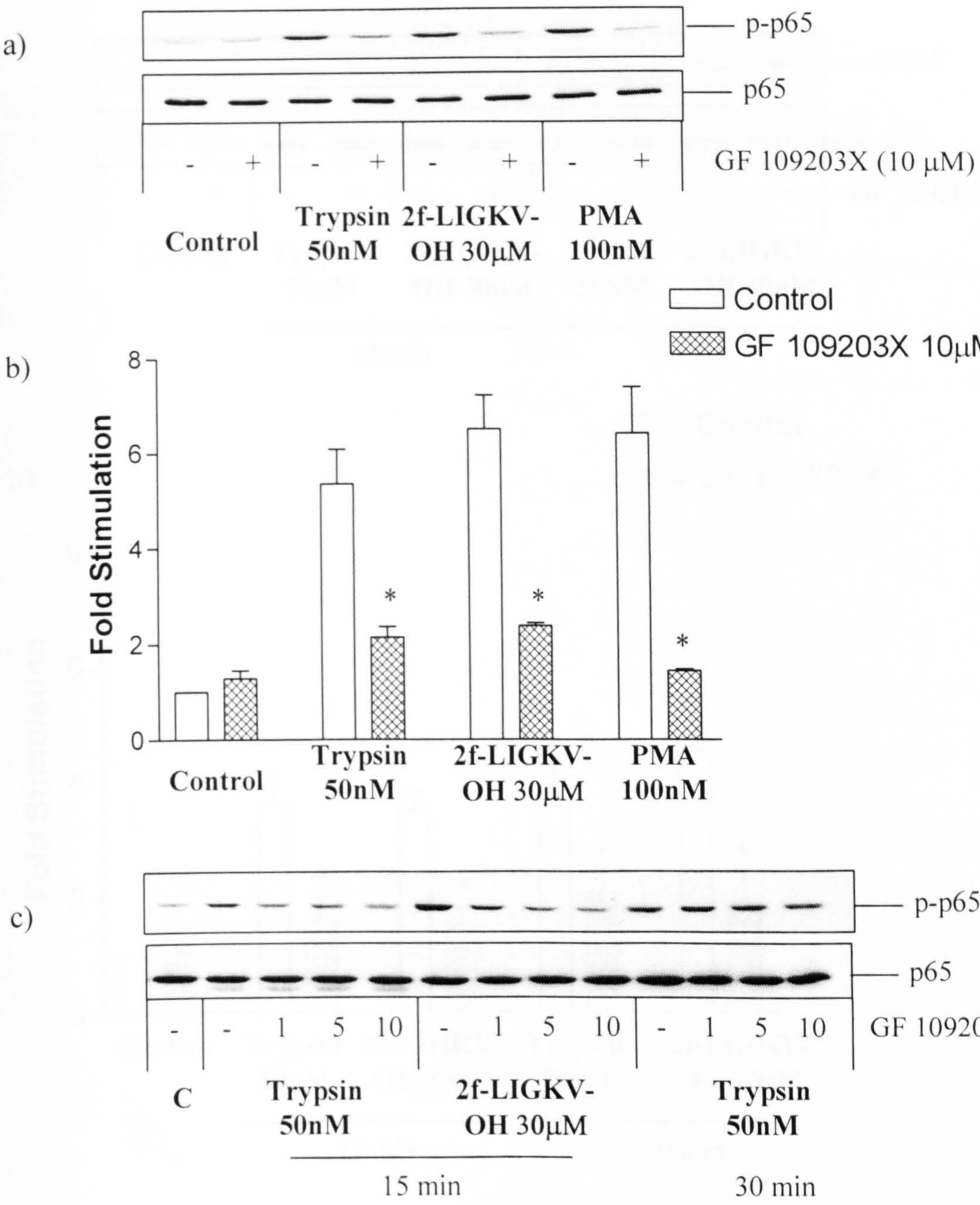


Figure 4.9. The effects of GF 109203X on PAR-2–mediated phosphorylation of p65 NFκB in clone G cells

Cells were rendered quiescent for 18 hours and preincubated with the respective concentrations of GF 109203X for 45 min prior to stimulation with trypsin (50nM), 2f-LIGKV-OH (30μM) or PMA (100nM) for (a) 15 min (c) 15 and 30min. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2). Blots shown are representative of (a) 3 and (c) 2 individual experiments. (b) Blots of (a) were quantified using densitometry and expressed as mean ± s.e.m. (fold stimulation). *$P<0.05$ compared with agonist-stimulated control.

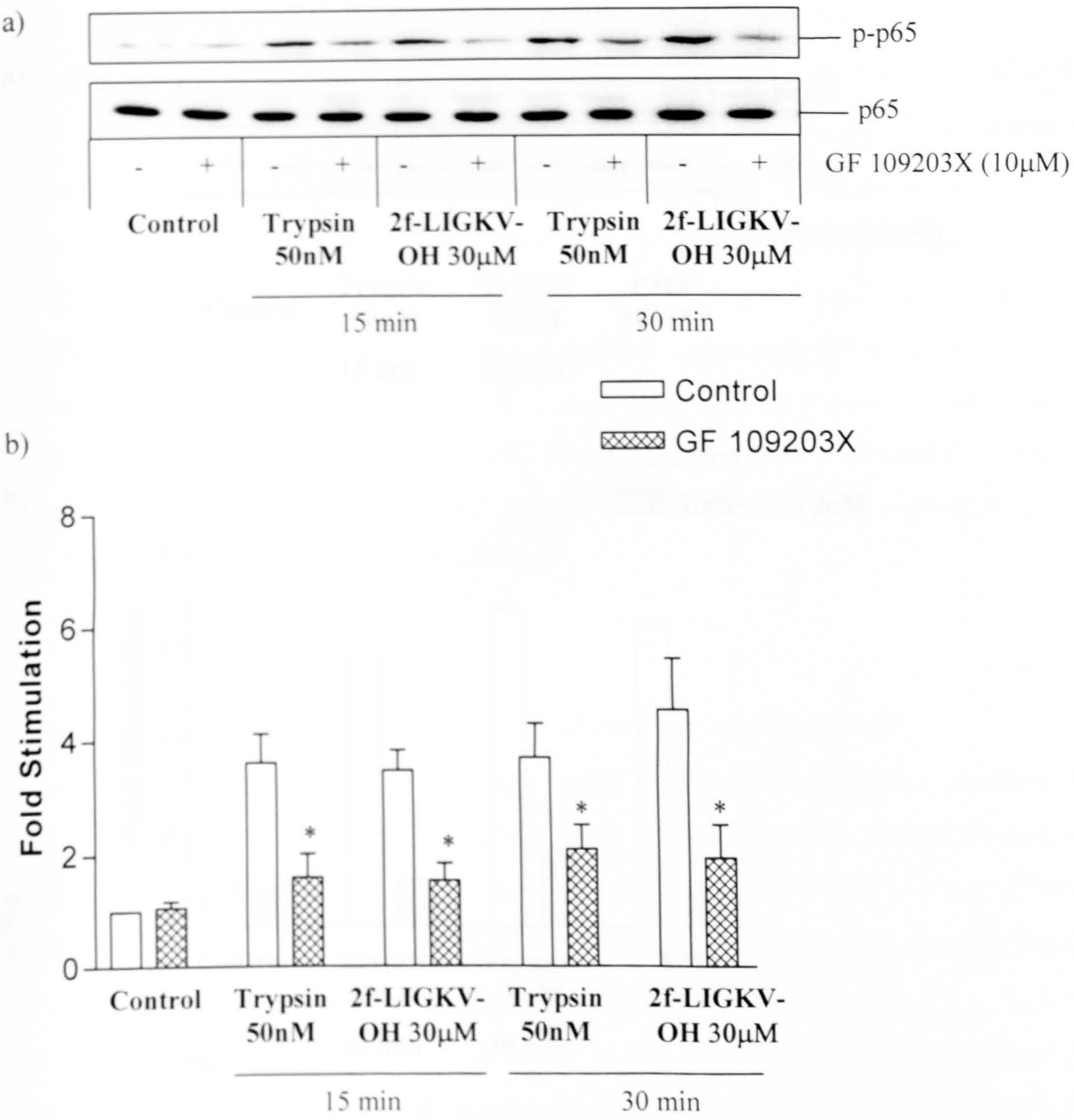
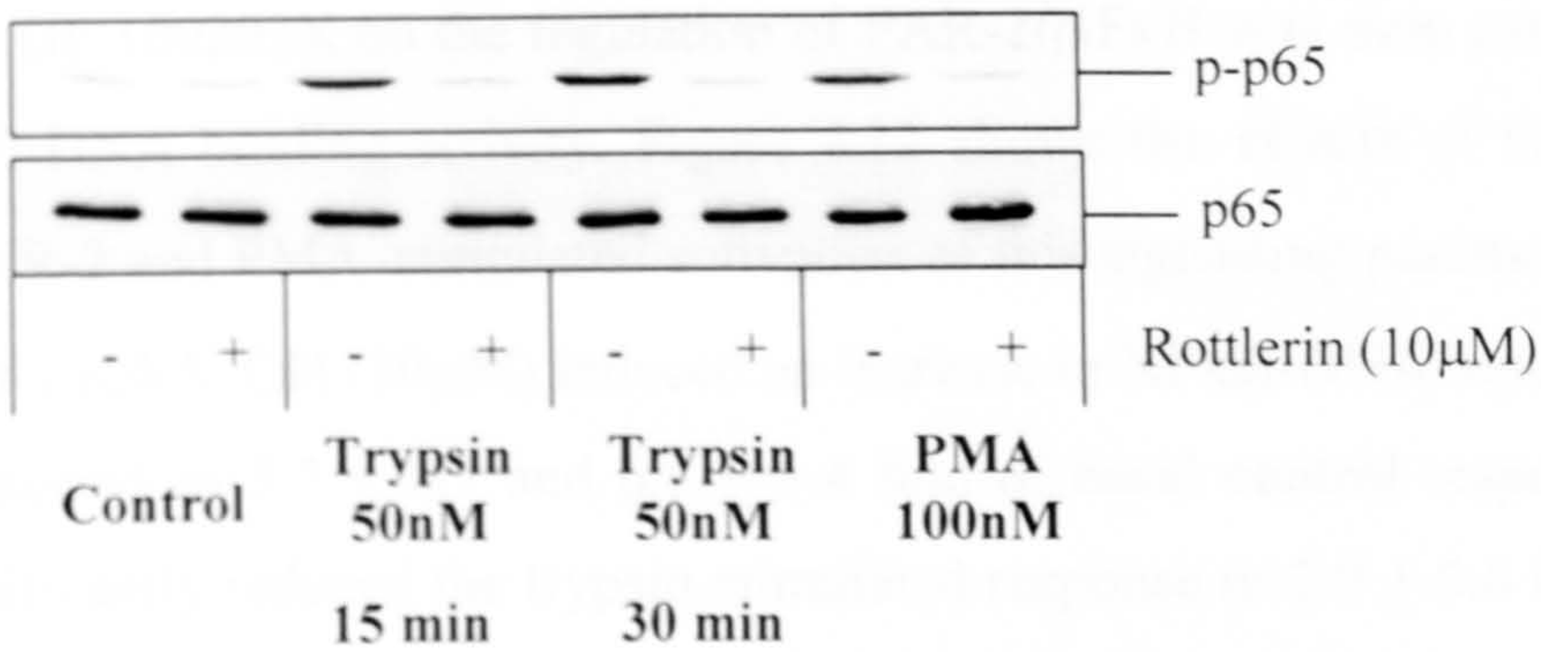


Figure 4.10. The effects of GF 109203X on PAR-2–mediated phosphorylation of p65 NFκB in NHEK

Cells were rendered quiescent for 18 hours and preincubated with 10μM of GF 109203X for 45 min prior to stimulation with trypsin (50nM), 2f-LIGKV-OH (30μM) or PMA (100nM) for the indicated times. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2). Blots shown are representative of three individual experiments. (b) Blots were quantified using densitometry and expressed as mean ± s.e.m. (fold stimulation). *$P<0.05$ compared with agonist-stimulated control.

a)



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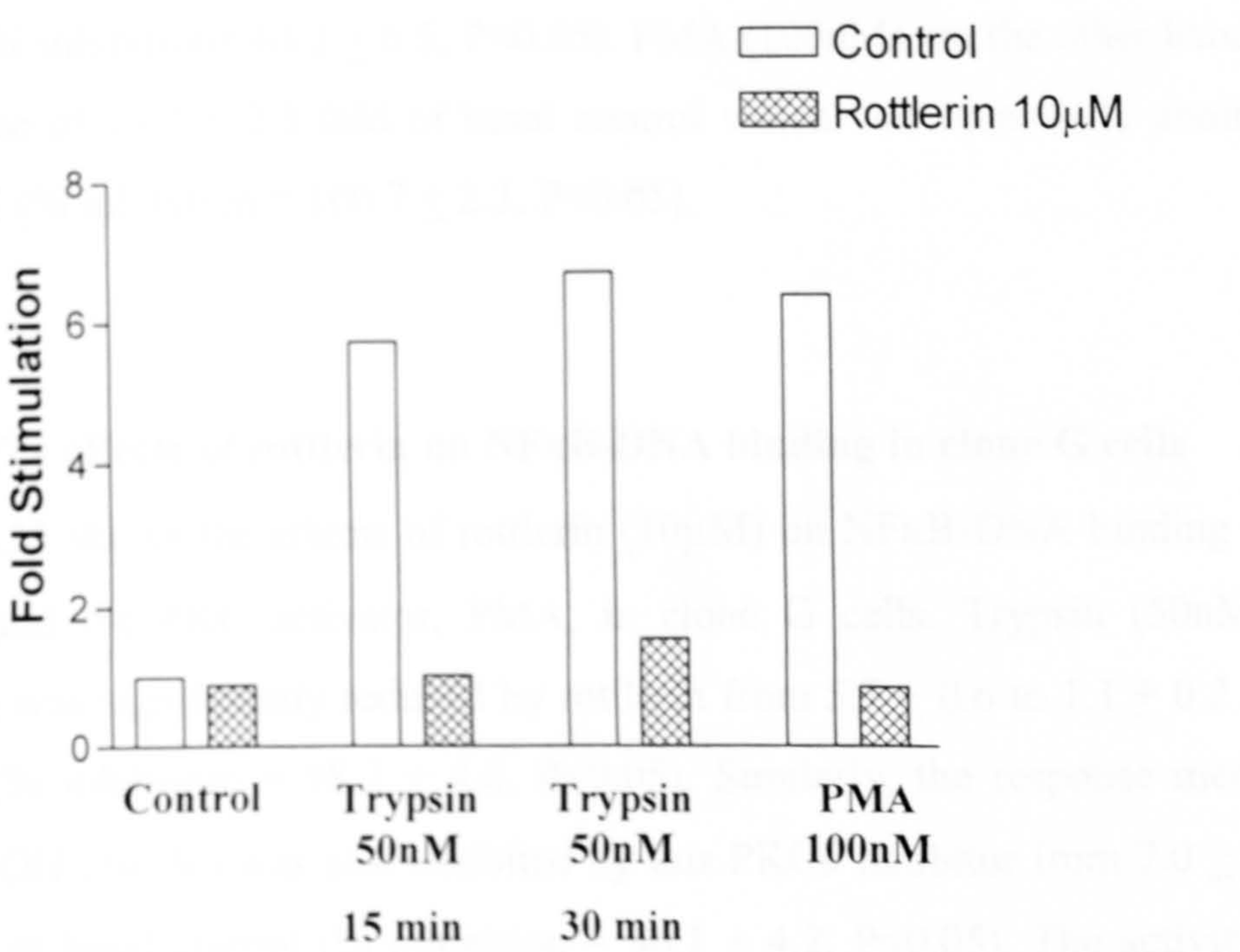


Figure 4.11. The effects of rottlerin on PAR-2–mediated phosphorylation of p65 NFκB in clone G cells

Cells were rendered quiescent for 18 hours and preincubated with 10μM of rottlerin for 45 min prior to stimulation with trypsin (50nM) or PMA (100nM) for the times indicated. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and visualised by Western blotting (Section 2.6.2). (a) Blots shown are representative of two individual experiments. (b) Blots were quantified using densitometry and expressed as mean ± s.e.m. (fold stimulation).

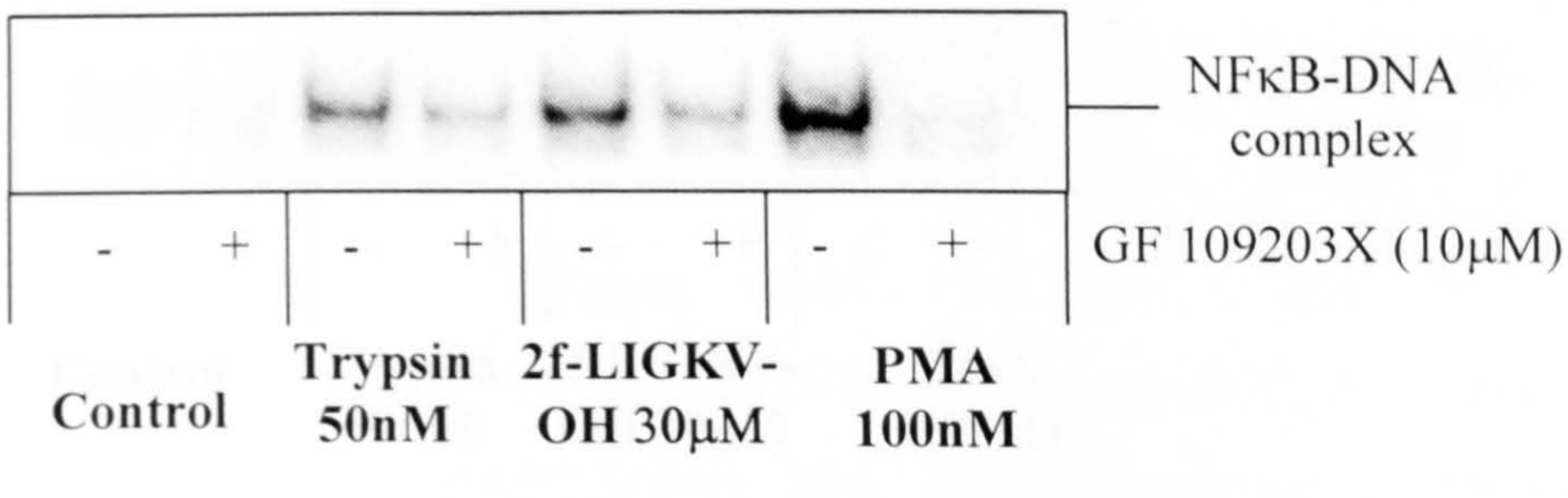
4.3.4 The effects of GF 109203X on NFκB-DNA binding in clone G cells

The effects of GF 109203X on the regulation of PAR-2/NFκB was then assessed at the level of NFκB-DNA binding activity. Figure 4.12 shows the effects of GF 109203X (10μM) on PAR-2 and PMA–stimulated activation of this signalling parameter. Trypsin (50nM) and 2f-LIGKV-OH (30μM) induced an increase in NFκB-DNA binding activity which was detected as 5.7 ± 0.7 and 6.8 ± 1.4 fold of basal control respectively. GF 109203X significantly reduced the trypsin-stimulated response to 2.9 ± 0.6 fold of basal control (% inhibition= 61.3 ± 4.1, $P<0.01$) and that of AP to 3.2 ± 0.9 fold of basal control (% inhibition= 64.2 ± 6.5, $P<0.05$). PMA (100nM), on the other hand, stimulated a response of 10.7 ± 2.3 fold of basal control which was essentially abolished by GF 109203X (% inhibition = 100.7 ± 2.3, $P<0.05$).

4.3.5 The effects of rottlerin on NFκB-DNA binding in clone G cells

Figure 4.13 shows the effects of rottlerin (10μM) on NFκB-DNA binding mediated by PAR-2 and the PKC activator, PMA, in clone G cells. Trypsin (50nM)-stimulated response was significantly reduced by rottlerin from 5.7 ± 0.6 to 1.1 ± 0.2 fold of basal control (% inhibition = 98.2 ± 4.8, $P<0.05$). Similarly, the response mediated by 2f-LIGKV-OH (30μM) was also inhibited by this PKCδ inhibitor from 7.0 ± 1.3 to 1.1 ± 0.3 fold of basal control (% inhibition = 96.1 ± 4.2, $P<0.05$). The activity induced by 100nM of PMA (7.3 ± 1.3 fold of basal control) was also largely reduced to 2.3 ± 0.7 fold of basal control with % inhibition = 82.1 ± 6.4 ($P<0.05$).

a)



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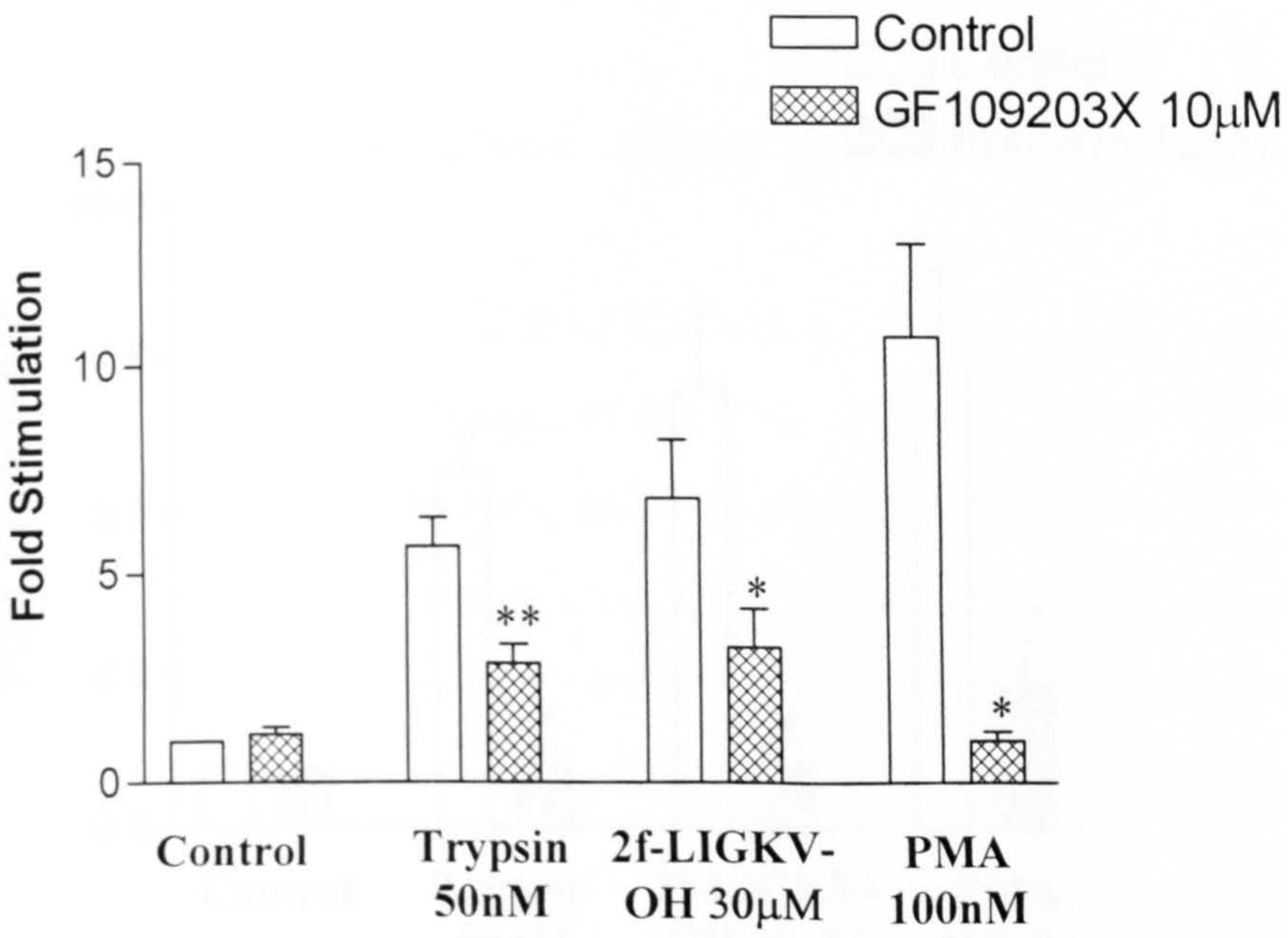
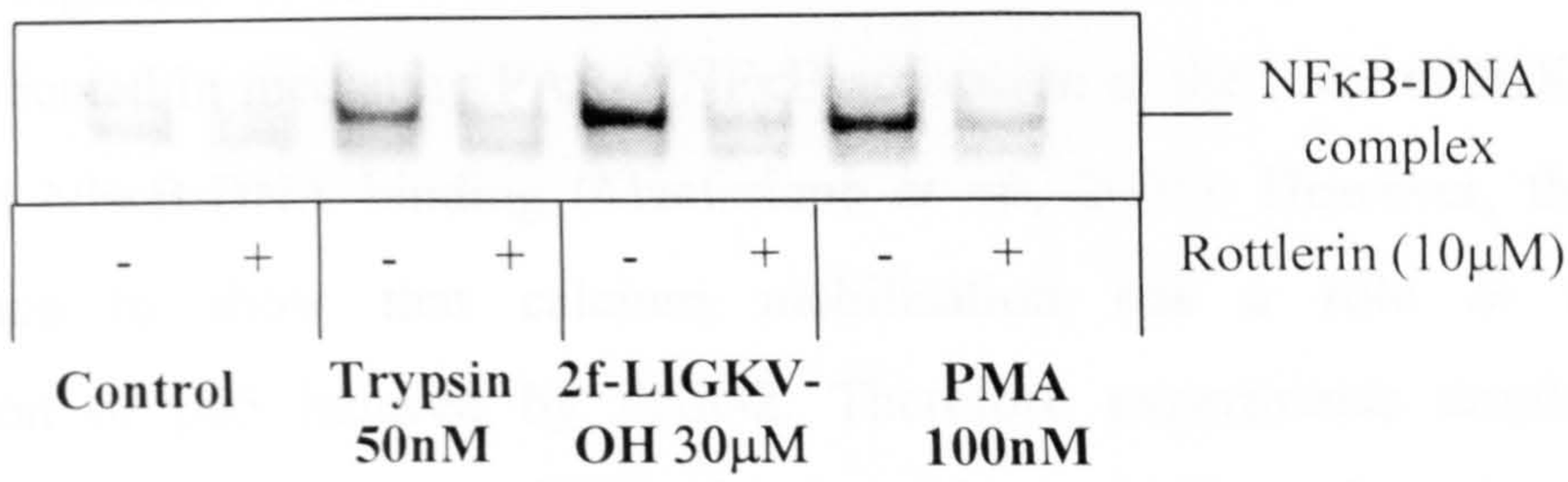


Figure 4.12. The effects of GF 109203X on PAR-2-stimulated NFκB-DNA binding

Cells were grown to confluence before being rendered quiescent with serum-free medium for 18 hours before preincubated with 10μM of GF 109203X for 45 min. Following this, cells were treated with trypsin (50nM), 2f-LIGKV-OH (30μM) or PMA (100nM) for 60 min. Nuclear extracts were prepared and the activity was measured by EMSA (Section 2.7). (a) The autoradiogram shown is the representative of two others. (b) Autoradiograms were quantified using densitometry and expressed as mean ± s.e.m. (fold stimulation). *P<0.05, **P<0.01 compared with agonist-stimulated control.

a)



b)

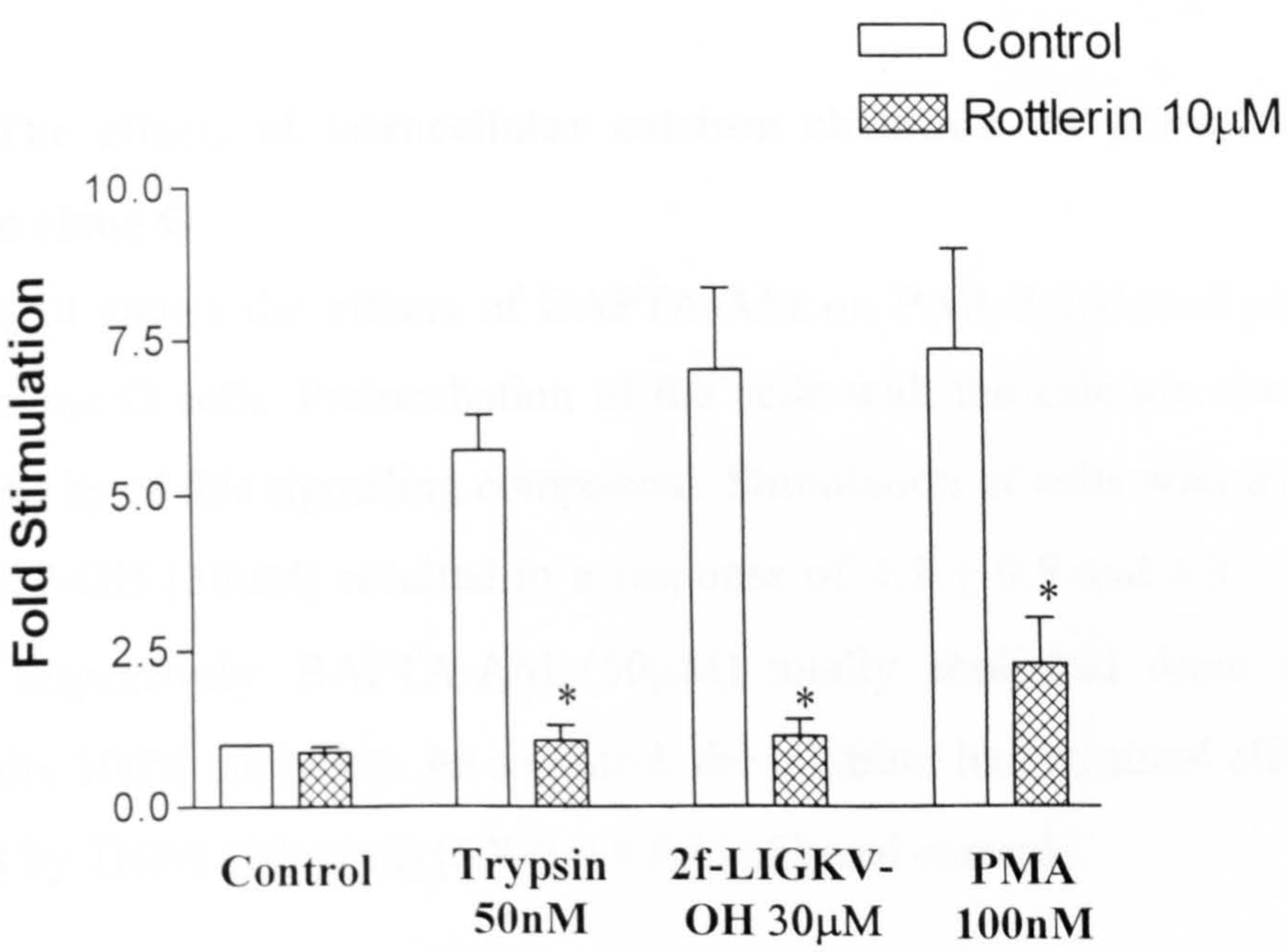


Figure 4.13. The effects of rottlerin on PAR-2-stimulated NFκB-DNA binding

Cells were grown to confluence before being rendered quiescent with serum-free medium for 18 hours and preincubated with rottlerin (10μM) for 45 min prior to stimulation with trypsin (50nM), 2f-LIGKV-OH (30μM) or PMA (100nM) for 60 min. Nuclear extracts were prepared and the NFκB-DNA binding was assessed by EMSA as outlined in Section 2.7. (a) The autoradiogram shown is the representative of two others. (b) Autoradiograms were quantified using densitometry and expressed as mean ± s.e.m. (fold stimulation). *$P<0.05$ compared with agonist-stimulated control.

4.4 The Roles of Intracellular Calcium

The upstream regulator of calcium-dependent PKC isoforms, intracellular calcium, has also been implicated in mediating PAR-2/NFκB activation at the level of IKK complex activation and NFκB-DNA binding (Macfarlane *et al.*, 2005). However, there is no direct evidence to show that calcium mobilisation has a role in mediating phosphorylation of p65 induced by PAR-2. Therefore experiments employing the intracellular calcium chelator, BAPTA-AM, have been performed to investigate if intracellular calcium has any role in this context in addition to NFκB-DNA binding.

4.4.1 The effects of intracellular calcium chelation on phosphorylation of p65 NFκB in clone G

Figure 4.14 shows the effects of BAPTA-AM on PAR-2–induced phosphorylation of p65 in clone G cells. Preincubation of the cells with the calcium chelator reduced the basal activity of this signalling component. Stimulation of cells with trypsin (50nM) and 2f-LIGKV-OH (30μM) resulted in a response of 4.8 ± 0.5 and 6.3 ± 0.5 fold of basal control respectively. BAPTA-AM (50μM) totally abolished these responses, giving essentially 100% inhibition. As a control, the inhibitor had minimal effect on the activity induced by TNFα (20ng/ml) (5.8 ± 0.4 fold of basal control).

4.4.2 The effects of intracellular calcium chelation on NFκB-DNA binding activity in clone G

The role of intracellular calcium was then investigated at the level of NFκB-DNA binding induced by PAR-2 as illustrated in Figure 4.15. An activity of 5.1 ± 0.2 fold of basal control was detected upon stimulation of clone G cells with 50nM of trypsin, and this response was again suppressed in the presence of BAPTA-AM (50μM) to 1.9 ± 0.2 fold of basal control (% inhibition = 79.4 ± 4.1, $P < 0.05$). Similar inhibition profile was also observed in the case of 2f-LIGKV-OH (30μM), in that the response was observed to be reduced significantly from 5.7 ± 0.2 fold of basal control to 2.2 ± 0.2 fold of basal

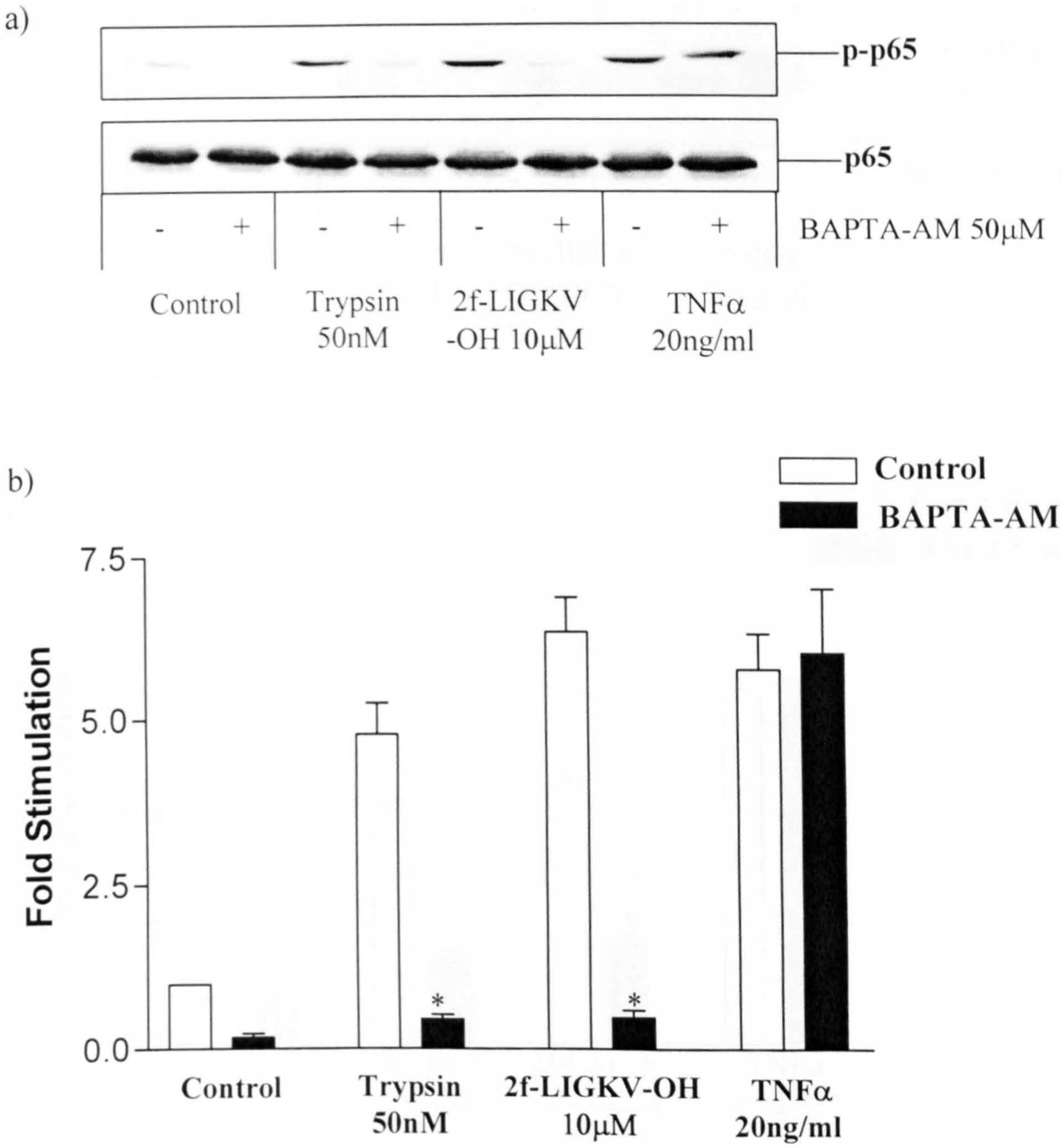
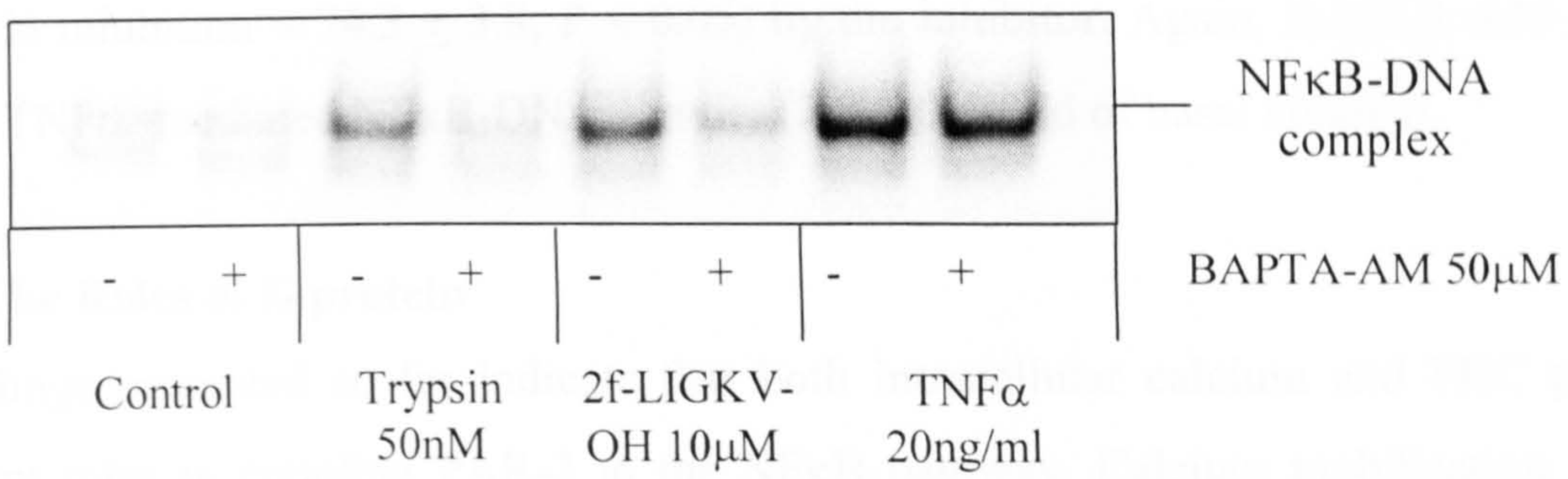


Figure 4.14 The effects of BAPTA-AM on PAR-2 and TNFα–mediated phosphorylation of p65 NFκB in clone G cells.

Cells were rendered quiescent for 18 hours prior to preincubation with 50μM of BAPTA-AM for 30 min. Following this, the cells were exposed to trypsin (50nM), 2f-LIGKV-OH (30μM) or TNFα (20ng/ml) for a further 30 min. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2). (a) The blot shown is the representative of 3 individual experiments. (b) Blots were quantified using densitometry and expressed as mean ± s.e.m. (fold stimulation). *$P<0.05$ compared with agonist-stimulated control.

a)



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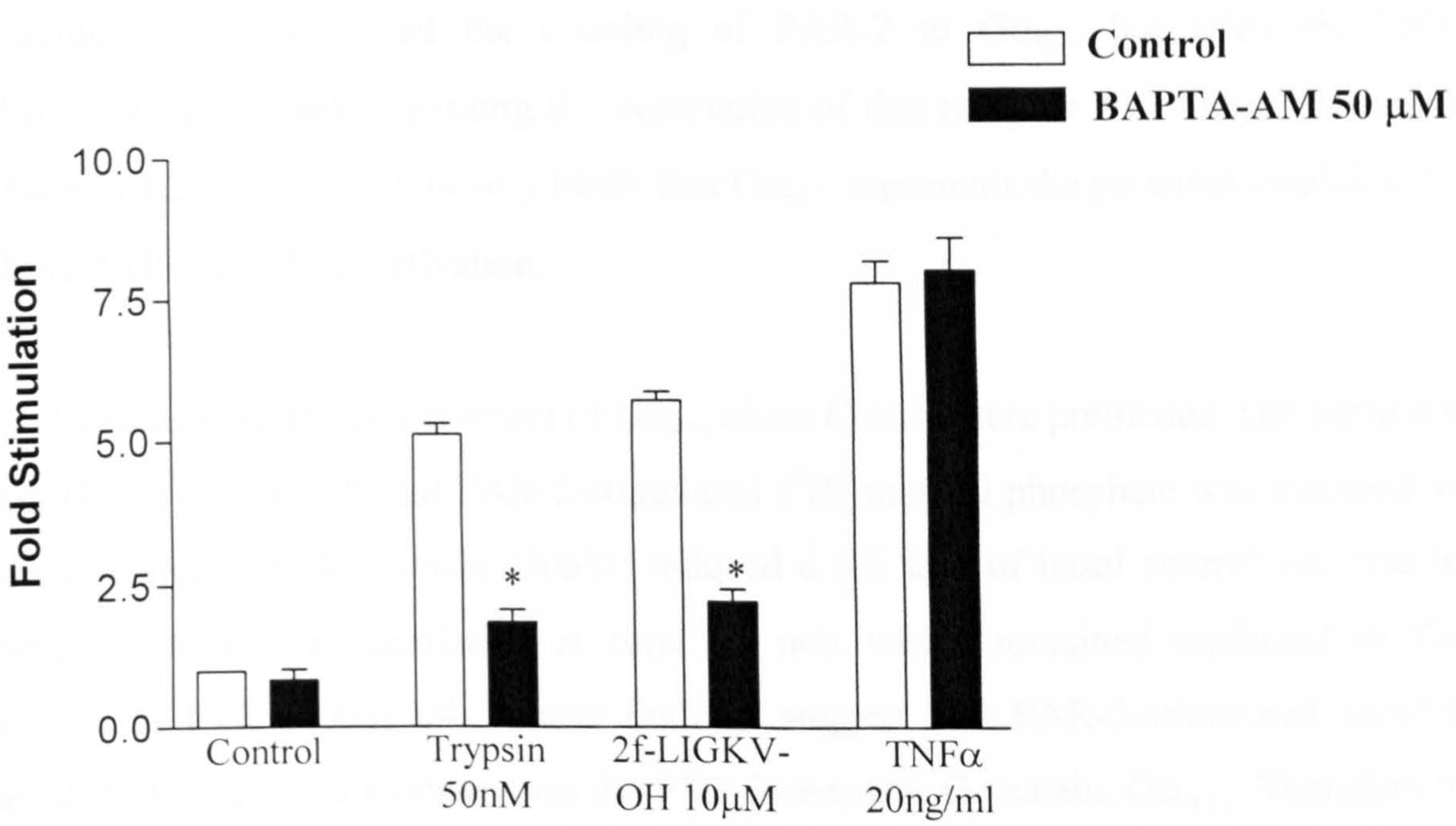


Figure 4.15 The effects of BAPTA-AM on PAR-2 and TNFα–mediated NFκB-DNA binding in clone G cells

Cells were rendered quiescent for 18 hours and preincubated with 50μM of BAPTA-AM for 30 min before stimulation with trypsin (50nM), 2f-LIGKV-OH (30μM) or TNFα (20ng/ml) for a further 60 min. Crude nuclear lysates were prepared and processed as previously described (Sections 2.7.1 & 2.7.2), and the binding was assessed by using EMSA (Section 2.7.4). (a) The autoradiogram shown is the representative of 3 individual experiments. (b) Autoradiograms were quantified using densitometry and expressed as mean ± s.e.m. (fold stimulation). *$P<0.05$ compared with agonist-stimulated control.

control (% inhibition = 74.3 $\pm$ 3.8, $P < 0.05$) by the inhibitor. Again, BAPTA-AM did not alter TNFα–mediated NFκB-DNA activity (7.8 $\pm$ 0.4 fold of basal control).

4.5 The Roles of G protein

The findings presented so far indicate that both intracellular calcium and PKC play significant roles in coupling PAR-2 to the NFκB pathway. Calcium mobilisation and PKC activation represent the classical downstream effectors of PLC, an enzyme that can be stimulated upon $G\alpha_{q/11}$ or $G\alpha_{i/o}$ activation (discussed in Section 1.1.3). A large body of evidence has suggested the coupling of PAR-2 to $G\alpha_{q/11}$ but relatively little information is available regarding the association of this receptor with $G\alpha_{i/o}$ (discussed in Section 1.2.2.4). Thus it is very likely that $G\alpha_{q/11}$ represents the potential candidate in linking PAR-2 to NFκB activation.

In order to rule out the involvement of $G\alpha_{i/o}$, clone G cells were pretreated with pertussis toxin (PTX) (100ng/ml) and PAR-2-stimulated [^{3}H]-inositol phosphate was assessed as shown in Figure 4.16. Trypsin (30nM) induced a 6.6 fold of basal control increase in inositol phosphate accumulation at time 60 min which remained unaltered in the presence of PTX (100ng/ml). These findings suggest that PAR-2-stimulated inositol phosphate formation is mediated via the PTX-insensitive G protein, $G\alpha_{q/11}$. Therefore in order to pursue the postulation that $G\alpha_{q/11}$ is the key player in PAR-2/NFκB signalling cascade, several approaches including overexpressing regulators of G protein signalling proteins (RGS), small interfering RNA (siRNA) technology and pharmacological inhibition of $G\alpha_{q/11}$ using compound YM 254890 were employed to test this hypothesis.

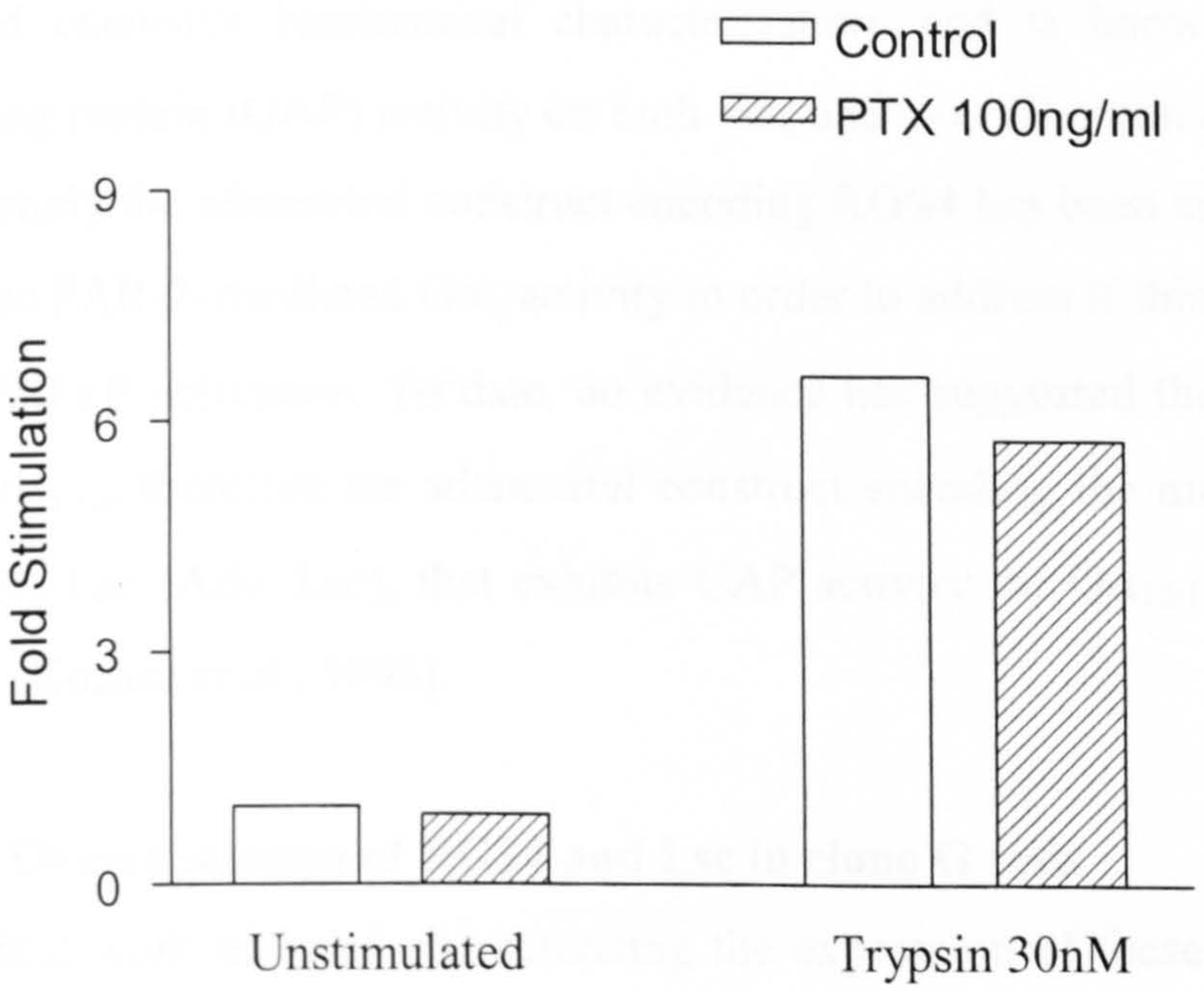


Figure 4.16. The effects of PTX pretreatment on PAR-2–mediated [^{3}H]-inositol phosphate accumulation in clone G

Cells were grown to 80% confluency, prelabelled with [^{3}H]-myo-inositol in addition to PTX pre-treatment for 18 hours before stimulation with agonist for 60 min. Accumulation of total [^{3}H]-inositol phosphate was measured as described (Section 2.5). Each value represents the mean $\pm$ s.e.m. from two separate experiments performed in triplicate.

4.5.1 Regulators of G protein signalling (RGS) proteins

The extent of G protein activation will dictate the magnitude and kinetics of the downstream effectors stimulation. RGS proteins are key elements that negatively regulate G protein activation and are important for signal termination (reviewed in Hollinger & Hepler, 2002). RGS4 as one of the earliest RGS proteins identified, has received extensive biochemical characterisation, and is known to exhibit GTPase-activating protein (GAP) activity on both $G\alpha_i$ and $G\alpha_q$ (Berman *et al.*, 1996). Therefore in this study the adenoviral construct encoding RGS4 has been employed as the GAP to attenuate PAR-2–mediated $G\alpha_q$ activity in order to address if this G protein has a role in PAR-2/NFκB activation. To date, no evidence has suggested the association of PAR-2 with $G\alpha_{12/13}$, therefore the adenoviral construct encoding the murine homolog of p115 RhoGEF, Lsc (Adv. Lsc), that exhibits GAP activity for $G\alpha_{12/13}$ was also utilised as a control (Kozasa *et al.*, 1998).

4.5.1.1 Overexpression of RGS4 and Lsc in clone G cells

The initial work included characterising the expression of these RGS proteins in clone G. Cells were infected with the adenoviral constructs encoding RGS4 and Lsc in the order of increasing concentration to ascertain the levels of protein expression. As RGS4 and Lsc have been expressed as Flag-tagged and His-tagged proteins respectively, protein expression levels can be identified using immunoblotting with specific antibodies. Figure 4.17(a) shows the levels of RGS4 expression for cells with infections up to 1000 pfu/cell. No apparent protein expression was detected in the control or infection at 200 pfu/cell, whereas infection of 500-1000 pfu/cell produced considerable RGS4 expression. The levels of Lsc expression with infection up to 500 pfu/cell is shown in Figure 4.17(b). Low levels of protein expression were obtained in the control and infection at 30 pfu/cell, whereas apparent Lsc expression levels could be detected at infection 100-500 pfu/cell.

4.5.1.2 The effects of RGS4 and Lsc overexpression on PAR-2–induced [^{3}H]–inositol phosphate accumulation

Having confirmed the expression of RGS4 and Lsc in clone G cells using the respective adenoviral constructs, the effects of overexpressing these RGS proteins on PAR-2–stimulated inositol phosphate accumulation were investigated as shown in Figure 4.18. Trypsin (30nM) stimulated a 4.5 $\pm$ 0.6 fold of basal control increase in response compared to the basal values. Overexpression of RGS4 with infection of the adenovirus up to 1000 pfu/cell, however, did not alter trypsin-induced accumulation of [^{3}H]-inositol phosphate (panel (a)). The effects of Lsc on PAR-2–mediated [^{3}H]-inositol phosphate accumulation is shown in panel (b). Interestingly, overexpressing Lsc with infection at MOI of 200, 500 and 1000 pfu/cell significantly potentiated the response induced by trypsin (30nM) from 4.6 $\pm$ 1.0 to 9.7 $\pm$ 0.9, 8.4 $\pm$ 1.7 and 9.6 $\pm$ 2.0 fold of basal control respectively ($P<0.05$ in all cases).

4.5.1.3 The effects of RGS4 overexpression on PAR-2–induced p42/44, p38 MAPK and p65 phosphorylation in clone G cells

The effects of Adv. RGS4 on other PAR-2–mediated signalling cascades were also investigated. Figure 4.19 shows the effects of Adv. RGS4 on PAR-2 -mediated MAP kinases and NFκB phosphorylation. Trypsin (30nM) stimulated the activation of p42/44 MAP kinase, and overexpression of this RGS protein, however, did not alter the response mediated by trypsin (panel (a)). Trypsin also induced the phosphorylation of p38 MAPK, RGS4 overexpression slightly potentiated the response of trypsin (panel (b)). In addition, infection of the cells with 1000 pfu/cell of Adv. RGS4 also enhanced the basal activity of this MAP kinase. The phosphorylation of NFκB was detected when the cells were treated with 30nM trypsin (panel (c)) and by overexpressing RGS4 with MOI from 200 to 1000 pfu/cell, the responses mediated by trypsin were slightly potentiated. Again, Adv. RGS4 (200-1000 pfu/cell) did enhance the basal of NFκB activity.

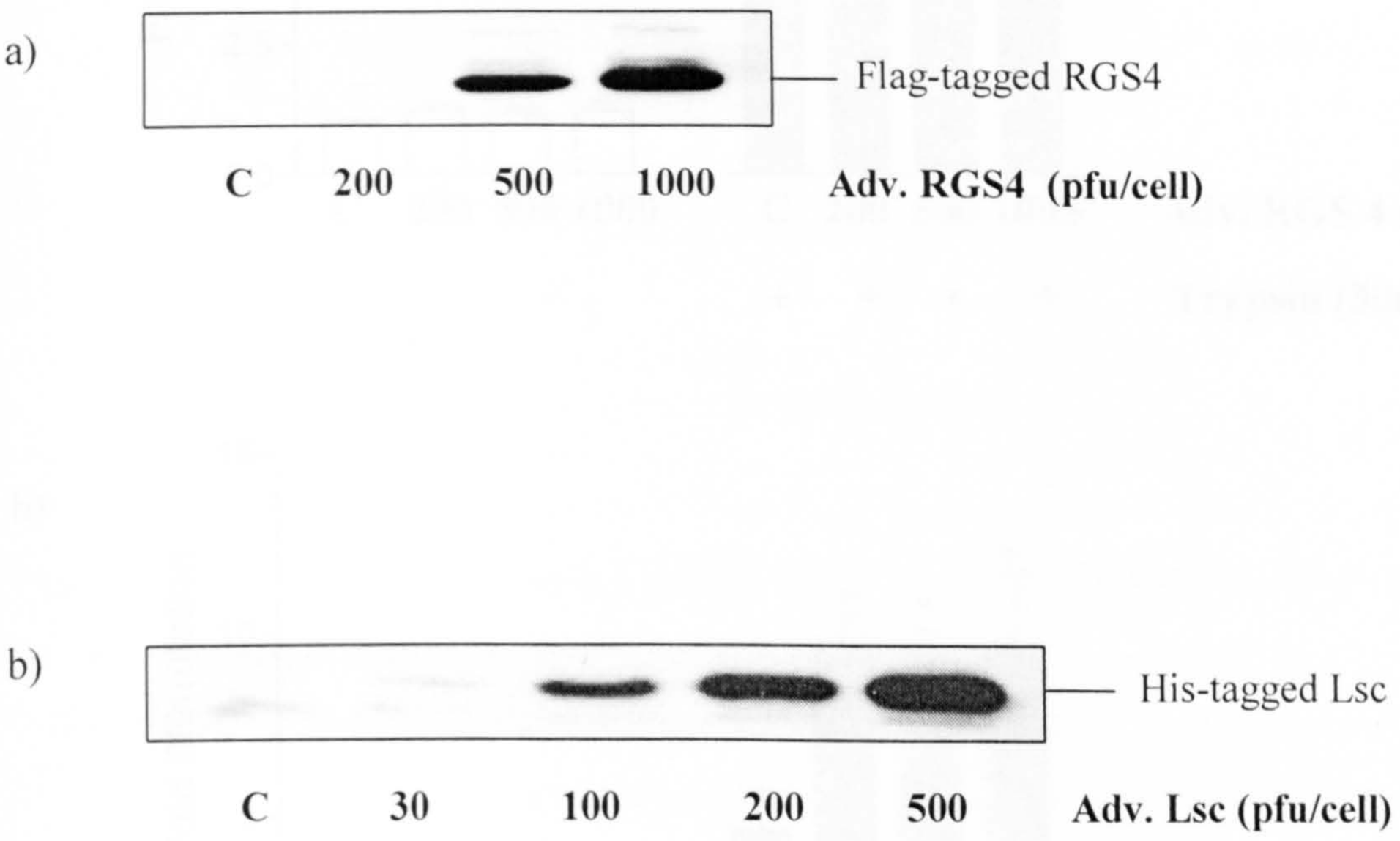
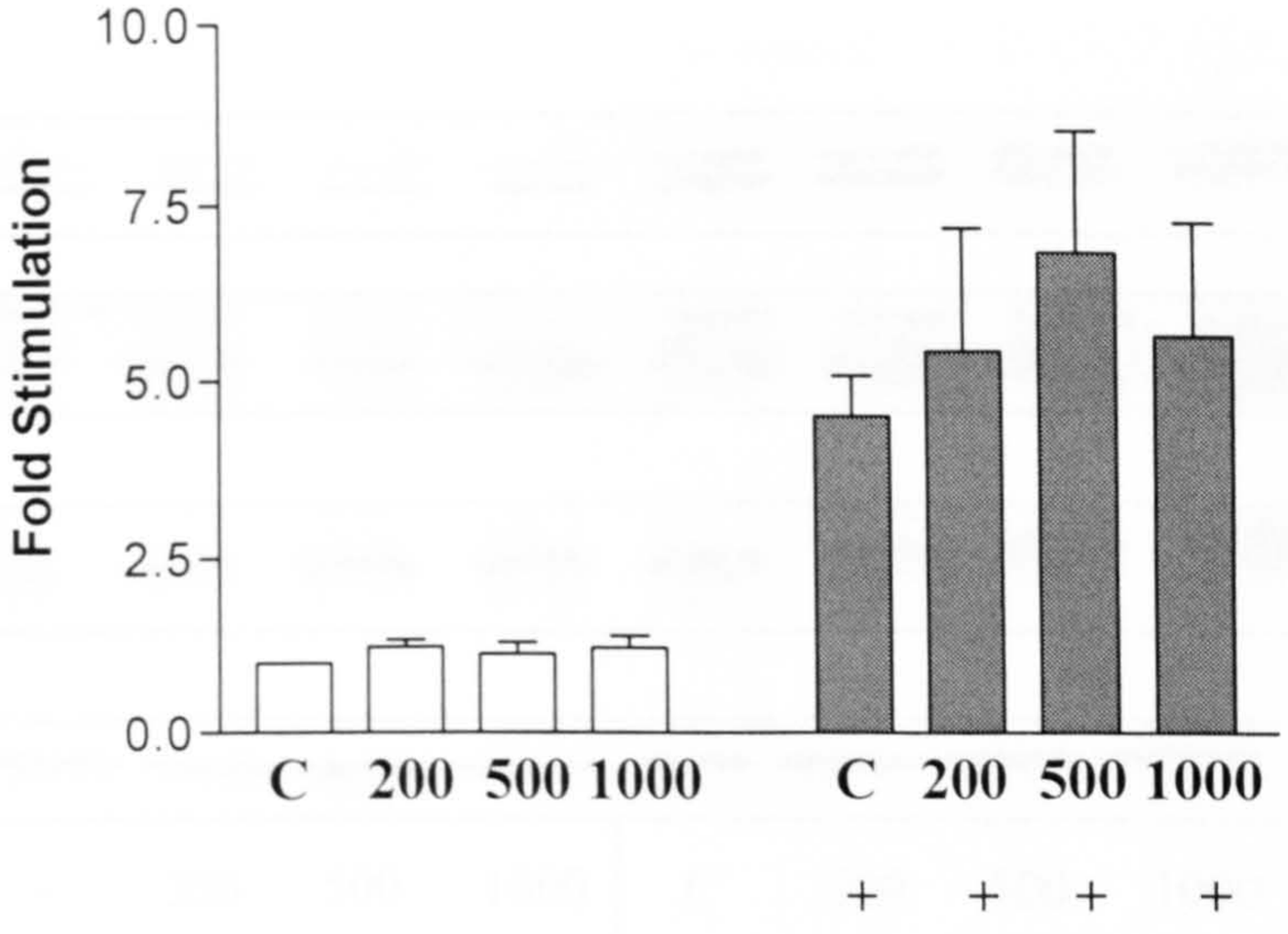


Figure 4.17. Overexpression of RGS4 and Lsc in clone G cells with recombinant adenoviral constructs

Cells were grown to 80% confluency and infected with increasing concentrations of adenoviral constructs encoding RGS4 and Lsc genes respectively for 48 hours as described (Section 2.4.5). Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2). The expression of (a) RGS4 was verified with an anti-FLAG antibody whereas (b) the level of Lsc expression was ascertained with an anti-His antibody. Blots shown are representative of 3 experiments.

a)



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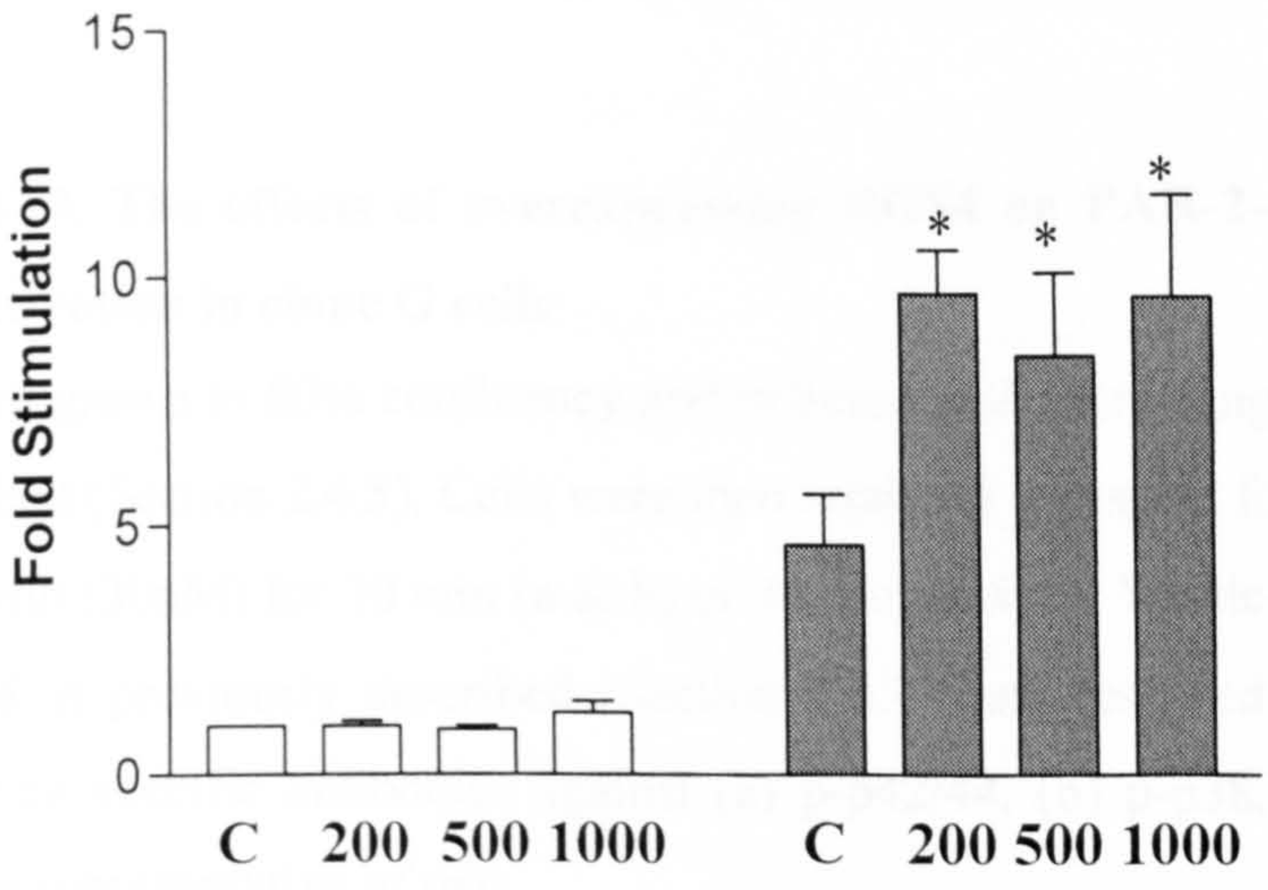


Figure 4.18. The effects of RGS4 and Lsc overexpression on PAR-2–mediated [^{3}H]-inositol phosphate accumulation in clone G cells

Cells were grown to 80% confluency and infected with increasing concentrations of adenoviral constructs encoding genes for (a) RGS4 and (b) Lsc respectively for 48 hours as described (Section 2.4.5). Cells were then prelabelled with [^{3}H]-myo-inositol for 18 hours before stimulation with agonist for 60 min. Accumulation of total [^{3}H]-inositol phosphate was measured as described (Section 2.5). Each value represents the mean $\pm$ s.e.m. from three separate experiments performed in triplicate. * $P<0.05$ compared with trypsin-stimulated control (+).

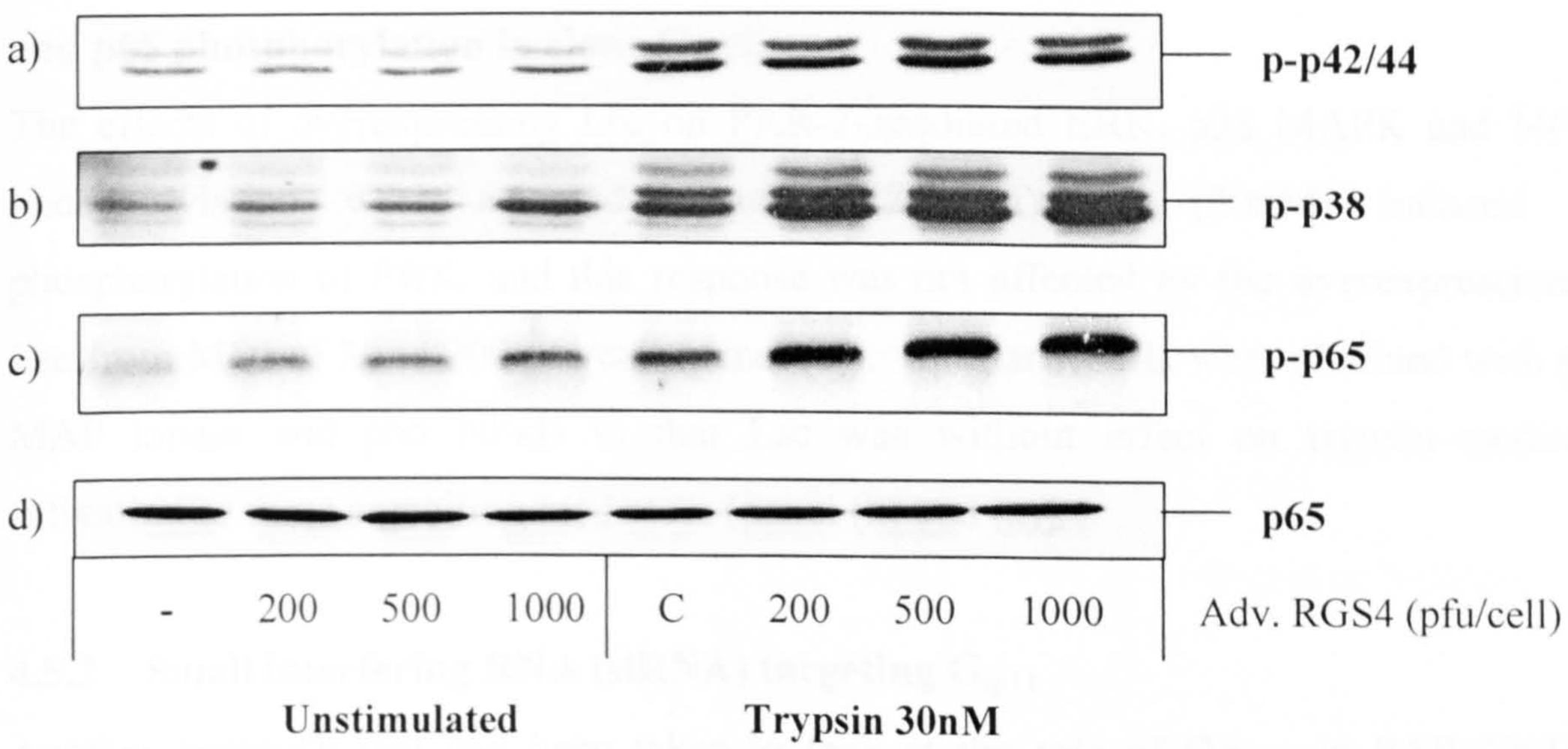


Figure 4.19. The effects of overexpressing RGS4 on PAR-2–mediated MAP kinases and NFκB activation in clone G cells

Cells were grown to 80% confluency and infected with increasing concentrations of Adv. RGS4 as described (Section 2.4.5). Cells were then rendered quiescent for 18 hours prior to stimulation with trypsin (30nM) for 10 min (a & b) or 30 min (c & d). Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2) using specific antibodies against (a) p-p42/44, (b) p-p38, (c) p-p65 and (d) p65. Blots shown are representative of two.

4.5.1.4 The effects of Lsc overexpression on PAR-2–induced p42/44, p38 MAPK and p65 phosphorylation in clone G cells

The effects of overexpressing Lsc on PAR-2–mediated ERK, p38 MAPK and NFκB phosphorylation were assessed (Figure 4.20). Trypsin (30nM) induced the phosphorylation of ERK, and this response was not affected by the overexpression of Lsc from MOI of 30 to 200 pfu/cell (panel (a)). Similar results were obtained with p38 MAP kinase and p65 NFκB in that Lsc was without effect on trypsin–mediated activation of these signalling mediators (panel (b) and (c)).

4.5.2 Small interfering RNA (siRNA) targeting $G_{q/11}$

Another approach that had been taken to look at the role of $G\alpha_{q/11}$ in PAR-2/NFκB signalling axis was small interfering RNA (siRNA). The siRNA targeting the overlapping regions of G_q and G_{11} with the sequence of 5'-AAGATGTTCGTGGACCTGAAC-3' was utilised as a tool to knock down the expression of these G proteins (Barnes *et al.*, 2005). The initial attempt of transiently transfecting clone G cells with siRNA using oligofectamine was not successful as the transfection efficiency appeared to be too low for any functional experiments (data not shown). In order to resolve this issue, an adenoviral construct encoding the respective siRNA sequence tagged with GFP was generated and the effects of Adv. siRNA upon PAR-2 signalling were determined.

4.5.2.1 Adenoviral construct encoding siRNA sequence targeting $G\alpha_{q/11}$

Figure 4.21 shows the results of infecting clone G cells with an increasing MOI of Adv. siRNA. Cells were infected with 1-100 pfu/cell of the adenoviral construct. Following this, the infection efficiency and functional expression of siRNA were ascertained by examining the levels of GFP (panel (a)) and $G\alpha_{q/11}$ (panel (b)) expression. It is clearly shown that there was increasing expression of GFP in a concentration-dependent manner. However, the endogenous levels of $G\alpha_{q/11}$ remained unaltered in the range of MOI tested. Similarly, PAR-2–induced phosphorylation of p65 NFκB was not affected even with infection of the Adv. siRNA up to 200 pfu/cell.

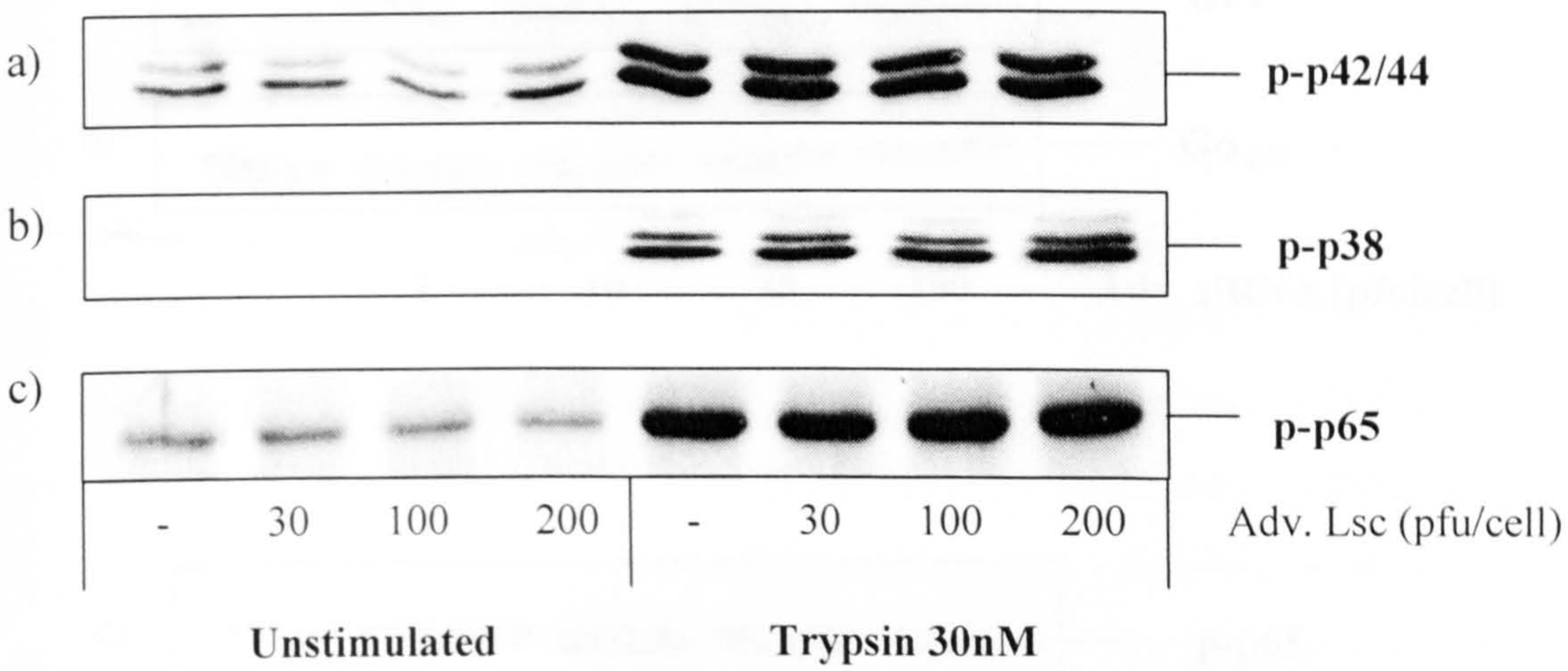
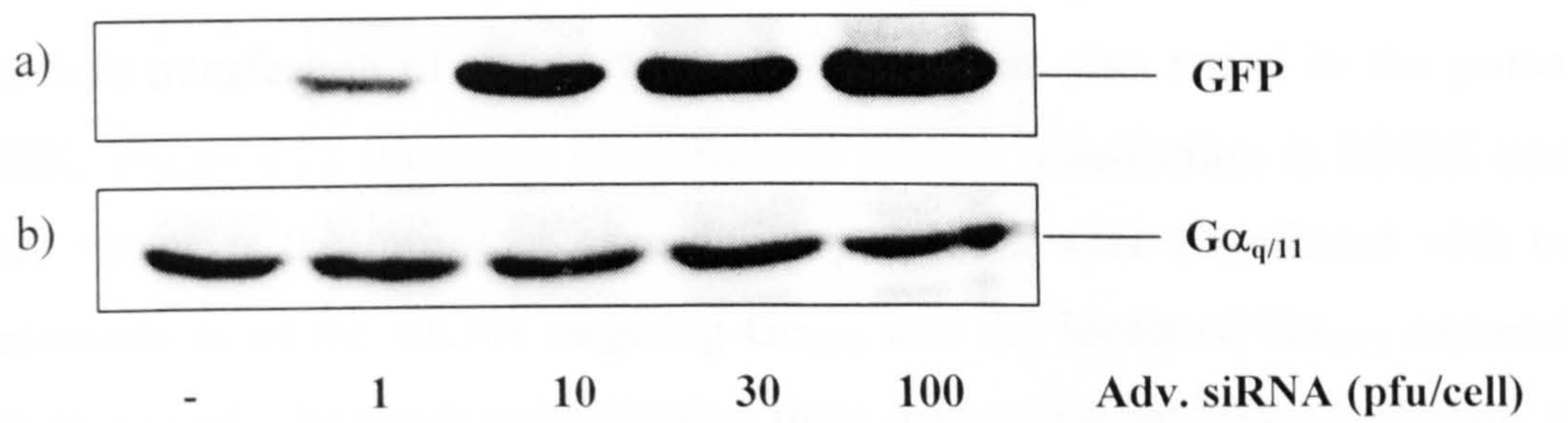


Figure 4.20. The effects of overexpressing Lsc on PAR-2-mediated MAP kinases and NFκB activation in clone G cells

Cells were grown to 80% confluency and infected with increasing concentrations of Adv. Lsc as described (Section 2.2.2). Cells were rendered quiescent for 18 hours prior to stimulation with agonist for 10 min (a & b) and 30 min (c) respectively. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2) using specific antibodies against (a) p-p42/44, (b) p-p38, and (c) p-p65. Blots shown are representative of two.



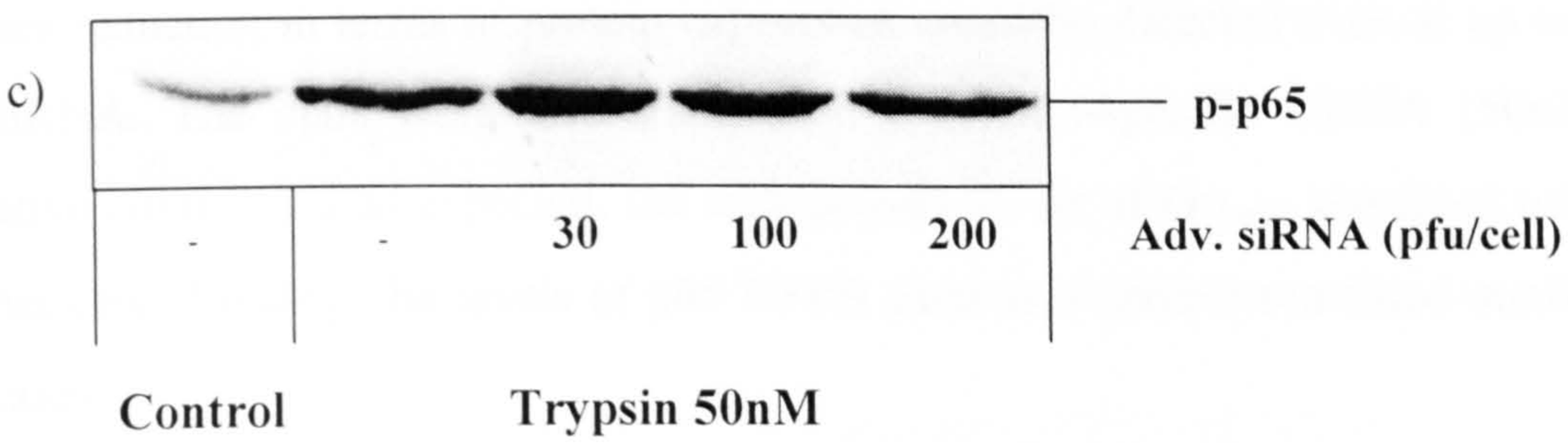


Figure 4.21. The effects of Adv. siRNA targeting Gα$_{q/11}$ in clone G cells

Cells were infected with increasing concentrations of the Adv. siRNA targeting Gα$_{q/11}$ for 48 hours prior to quiescence and stimulation with 50nM of trypsin for 15 min. Whole cell lysates were prepared and processed as previously described (Section 2.6.1) and resolved by Western blotting (Section 2.6.2) using specific antibodies against (a) GFP, (b) Gα$_{q/11}$ and (c) p-p65. Blots shown are representative of two.

4.5.2.2 Transient transfection of siRNA targeting $G\alpha_{q/11}$ in NHEK

Transient transfection of siRNA targeting $G\alpha_{q/11}$ was also tested in the primary cells, NHEK. Figure 4.22 illustrates the effects of siRNA transfection in NHEK using lipid-based vector as the means of gene delivery. NHEK were transfected with increasing concentrations of the siRNA targeting $G\alpha_{q/11}$ and the levels of $G\alpha_{q/11}$ expression were then examined. The result indicates that there was a concentration-dependent inhibition of $G\alpha_{q/11}$ expression in transfected cells, and maximal suppression of the protein level was observed to be at 30-50nM of siRNA, giving approximately 80% inhibition. No further reduction in terms of protein expression could be detected even at up to 100nM of siRNA. The cells were also transfected with non-specific siRNA (50nM) as a negative control and as expected, the endogenous levels of $G\alpha_{q/11}$ remained unchanged in this case. Notably, the levels of p65 NFκB used as a control remained unaffected in all cases.

4.5.2.3 The effects of transiently transfected siRNA targeting $G\alpha_{q/11}$ on PAR-2-induced p65 phosphorylation in NHEK

After acquiring information regarding the optimal concentration of siRNA required to produce maximal knock-down of $G\alpha_{q/11}$ expression, Figure 4.23 shows the effects of siRNA transfection on PAR-2–stimulated phosphorylation of p65. Transfection of NHEK with 50nM of either siRNA targeting $G\alpha_{q/11}$ or non-specific siRNA which serves as a control did not affect the basal activity of this signalling component. Upon stimulation with 50nM of trypsin for 15 or 30 min, responses of approximately 4 fold of basal control were obtained. Surprisingly, these responses remained unchanged in the presence of both types of siRNA.

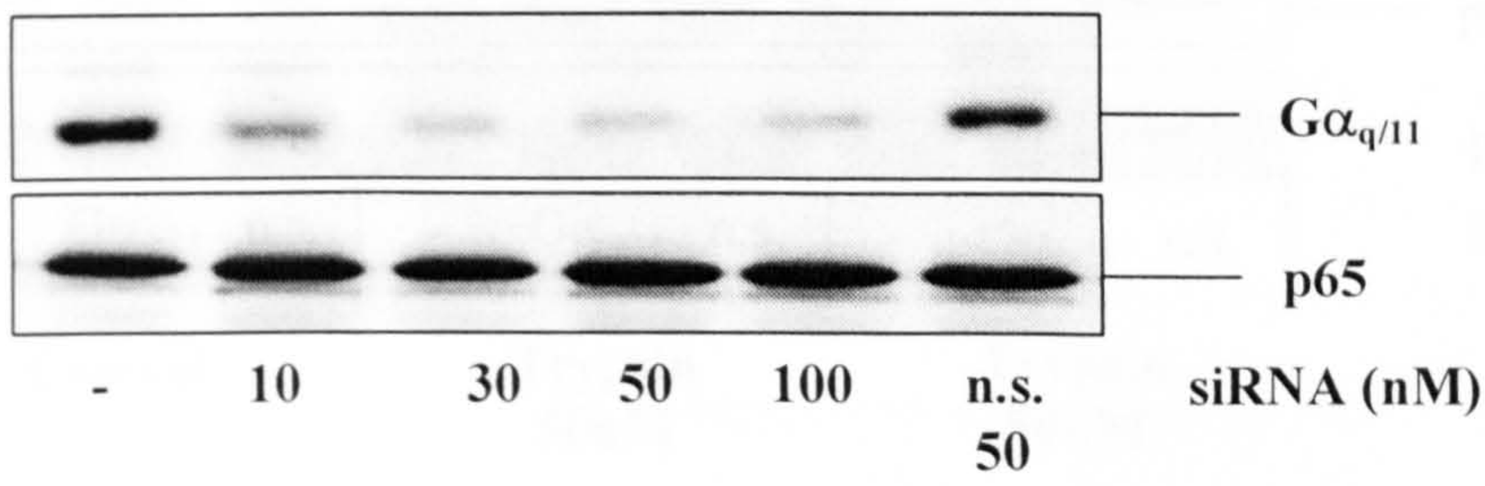


Figure 4.22. The effects of siRNA on the expression of $G\alpha_{q/11}$ in NHEK

NHEK were transiently transfected with increasing concentrations of siRNA targeting $G\alpha_{q/11}$ and non-specific siRNA (n.s.) for 72 hours. Whole cells lysates were then prepared as outlined (Section 2.6.1) and resolved by Western blotting (Section 2.6.2). Blots shown are representative of two.

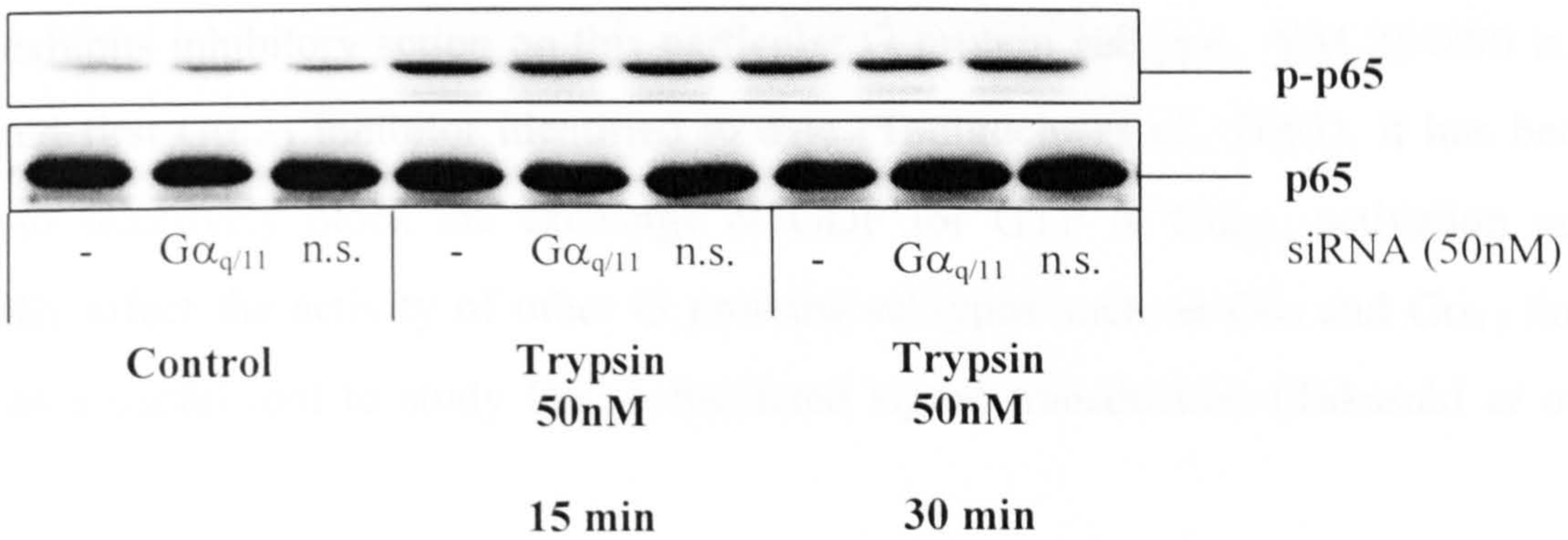


Figure 4.23. The effects of siRNA targeting $G\alpha_{q/11}$ on PAR-2–induced p65 phosphorylation in NHEK

Cells were transiently transfected with 50nM of the respective siRNAs for 48 hours prior to quiescence (n.s. = non-specific). Following this, cells were stimulated with trypsin (50nM) for the indicated times. Whole cell lysates were then processed as outlined (Section 2.6.1) and resolved by Western blotting (Section 2.6.2). Blots shown are representative of two experiments.

4.5.3 Pharmacological inhibition of $G\alpha_{q/11}$ activity using YM 254890

Another approach that has been employed in this study to investigate the role of $G\alpha_{q/11}$ in linking PAR-2 to NFκB cascade is the pharmacological tool, compound YM 254890, which exhibits inhibitory action on this particular G protein subtype. YM 254890 is a novel and first $G\alpha_{q/11}$ inhibitor identified to date (Taniguchi *et al.*, 2003). It has been shown to selectively block the exchange of GDP for GTP in $G\alpha_{q/11}$ activation and minimally affect the activity of other G proteins subtypes such as $G\alpha_i$ and $G\alpha_{15}$ thus serves as a useful tool to study $G\alpha_{q/11}$–mediated signal transduction (Takasaki *et al.*, 2004).

4.5.3.1 YM 254890 inhibits PAR-2-stimulated [^{3}H]-inositol phosphate accumulation in clone G cells

The initial characterisation of the effects of YM 254890 in clone G cells involved assessing the effects of this inhibitor on PAR-2–induced [^{3}H]-inositol phosphate production. Figure 4.24 shows the effects of YM 254890 over a range of 0.1 - 300nM on trypsin (50nM) and 2f-LIGKV-OH (30μM)–stimulated [^{3}H]-inositol phosphate production in clone G cells. Exposure of cells to either trypsin or the PAR-2 AP resulted in approximately a 22 fold increase in response. The magnitude of this response decreased with increasing concentrations of YM 254890, with maximal inhibition observed at ~100nM of the inhibitor, observed as approximately 95% inhibition or suppression of the response to ~2 fold of basal control. Notably, the IC_{50} exhibited by YM is 3.2 ± 0.6 nM and 2.6 ± 0.3 nM for the case of trypsin and AP respectively.

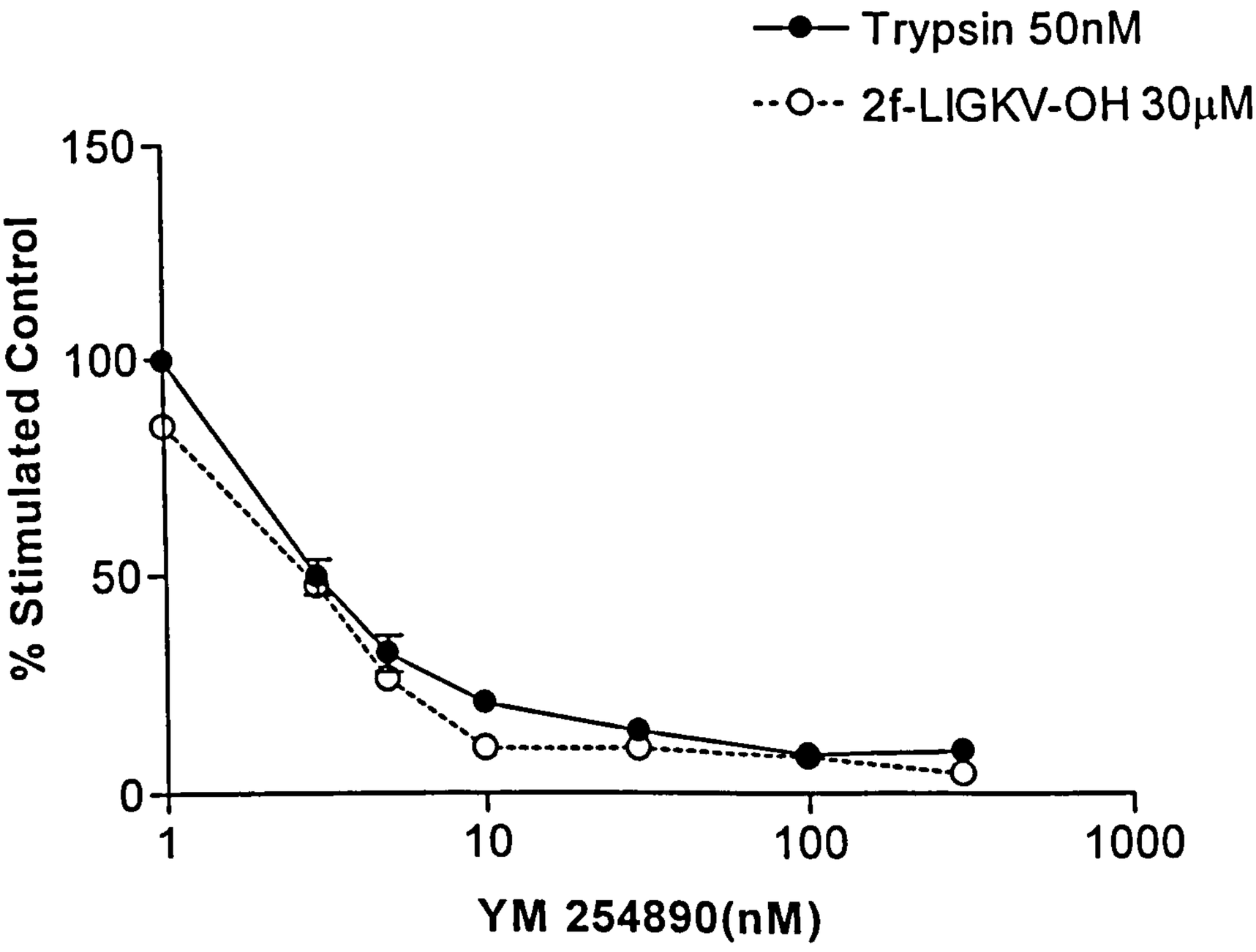


Figure 4.24. The effects of YM 254890 on PAR-2–mediated [^{3}H]-inositol phosphate accumulation in clone G cells

Cells were prelabelled with [^{3}H]-myo-inositol for 18 hours and incubated with 10mM of LiCl (10mM) for 15 min. Cells were then preincubated with increasing concentrations of YM 254890 for 15 min prior to stimulation with either trypsin (50nM) or 2f-LIGKV-OH (30μM) for a further 60 min. Accumulation of total [^{3}H]-inositol phosphate was measured as described (Section 2.5). Each value represents the mean ± s.e.m. (% stimulated control) from three separate experiments performed in triplicate.

4.5.3.2 The effects of YM 254890 on PAR-2–induced p65 phosphorylation in clone G cells

The role of $G\alpha_{q/11}$ in PAR-2/NFκB axis was then investigated using YM 254890 (1μM) at the level of p65 phosphorylation as illustrated in Figure 4.25. At time 15 min, the responses induced by trypsin (50nM) and 2f-LIGKV-OH (30μM) were significantly inhibited by YM 254890 (trypsin: 3.8 ± 0.3 to 1.6 ± 0.5 fold of basal control, % inhibition = 82.4 ± 16.8; AP: 4.0 ± 0.1 to 2.1 ± 0.5 fold of basal control, % inhibition = 63.7 ± 14.5, $P<0.05$ in both cases). Similar magnitude of responses were obtained when the cells were treated with the agonists for 30 min (~4 fold of basal control). These responses, however, were reduced only to a minor extent but not significantly (~20% inhibition) in both trypsin and AP-stimulated cells. PMA (100nM)-stimulated phosphorylation of p65 (4.1 ± 0.6 fold of basal control) remained unaltered by YM 254890.

4.5.3.3 The effects of YM 254890 on PAR-2–stimulated NFκB-DNA binding in clone G cells

YM 254890 was then utilised to elucidate the role of $G\alpha_{q/11}$ in PAR-2–stimulated NFκB-DNA binding in clone G cells as shown in Figure 4.26. Trypsin (50nM)-stimulated a response of 3.7 ± 0.3 fold of basal control which appeared to be minimally affected by 1μM of YM 254890. A similar profile was also observed in the case of PAR-2 AP in that the $G\alpha_{q/11}$ inhibitor had no effect on 2f-LIGKV-OH (30μM)–induced NFκB-DNA binding. PMA (100nM) or TNFα (20ng/ml) which have been employed as positive controls in this study, when added to cells, produced a response of approximately 7 fold of basal control which again remained unaltered by YM 254890.

4.5.3.4 The effects of YM 254890 on PAR-2–induced NFκB–driven transcription

PAR-2–stimulated transcription mediated via NFκB was then assessed in the presence of YM 254890 (1μM) as indicated in Figure 4.27. Addition of trypsin (50nM) to the cells

resulted in an increase of NFκB–driven transcription (23.6 ± 1.1 fold of basal control). The $G\alpha_{q/11}$ inhibitor significantly reduced the magnitude of this response to 12.9 ± 0.7 fold of basal control (% inhibition= 46.8 ± 5.1, P<0.01). Similarly, the NFκB–mediated luciferase expression stimulated by 2f-LIGKV-OH (30μM) (18.5 ± 1.2 fold of basal control) was also significantly suppressed by YM 254890 to 10.2 ± 1.0 fold of basal control (% inhibition = 47.6 ± 2.2, P<0.01). As expected, YM 254890 had no effect on both TNFα (20ng/ml) and PMA (100nM)–stimulated responses (7.0 ± 0.3 and 68.0 ± 1.4 fold of basal control respectively) in this context.

4.5.3.5 The effects of YM 254890 on PAR-2–mediated p65 phosphorylation in NHEK

The role of $G\alpha_{q/11}$ in coupling PAR-2 to p65 NFκB phosphorylation was also investigated in the primary cells, NHEK, in parallel with clone G using YM 254890. Figure 4.28 shows the effects of YM 254890 (1μM) on PAR-2-stimulated p65 phosphorylation at both 15 and 30 min post stimulation. Trypsin (50nM) and 2f-LIGKV-OH (30μM) induced the phosphorylation of p65 with similar magnitudes of response at time 15 min (5.0 ± 0.2 and 5.4 ± 0.2 fold of basal control respectively). These responses were significantly inhibited by YM 254890 (trypsin: 3.3 ± 0.3 fold of basal control, % inhibition = 41.3 ± 6.8; AP: 3.3 ± 0.5 fold of basal control, % inhibition = 47.0 ± 8.8, P<0.05 in both cases). At 30 min, phosphorylation of p65 stimulated by trypsin was also significantly reduced by YM 254890 (4.6 ± 0.4 to 3.0 ± 0.4 fold of basal control, % inhibition = 43.9 ± 7.1, P<0.05), whereas the response induced by PAR-2 AP at this time point although largely inhibited by YM 254890 did not reach statistical significance (6.0 ± 1.5 to 2.3 ± 0.3 fold of basal control, % inhibition = 66.6 ± 15.1). Again, YM 254890 did not affect the response stimulated by PMA (100nM) (8.3 ± 0.3 fold of basal control).

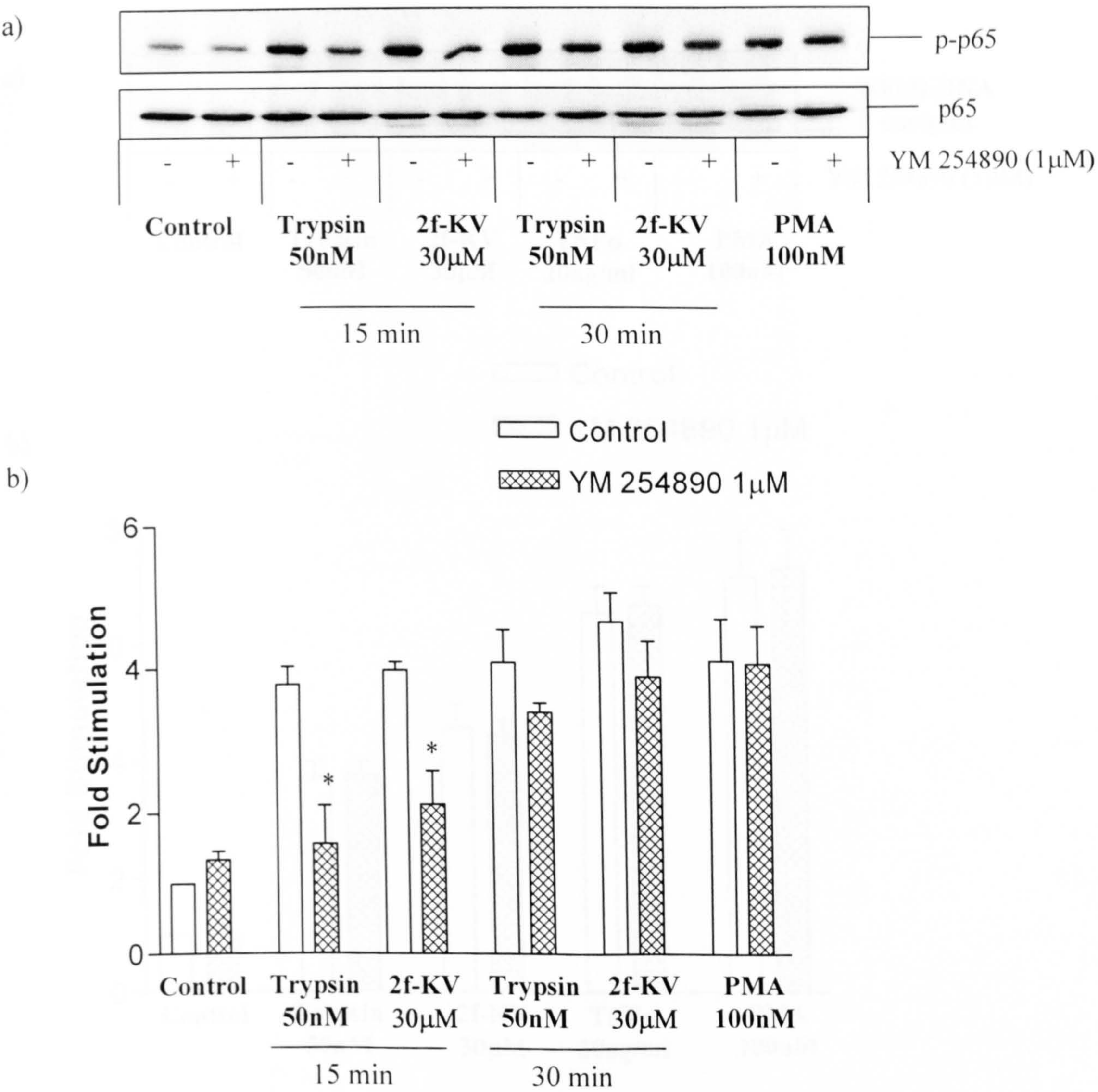
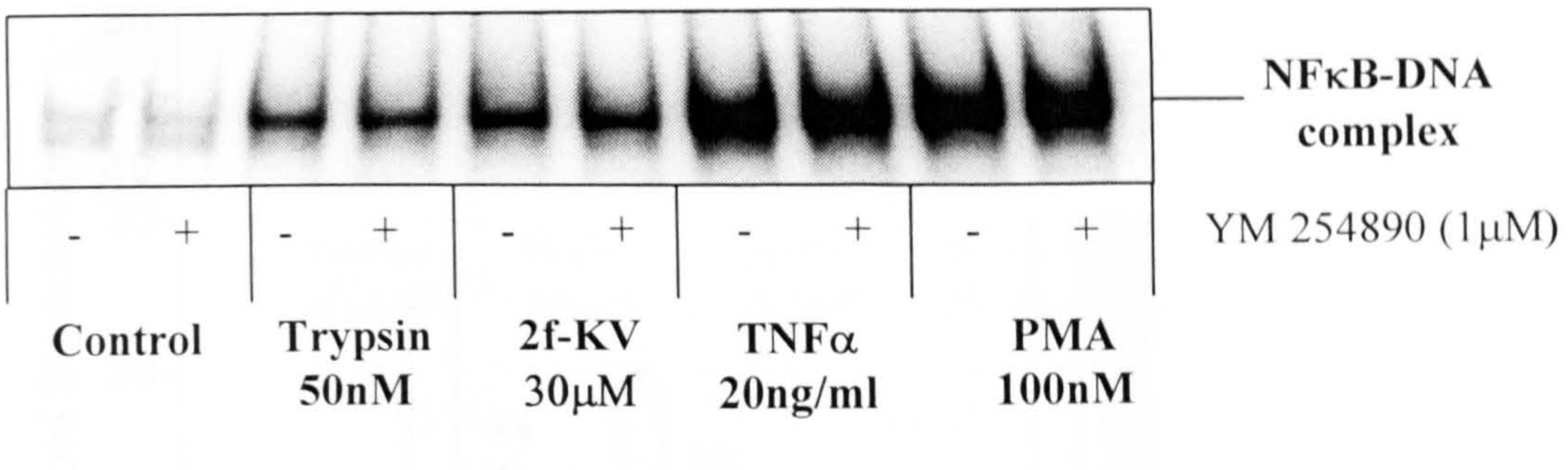


Figure 4.25. The effects of YM 254890 on PAR-2–mediated phosphorylation of p65 in clone G cells

Cells were rendered quiescent for 18 hours and preincubated with YM 254890 (1μM) for 15 min prior to stimulation with trypsin (50nM), 2f-LIGKV-OH (2f-KV) (30μM) or PMA (100nM) for the indicated times. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and visualised by Western blotting (Section 2.6.2). (a) Blots shown are representative of 3 individual experiments. (b) Blots were quantified by densitometry and expressed as mean ± s.e.m. (fold stimulation). *$P<0.05$ compared with agonist-stimulated control.

a)



b)

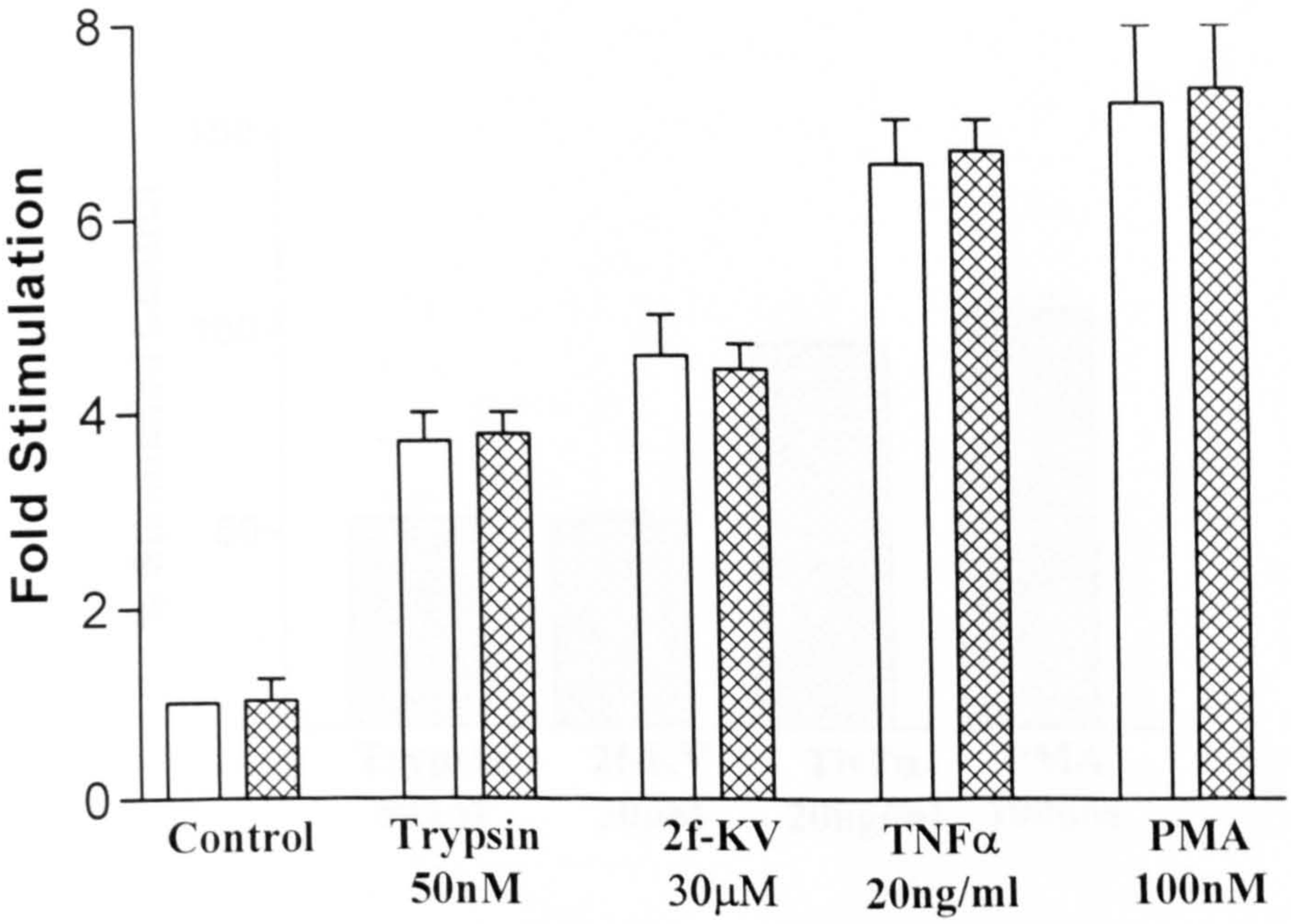
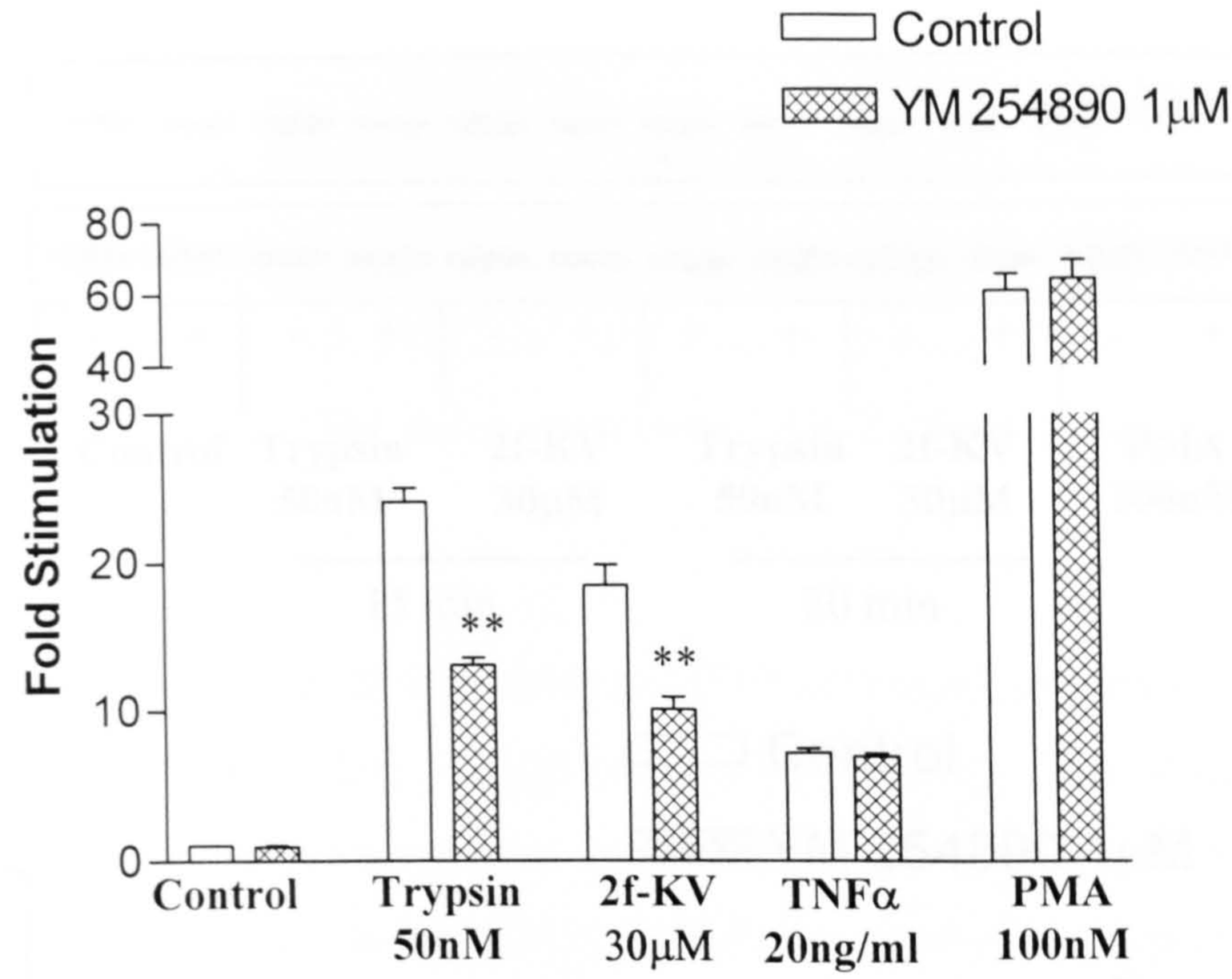


Figure 4.26. The effects of YM 254890 on PAR-2-stimulated NFκB-DNA binding

Cells were grown to confluence before being rendered quiescent with serum-free medium for 18 hours and preincubated with 1μM of YM 254890 for 15 min. Following this, cells were treated with trypsin (50nM), 2f-LIGKV-OH (2f-KV) (30μM), TNFα (20ng/ml) or PMA (100nM) for 60 min. Nuclear extracts were prepared and the NFκB-DNA binding was investigated by EMSA (Section 2.7). (a) The autoradiogram shown is the representative of two others. (b) Autoradiograms were quantified by densitometry and expressed as mean ± s.e.m. (fold stimulation).

a)



b)

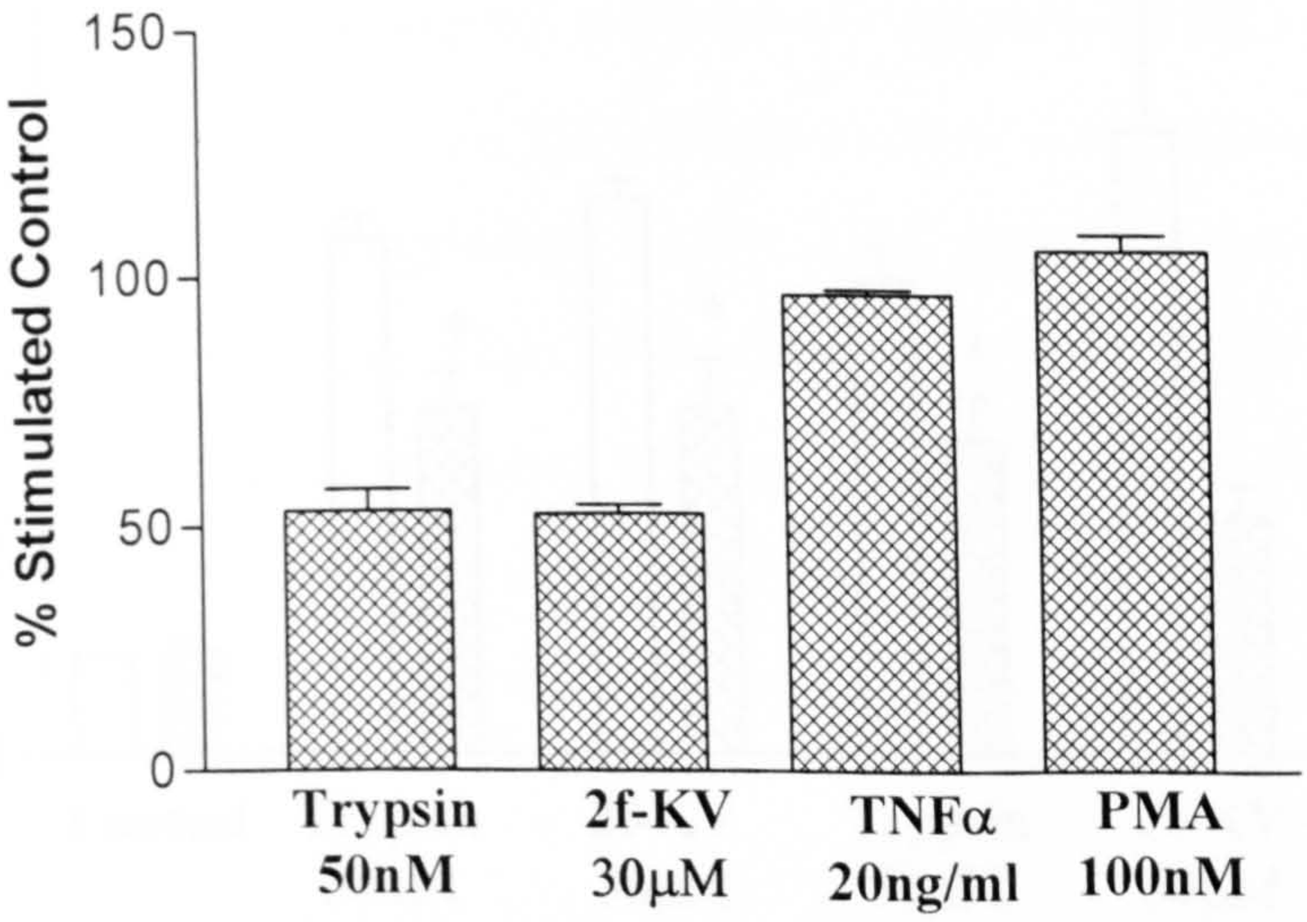


Figure 4.27. The effects of YM 254890 on PAR-2–induced NFκB–driven transcriptional activation in clone G cells

Cells were grown to confluency and rendered quiescent for 18 hours prior to preincubation with YM 254890 (1μM) for 15 min and stimulation with various agonists for 6 hours (2f-KV=2f-LIGKV-OH). Cells were then assayed as previously described (Section 2.8). Data shown are expressed as (a) fold over unstimulated basal, (b) % stimulated control; with each value represents the mean ± s.e.m. from four separate experiments. **P<0.01 compared with agonist-stimulated control.

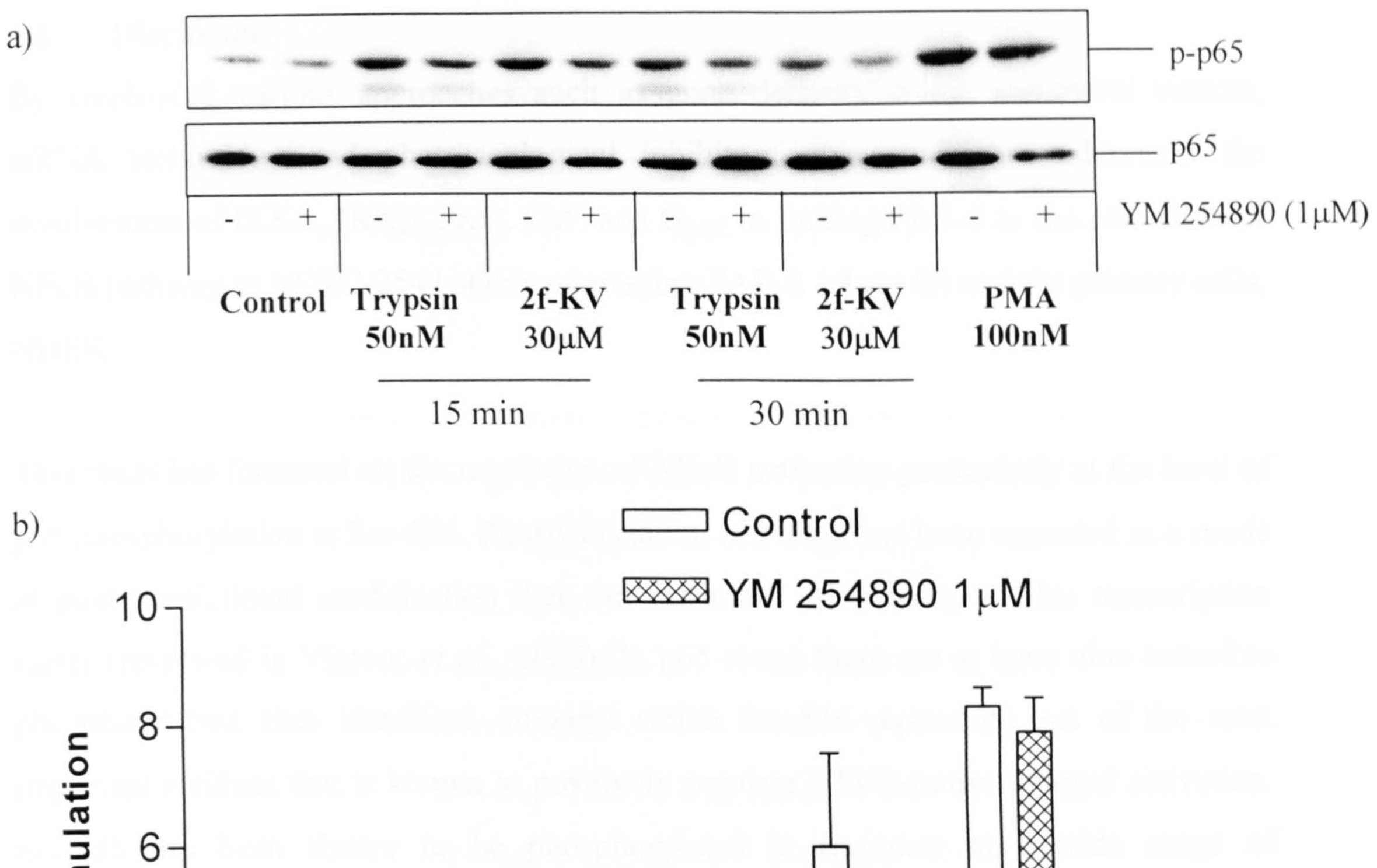


Figure 4.28. The effects of YM 254890 on PAR-2–mediated phosphorylation of p65 NFκB in NHEK

Cells were rendered quiescent for 18 hours and preincubated with YM 254890 (1μM) for 15 min prior to stimulation with trypsin (50nM), 2f-LIGKV-OH (2f-KV) (30μM) or PMA (100nM) for the indicated times. Whole cell lysates were prepared and processed as previously described (Section 2.6.1), and resolved by Western blotting (Section 2.6.2). Blots shown are representative of 3 individual experiments. (b) Blots were quantified by densitometry and expressed as mean ± s.e.m. (fold stimulation). *$P<0.05$ compared with agonist-stimulated control.

4.6 Discussion

By employing various approaches such as gene delivery using adenoviral vectors, siRNA technology and pharmacological inhibitors, this study has addressed the involvement of IKKα, IKKβ, PKC, Ca^{2+} and $G_{q/11}$ in linking PAR-2 to the activation of NFκB pathway in NCTC-2544 stably expressing PAR-2 (clone G) and the primary cells, NHEK.

This study has focussed on the regulation of NFκB activation particularly at the level of p65 phosphorylation at Ser-536. Phosphorylation of NFκB has been regarded as a mode of post-translational modification that can modulate the activity of this transcription factor (reviewed in Viatour *et al.*, 2005). In p65 alone there are at least nine inducible phosphorylation sites identified, amongst which Ser-536 represents one of the most important residues that is known to positively regulate NFκB-transcriptional activation. Ser-536 has been shown to be phosphorylated in response to a wide range of proinflammatory stimuli including TNFα, LPS, lymphotoxin and T-cell costimulation (Sakurai *et al.*, 1999; Yang *et al.*, 2003; Jiang *et al.*, 2003; Mattioli *et al.*, 2004). To date relatively little is known regarding the regulation of PAR-2/NFκB activation especially at the level of p65 phosphorylation, therefore this study sought to identify the upstream mediators involved in this context and thus NFκB-transcriptional activity.

In both clone G and NHEK, Adv. $IKK\beta^{+/-}$ significantly reduced PAR-2–mediated IκBα loss and p65 phosphorylation whilst Adv. $IKK\alpha^{+/-}$ appeared to play minimal role in this context. In addition, PAR-2-stimulated NFκB-DNA binding and transcriptional activation were markedly inhibited only by Adv. $IKK\beta^{+/-}$. These data clearly indicate that IKKβ plays a selective role in the PAR-2/NFκB signalling axis in keratinocytes whereas IKKα seems dispensable in this context.

In the NFκB pathway, almost all known inducers of NFκB such as cytokines cause the cellular degradation IκBα (reviewed in Ghosh & Karin, 2002). The results obtained in

this study indicate that PAR-2 stimulates the classical NFκB pathway by removing IκBα to permit liberation of this transcription factor via the action of IKKβ and not IKKα, which is consistent with the literature as biological and genetic studies have indicated that IKKβ is the dominant kinase involved in inducing cellular degradation of IκBα (Ghosh & Karin, 2002).

Similarly, this study has also provided evidence that IKKβ plays a selective role in mediating PAR-2–induced p65 phosphorylation on Ser-536 whereas IKKα has no apparent role in this context. Previous literature has documented the ability of both catalytic subunits in phosphorylating Ser-536 of p65 in different experimental models (Jiang *et al.*, 2003; Sakurai *et al.*, 2003; Jeong et *al.*, 2005; Mattioli *et al.*, 2004). Notably, some other studies have implicated the distinct roles of these kinases in that IKKβ is involved in mediating IκBα degradation whereas IKKα regulates phosphorylation of p65 (O' Mahony *et al.*, 2004). The results presented here fit into the model suggested by other groups in that IKKβ, being the best characterised kinase for Ser-536 of p65, plays dual roles in the NFκB cascade by mediating IκBα degradation and phosphorylation of p65 whereas IKKα is not essential in this context (Sakurai *et al.*, 1999; Schwabe *et al.*, 2001). Such effects by IKKβ could be modulated directly by this kinase subunit or via other kinases which are under the regulation of IKKβ and certainly more investigation is needed to shed light on this matter. Another important implication from this study is that as the IKKα mutant did not affect NFκB-DNA binding and transcriptional activation stimulated by PAR-2 in clone G, this receptor does not utilise the non-canonical, IKKα-dependent pathway to signal to NFκB cascade via processing of the precursor protein p100. Thus far, this is the first report describing the selective role of IKKβ in mediating PAR-2-induced IκBα loss and phosphorylation of p65 in keratinocytes.

Notably, previous findings have proposed the activation of both IKKα and IKKβ in response to PAR-2 stimulation in clone G cells (Kanke *et al.*, 2001; Macfarlane *et al.*,

2005). Although findings from the present study have ruled out the role of IKKα in the PAR-2/NFκB pathway, IKKα has been shown to mediate epidermal differentiation independent of NFκB pathway (Hu *et al.*, 2001). Thus it remains to be determined if PAR-2-stimulated IKKα activation has any functional significance in skin biology and/or other downstream signalling cascade(s).

The upstream activators of the IKK complex activation and p65 phosphorylation in PAR-2/NFκB signalling remain largely unknown. As previous reports have supported the profound roles of PKC and intracellular calcium in PAR-2-stimulated IKK complex activation and NFκB-DNA binding, this study then investigated the effects of these signalling components on the phosphorylation of p65 (Kanke *et al.*, 2001, Macfarlane *et al.*, 2005).

The PKC isoforms are classified into three groups, namely the conventional (PKCα, PKCβ and PKCγ), novel (PKCδ, PKCε, PKCη, PKCμ and PKCθ) and atypical (PKCζ, PKCι and PKCλ). The conventional isoforms are both calcium and DAG-responsive; the novel group is only DAG-dependent whereas the atypical class is not regulated by either calcium or DAG (reviewed in Liu & Heckman, 1998). A large body of evidence has indicated the involvement of PKC isoforms in response to a wide range of stimuli in regulating NFκB activation, and members from each class have been implicated (Folgueira *et al.*, 1996; Anrather *et al.*, 1999; Lallena *et al.*, 1999; Vancurova *et al.*, 2001; Asehnoune *et al.*, 2005). Several PKC isotypes have been identified in the skin, including the conventional PKCα and PKCβ; the novel PKCδ, PKCε, PKCη and PKCμ; as well as the atypical PKCζ (Dlugosz *et al.*, 1992; Fisher *et al.*, 1993; Rennecke *et al.*, 1999). It has been postulated that PKC isoforms can directly activate IKK complex by inducing phosphorylation of the catalytic subunits, as exemplified by PKCα and PKCζ (Lallena *et al.*, 1999), thus it is rational to investigate if PKC lies upstream of the IKK complex in the PAR-2/NFκB cascade. In order to do this, GF 109203X and

rottlerin which are broad range and selective PKC inhibitors respectively have been utilised in this study.

GF 109203X is a potent compound that inhibits several Ca^{2+}-dependent and independent PKC isoforms including PKCα, PKCβ, PKCγ, PKCδ and PKCε (Toullec *et al.*, 1991). Interestingly, GF 109203X only partially inhibited p65 phosphorylation induced by PAR-2 at 15 min but exerted minimal effect at 30 min in clone G. This indicates that those conventional and novel PKC isoforms present in keratinocytes which are under the regulation of GF 109203X such as PKCα, PKCβ, PKCδ and PKCε might regulate p65 phosphorylation in a cooperative manner with other as yet unidentified components. The inhibitory effect of GF 109203X on PAR-2–mediated NFκB-DNA binding was also incomplete, again suggesting the existence of other regulatory mechanisms. A slightly different profile was obtained in NHEK in that p65 phosphorylation at both time points was sensitive to GF 109203X, indicating that the phosphorylation of p65 in the primary cells is relatively more PKC-dependent than that of clone G. In order to further elucidate if PKCδ is the key player in this case, rottlerin which is a selective PKCδ inhibitor has been utilised (Gschwendt *et al.*, 1994). Notably, rottlerin inhibited PAR-2-stimulated p65 phosphorylation and NFκB-DNA binding, strongly suggesting the involvement of this PKC isotype.

On the other hand, intracellular calcium which serves as an activator for selective PKC isoforms has previously been shown to mediate PAR-2-induced NFκB-DNA binding (Macfarlane *et al.*, 2005). This study presented the novel evidence that in addition to inhibiting the binding of NFκB to DNA, intracellular calcium chelation also abrogated phosphorylation of p65 stimulated by PAR-2 agonists, indicating the involvement of a highly calcium-responsive element in this case. However, this does not agree with the observation of PKCδ which is a calcium-independent component in having a role in the PAR-2/NFκB signalling, but in fact points to other calcium-dependent PKC isoforms such as PKCα and PKCβ. This remains difficult to explain. One possible explanation is

that rottlerin may inhibit other components such as CaMKIII which has been reported elsewhere (Parmer *et al.*, 1997). In fact calcium/calmodulin–dependent kinase (CaMK) such as CaMKIII could possibly lie in the axis linking PAR-2 to the NFκB cascade as other CaMK isoforms such as CaMKII and CaMKIV have been shown to phosphorylate p65 (Nairn & Picciotto, 1994; Bird *et al.*, 1997; Bae *et al.*, 2003). However, this might not seem very likely as previous work in the lab has proposed that the PAR-2/NFκB pathway appeared to be calcineurin and CaMKII–independent (Macfarlane *et al.*, 2005). Taken together, the identity of the PKC isotypes involved remains to be investigated and perhaps dominant negative proteins or RNAi technology to specifically knock-down the respective components would help to clarify this matter.

Following the establishment of the roles of intracellular calcium and PKC in linking PAR-2 to NFκB signalling, several approaches were taken in order to investigate the possible upstream mediators. It is a well-known fact that calcium mobilisation and PKC activation serve as the downstream effectors of PLCβ. In fact, previous findings have reported that inhibition of PLCβ activity abrogated PAR-2/NFκB activation (Macfarlane *et al.*, 2005). Therefore the further upstream key player in coupling PAR-2 to NFκB cascade would be the G protein that can effect PLCβ activation. Consistent with previous studies, the results obtained indicate that PAR-2-stimulated [^{3}H]-inositol phosphate accumulation is PTX-insensitive, implying the contribution of $G\alpha_{q/11}$ in this context (Nystedt *et al.*, 1994, Santulli *et al.*, 1995, Böhm *et al.*, 1996b). In order to investigate the role of $G\alpha_{q/11}$ in the regulation of PAR-2/NFκB signalling, this project has utilised adenovirus encoding RGS proteins, siRNA technology and the pharmacological tool, YM 254890, to modulate $G\alpha_{q/11}$ activity.

RGS4 which is a well-known GAP for $G\alpha_{q/11}$ and $G\alpha_i$ has been employed to modulate PAR-2-associated $G\alpha_{q/11}$ activity (Berman *et al.*, 1996). To date no evidence has suggested the coupling of PAR-2 to $G\alpha_{12/13}$, thus the GAP protein for $G\alpha_{12/13}$, Lsc, has been utilised as a control in the absence of an equivalent control (Kozasa *et al.*, 1998).

Surprisingly, infection of the cells with Adv. RGS4 up to 1000 pfu/cell did not alter the accumulation of inositol phosphate stimulated by PAR-2 agonists. These data highlighted that RGS4 did not serve as the GAP protein for PAR-2 associated $G\alpha_{q/11}$. Adv. Lsc also did not affect the activity of the signalling components measured, except in increasing PAR-2-induced inositol phosphate accumulation through its non-specific action(s).

RGS4 has been shown to inhibit $G\alpha_{q/11}$ activation associated with multiple GPCRs including muscarinic M_3, bombesin and cholecystokinin receptor (Rumenapp *et al.*, 2001; Xu *et al.*, 1999; Tovey & Willars, 2004). However, recent studies have highlighted the possibility that RGS proteins could be selective in modulating different types of $G\alpha_{q/11}$-coupled receptors (Zeng *et al.*, 1998; Xu *et al.*, 1999, Tovey & Willars, 2004). For example, in a study which utilised RGS2, RGS3 and RGS4 which were all GAP proteins against $G\alpha_{q/11}$, only RGS2 and RGS3 showed inhibitory effects on signalling transduced by $G\alpha_{q/11}$-coupled-muscarinic M_3 receptor, but not RGS4 (Tovey & Willars, 2004). Zeng *et al.* (1998) reported that the amino-terminal of RGS4 is critical in conveying selective $G\alpha_{q/11}$-coupled receptor inhibition. A later study proposed that selective-receptor inhibition of the RGS proteins could be a direct consequence of the physical interactions between the receptor and the RGS protein (Bernstein *et al.*, 2004). The authors reported that RGS2 was selectively recruited to M_1 but not M_2 muscarinic receptors via its amino terminal to exert a negative regulatory effect on G protein coupling. This paradigm could be extended to other GPCRs, for example PAR-2, as the data reported here implies that RGS4 played no role in modulating signals transduced by $G\alpha_{q/11}$-coupled PAR-2. Nevertheless, the identity(ies) of the RGS protein(s) that can selectively interact with PAR-2 require further investigation. $G\alpha_{12/13}$ has always been implied in cell morphology and actin cytoskeleton changes (Riobo & Manning, 2005). The lack of effect of Adv. Lsc on PAR-2-mediated signalling indicates that unlike PAR-1 (Section 1.2.1.3), PAR-2 does not signal through $G\alpha_{12/13}$ thus consistent with the fact that limited evidence has supported the role of $G\alpha_{12/13}$ in governing PAR-2 signalling.

Since utilising RGS4 as an inhibitory tool to modulate PAR-2–mediated responses was unsuccessful, an alternative approach is to employ siRNA targeting $G\alpha_{q/11}$. Messenger RNA (mRNA) transcribed from DNA will serve as the template for protein synthesis during the process of translation. RNA interference (RNAi) is a post-transcriptional, specific gene-silencing technique achieved via the degradation of the target mRNA and is now recognised as a novel tool to inhibit the protein expression of a targeted gene (reviewed in Stanislawska & Olszewski, 2005). In this study, the siRNA with the sequence 5'-AAGATGTTCGTGGACCTGAAC-3' targeting the overlapping regions of $G\alpha_q$ and $G\alpha_{11}$ between nucleotides 931 and 951 was utilised (Barnes *et al.*, 2005). Transient transfection of this siRNA has been previously shown to effectively knock-down $G\alpha_{q/11}$ expression and inhibited both angiotensin and muscarinic receptor–stimulated signal transduction (Barnes *et al.*, 2005; Atkinson *et al.*, 2006).

The initial characterisation involved transfecting the cells with increasing concentrations of $G\alpha_{q/11}$ siRNA in clone G cells to ascertain the down-regulation of protein expression (data not shown). Even at the concentration of 100nM, the endogenous expression of $G\alpha_{q/11}$ was only partially inhibited (~20%) compared to control. This is possibly due to poor transfection efficiency in clone G cells. In order to solve this problem, adenoviral construct encoding $G\alpha_{q/11}$ siRNA tagged with GFP was established.

Clone G cells were then infected with increasing concentrations of Adv. siRNA targeting $G\alpha_{q/11}$ and the levels of protein expression were determined. The results clearly show increasing GFP expression with increasing concentrations of Adv. siRNA. However, the expression of $G\alpha_{q/11}$ remained unchanged over the range of Adv. siRNA tested. This indicates that the infection has taken place but somehow siRNA did not act to reduce the protein level of $G\alpha_{q/11}$, an unexplained experimental defect. Therefore the attempt to deliver siRNA targeting $G\alpha_{q/11}$ in clone G cells using the adenoviral construct was not successful.

Transient transfection of $G\alpha_{q/11}$ siRNA was also tested in the primary keratinocytes, NHEK, to assess the transfection efficiency. The data obtained indicates substantial inhibition of $G\alpha_{q/11}$ expression with ~30nM of siRNA. As expected, the non-specific siRNA that has been employed as a positive control did not alter $G\alpha_{q/11}$ expression. When examined at the level of p65 phosphorylation, however, both $G\alpha_{q/11}$ and non-specific siRNA had no effect on the responses induced by trypsin. This implies that $G\alpha_{q/11}$ might not lie in the axis of PAR-2/NFκB. Another possibility is that the silencing effect of siRNA was not complete and the residual $G\alpha_{q/11}$ was sufficient to transmit the signal stimulated by PAR-2. Thus further studies using functional adenoviral constructs encoding siRNA targeting $G\alpha_{q/11}$ or cells derived from transgenic mice lacking $G\alpha_{q/11}$ would be useful to shed some light on this matter.

Another approach that has been employed to modulate $G_{q/11}$ activity is by using the pharmacological tool, YM 254890. YM 254890 is a cyclic depsipeptide isolated from the culture broth of *Chromobacterium sp.* QS3666 (Taniguchi *et al.*, 2003). Initial characterisation of this compound provided evidence of its inhibitory effect on ADP–induced platelet aggregation with an IC_{50} value of less than 0.4μM. Further investigation demonstrated that YM 254890 acts specifically on $G\alpha_{q/11}$ to block the exchange of GDP for GTP and had minimal effect on calcium mobilisation stimulated by $G\alpha_i$ or $G\alpha_{15}$ (Takasaki *et al.*, 2004). These results highlight the fact that YM 254890 is a novel, selective and first-of-its kind inhibitor for $G\alpha_{q/11}$ thus serves as a useful tool in assessing the signalling paradigm mediated by this G protein subtype.

The effects of YM 254890 on PAR-2 induced signalling were first tested at the level of total inositol phosphate accumulation. As production of IP_3 is the classical downstream component of $G\alpha_{q/11}$ activation, inhibition of this G protein can be reflected at the level of inositol phosphate accumulation. YM 254890 showed an inhibitory effect in a concentration-dependent manner with the IC_{50} value observed to be ~ 3nM, indicating that YM 254890 indeed serves as a potent inhibitor for PAR-2–associated $G\alpha_{q/11}$

activity. Also, the IC_{50} reported herein is about 50 times higher than the effects of YM 254890 on calcium mobilisation mediated by muscarinic and leukotriene receptors (Takasaki *et al.*, 2004).

Following this, PAR-2–stimulated NFκB activation at the levels of p65 phosphorylation, NFκB-DNA binding and transcriptional activation were assessed in the presence of 1μM YM 254890. Notably, PAR-2–stimulated phosphorylation of p65 was only partially abolished by YM 254890 at time 15 min, and the responses at 30 min were only marginally reduced. These results suggest that $G\alpha_{q/11}$ plays only a partial role in the early phase of p65 phosphorylation mediated by PAR-2, proposing that possibly only a sub population of p65 phosphorylation at Ser-536 is governed by $G_{q/11}$ and other yet to be identified regulatory mechanism(s) might be involved in this context. Unexpectedly, YM 254890 had no effect at all on NFκB-DNA binding in response to PAR-2 agonists, denoting that PAR-2–associated $G\alpha_{q/11}$ had no role in governing the binding of NFκB to DNA, and presumably to have no impact on the degradation of IκBα protein and translocation of this transcription factor into the nucleus.

Although YM 254890 did not affect NFκB-DNA binding, it clearly reduced PAR-2-stimulated NFκB-transcriptional activation. This could be explained by its effect on NFκB phosphorylation as post-translational modifications of NFκB such as phosphorylation or acetylation have been shown to secondarily mediate the transactivation ability of this transcription factor (Yang *et al.*, 2003; Doyle *et al.*, 2005). However, relatively little information is known regarding how phosphorylation at Ser-536 increases the transactivation ability of p65; possibilities such as interaction with other transcription factord and/or conformational change remain to be investigated.

This project has thus assessed the functional importance of $G\alpha_{q/11}$, calcium mobilisation, PKC and IKKβ on linking PAR-2, which is a GPCR, to the NFκB pathway. A large number of GPCRs have been shown to signal to NFκB cascade (discussed in Section

1.4.6). The general signalling paradigm that has been proposed for NFκB activation induced by GPCRs involves initiation of the signal through heterotrimeric G proteins which will then lead to distinct signalling pathways that will converge at the level of cellular IκBα degradation (reviewed in Ye, 2001). Unlike cytokine receptors, the precise regulation of GPCRs-mediated NFκB activation remains largely undefined. For example, the IKK complex which represents the key player for most cytokine-induced NFκB activation regime has only been implicated in a number of GPCRs such as PAR-1, M_3-muscarinic and bradykinin receptors (Rahman *et al.*, 2002, Todisco *et al.*, 1999; Xie *et al.*, 2000). Also, relatively little is known regarding phosphorylation of p65 induced by GPCRs. Thus this study has provided the direct evidence of IKKβ having a selective role in mediating PAR-2-stimulated NFκB activation including at the level of p65 phosphorylation.

In terms of heterotrimeric G proteins, almost each member from each class has been implicated in the regulation of NFκB activation (Ye, 2001). Several GPCRs have been shown to activate NFκB via $G\alpha_{q/11}$, for example PAR-1 and bradykinin receptor have been reported to stimulate NFκB-driven transcriptional activation through this G protein subtype (Rahman *et al.*, 2002, Xie *et al.*, 2000). Notably, the classical effectors of $G\alpha_{q/11}$, PKC and calcium mobilisation, have also been strongly implicated in stimulating NFκB activation at NFκB-DNA binding level and/or transcriptional activation for several other GPCRs including lysophosphatidic acid and neurotensin receptor, although the role of $G\alpha_{q/11}$ was not assessed in these studies (Shahrestanifar *et al.*, 1999; Zhao *et al.*, 2005). Therefore the signalling paradigm of PAR-2/NFκB reported herein shows a large degree of overlapping with those reported in the literature.

The $G\alpha_{q/11}/Ca^{2+}$/PKC/IKKβ axis delineated in this study, however, does not appear to be a linear signalling paradigm. This is because Ca^{2+}/PKC/IKKβ seem to have an impact on both p65 phosphorylation and NFκB-DNA binding; whereas $G\alpha_{q/11}$ activation has a role only on the former but not the latter.

One critical question arises as to whether or not $G\alpha_{q/11}$ is the sole source of PAR-2-induced calcium mobilisation. If calcium is the principal downstream component of $G\alpha_{q/11}$, both calcium and $G\alpha_{q/11}$ should share a similar functional profile in this context. However, in this case it appears that PAR-2 might utilise other ion channels to result in calcium mobilisation. For example, novel evidence has suggested the interaction between GPCRs and calcium channel, in that the β_2–adrenergic receptor was found to form a macrocomplex with the L-type calcium channel (Davare *et al.*, 2001). The multiprotein complex detected was found to consist of G protein, adenylyl cyclase and PKA; and the authors concluded that GPCRs such as β_2–adrenergic receptor is able to act as a scaffold protein to assemble the signalling molecules to achieve signal transduction rapidity as supported by later findings (Liu *et al.*, 2004). Thus it is possible that PAR-2 might directly associate with calcium channels in the presence or absence of other downstream effectors to induce calcium signalling.

One plausible explanation is that PAR-2 might signal to other G proteins to elevate intracellular calcium level, for example, $G\alpha_{16}$. $G\alpha_{16}$ is a family member of $G\alpha_{q/11}$, and previous literature has proposed that it is able to stimulate PLCβ activity through non-selective association with a wide range of $G\alpha_{q/11}$, $G\alpha_s$ or $G\alpha_i$-coupled receptors (Offermanns & Simon, 1995). However, this remains less likely as the expression of $G\alpha_{16}$ has only been detected in the cells of haematopoietic origin thus far. In addition, the total inositol phosphate accumulation induced by PAR-2 was almost completely abolished by YM 254890, highlighting that $G\alpha_{q/11}$ is the predominant player in activating PLCβ.

Phospholipase D (PLD) is a signalling molecule that is involved in hydrolysing phosphatidylcholine to phosphatidic acid (Waite, 1999). Phosphatidic acid can then act to release calcium from intracellular stores or serves as a source of DAG for PKC activation (English, 1996). It remains a possibility that PAR-2 might utilise this pathway as an additional regulatory feature to PLCβ to elevate the intracellular calcium level.

This might also explain the effects of PKC on PAR-2–induced NFκB-DNA binding that appeared to be independent of $G\alpha_{q/11}$.

Another possibility is that the concentration of YM 254890 employed in this project was not sufficient to totally abolish $G\alpha_{q/11}$-stimulated calcium mobilisation. In fact preliminary results of YM 254890 on PAR-2–mediated calcium signalling revealed an approximate 75% inhibition of trypsin–induced responses (Trevor Bushell, personal communication). The residual calcium mobilisation might be sufficient to exert functional roles on PAR-2/NFκB signalling paradigm.

If $G\alpha_{q/11}$ is not the only upstream regulatory component involved in PAR-2/NFκB signalling paradigm, there must exist other yet to be identified mechanisms in this context. Notably, several lines of evidence have suggested the involvement of more than one G protein in linking GPCRs to the NFκB pathway, for example S1P has been shown to employ $G\alpha_{q/11}$, $G\alpha_i$ and $G\alpha_{13}$, whereas muscarinic receptors have been reported to utilise both $G\alpha_q$ and $G\alpha_{13}$ to achieve such signal transmission (Siehler *et al.*, 2001; Shi & Kehrl, 2001). Nevertheless, by using PTX-pretreatment and Adv. Lsc in this project, the role of $G\alpha_i$ and $G\alpha_{12/13}$ in PAR-2 signalling has been excluded. Interestingly, in addition to $G\alpha_{q/11}$, PAR-1 has been shown to utilise $G\beta\gamma$ subunits to signal to the NFκB pathway (Rahman *et al.*, 2002). The authors postulated that in endothelial cells, PAR-1 stimulation leads to $G\alpha_{q/11}$ and the subsequent PKCδ activation in parallel to $G\beta\gamma$-induced PI3 kinase pathway, both of which then converge at Akt to stimulate NFκB activation. The functional role of PAR-2-associated $G\beta\gamma$ has not been investigated in this study, thus it remains to be elucidated if PAR-2 would resemble its close relative, PAR-1, in utilising a similar mechanism in activating NFκB.

In conclusion, this study provides insights into the regulation of PAR-2–mediated NFκB activation which involves $G\alpha_{q/11}$, intracellular calcium, PKC and IKKβ (see Figure

4.29). However, there might exist other regulatory mechanisms in this context and definitely more rigorous examination is needed to identify the missing link.

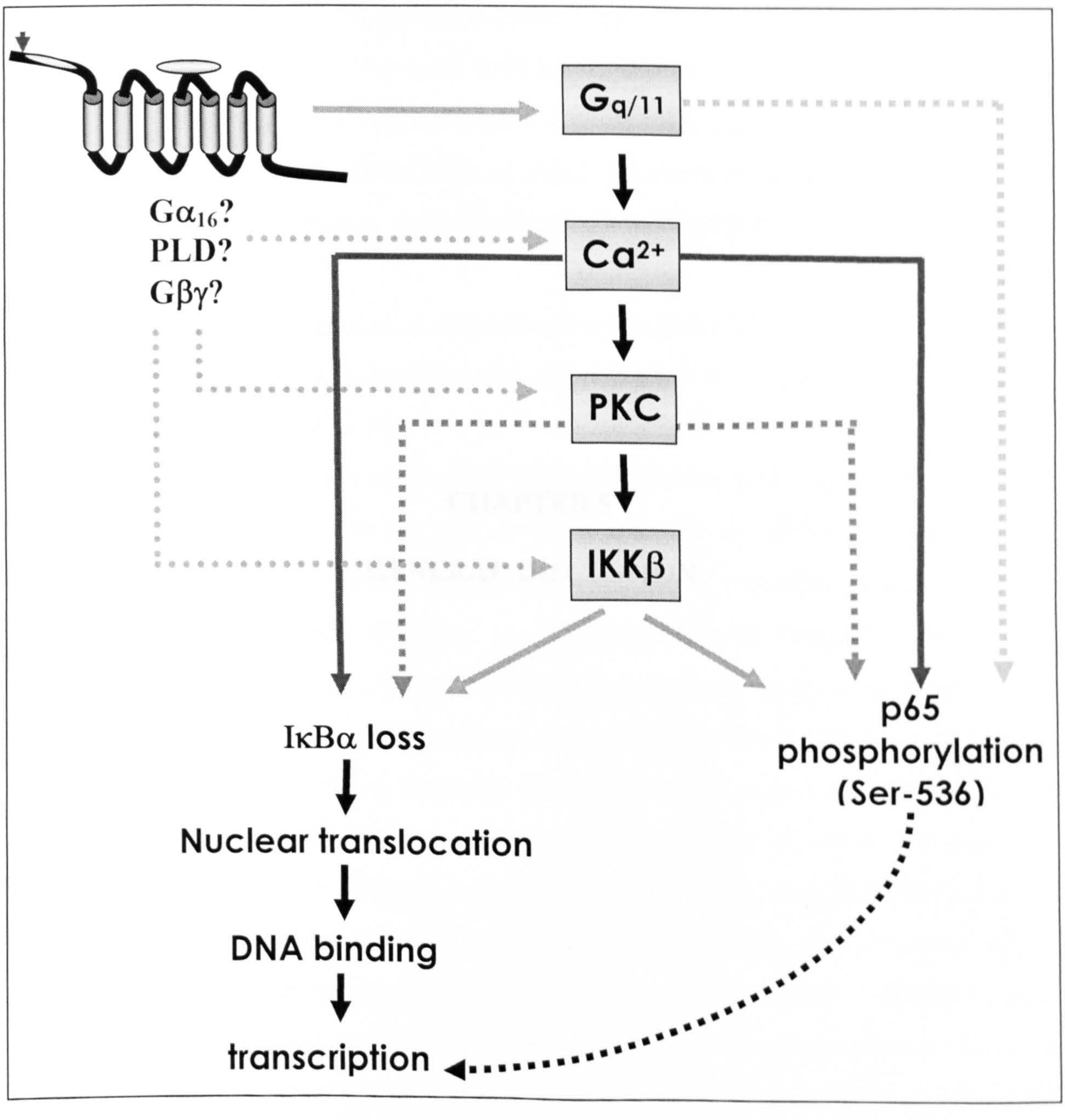


Figure 4.29. The regulation of PAR-2-mediated NFκB activation in keratinocytes.

CHAPTER 5

GENERAL DISCUSSION

In the skin, PAR-2 is present in the basal, spinous and granular layers of the epidermis and has been shown to exert several patho-physiological roles in this system (see Section 1.2.2.8). However, how PAR-2 exerts its effects in skin remains largely undefined. Therefore this study has investigated the kinetics of PAR-2–mediated signalling components including inositol phosphate accumulation, MAP kinases activation as well as the NFκB pathway in NCTC-2544 cells stably expressing PAR-2 or clone G (see Chapter 3). Furthermore, the regulation of NFκB cascade mediated by PAR-2 and the intermediary components involved were assessed in both transfected model system and the primary cells, normal human epidermal keratinocytes (NHEK) (see Chapter 4).

The results reported herein support the notion that PAR-2 is able to signal to a diverse array of signalling effectors, denoting that this receptor may utilise the discrete and complex signal transduction network to bring about its cellular effects. The ERK pathway represents the key modulator of cell proliferation; coupling of PAR-2 to this MAP kinase indicates that the receptor may employ this signalling cascade to effect mitogenic responses (Roux & Blenis, 2004). SAPKs, on the other hand, have been regarded as the important regulators in mediating cellular responses induced by extracellular stresses such as UV, γ-irradiation and reactive oxygen, to result in cell proliferation, transformation and apoptosis (Roux & Blenis, 2004; Nishina *et al.*, 2004). This study has also provided evidence that PAR-2 can signal to the SAPKs in keratinocytes, albeit a much lower magnitude of JNK activation was observed compared to that of p38 MAPK in clone G cells (see Chapter 3). In fact in NHEK, PAR-2 activation led to a more substantial phosphorylation of JNK than that in clone G along with p38 MAPK activation (unpublished data). The coupling of PAR-2 to SAPKs in a physiological environment indicates that PAR-2 can utilise these signalling components to mediate stress-induced cellular responses. Although several reports have described the roles of ERK and SAPKs in modulating different cellular aspects (see Section 3.4), relatively little is known about the direct roles of PAR-2-induced MAPK pathways in skin. Therefore further experiments are required to fully assess the biological importance of PAR-2/MAPKs in skin homeostasis.

The mechanisms of activation of ERK, p38 MAPK and JNK, however, were not investigated in this project. Despite the identification of a large number of upstream mediators for the MAPK pathways (see Section 1.3.1), the mechanism by which PAR-2 utilise to signal to MAPKs remain largely undefined (discussed in Section 1.3.2). Additionally, the activation kinetics of ERK, p38 MAPK and JNK reported herein and along with other studies point to the fact that in different models, PAR-2 might utilise distinct mechanisms to finely regulate signal transduction in order to achieve specific cellular responses (discussed in Section 3.4). For example, β-arrestins have been implicated in PAR-2-stimulated ERK activity (DeFea *et al.*, 2000, Stalheim *et al.*, 2005). These studies have suggested that β-arrestin as a scaffold protein is important in governing the subcellular compartmentalisation of ERK upon activation. DeFea *et al.* (2000) provided evidence that a PAR-2 mutant that was unable to interact with β-arrestin stimulated ERK activity through a distinct mechanism which led to nuclear translocation of this MAPK to effect cell proliferation. On the contrary, stimulation of wild type PAR-2 resulted in cytosolic retention of ERK through the formation of a macrocomplex containing the receptor, raf-1, β-arrestin and the activated ERK. Similarly, PAR-2 activation in β–arrestin deficient cells stimulated only transient phosphorylation of ERK as opposed to the prolonged activation profile observed in wild type cells (Stalheim *et al.*, 2005). These findings suggest that specific regulatory mechanisms of ERK will have impact on the spatiotemporal control of this signalling component thus directly affecting the cellular outcome (Torii *et al.*, 2004). Such a paradigm could be extended to other family members of MAPK; thus the delineation of the mechanisms and intermediary components involved is vital to understand the role of PAR-2 in human biological systems and certainly more work is needed to shed light on this matter.

This study has also looked into the regulation of the transcription factors, AP-1 and CRE, stimulated by PAR-2. The results presented herein demonstrate that PAR-2 is able to signal to these regulators of gene expression in clone G cells thus contributing to skin homeostasis. For example, AP-1 is the major transcription factor in epidermis and its

target genes include those affecting keratinocyte proliferation and differentiation (Eckert *et al.*, 1997; Eckert & Welter, 1996). By using the pharmacological tools which serve as inhibitors for the individual MAP kinase, this study has tried to ascertain the contribution of each pathway in the activation of AP-1 and CRE (see Chapter 3). Nevertheless more work is required to provide a clearer picture in this context. In addition, the elucidation of the composition of the transcriptional dimers activated and the targeted genes will shed light on how PAR-2 utilises AP-1 and CRE to modulate the physiology of skin.

NFκB is an ubiquitously expressed, rapid response transcription factor that is involved in immune and inflammatory reactions by inducing the expression of chemokines, cytokines, cell adhesion molecules, growth factors, and immunoreceptors (see Section 1.4.5). The coupling of PAR-2 to p65 NFκB in clone G and NHEK as shown in this study suggests that this receptor may have an impact on skin homeostasis via the NFκB cascade, as a large body of evidence has indicated the role of this transcription factor in balancing the proliferation and the differentiation of keratinocytes (reviewed in Kaufman & Fuchs, 2000). Moreover, various studies have supported the involvement of NFκB in skin pathology such as psoriasis, contact dermatitis and skin carcinogenesis (reviewed in Bell *et al.*, 2003). As PAR-2 has been implicated in such inflammatory skin disorders (see Chapter 1.2.2.8), it is likely that NFκB serves as one of the possible signalling candidates that PAR-2 utilises to exert pathological phenotype in epidermis. Notably, the activity of ERK and p38 MAPK has recently been shown to be upregulated in lesional psoriatic skin, indicating that these two components, as with NFκB, might represent the key mediators of skin pathological conditions (Johansen *et al.*, 2005). As PAR-2 can signal to all these signalling mediators in skin, it therefore represents a good candidate for therapeutic intervention in this context. Thus rigorous examination of how PAR-2 links to its diverging effector systems is eagerly awaited.

One of the most remarkable findings in this study is that PAR-2-stimulated NFκB activation appears to be only partially regulated by its respective G protein, $G\alpha_{q/11}$, and the involvement of other Gα proteins was not likely (see Chapter 4). One postulation for the observed phenomenon is the possibility of a G-protein-independent signalling paradigm. Classically, G proteins serve as the principal downstream effector system for GPCRs to permit signal transduction (see Chapter 1.1). However there is increasing evidence suggesting G-protein-independency in this context (Hall *et al.*, 1999; Hur & Kim, 2002). For example, the prototypical $G\alpha_s$-coupled receptor, adenosine A_{2A} receptor has been shown to activate MAP kinases in neither a $G\alpha_s$ nor $G\alpha_i$ dependent manner (Sexl *et al.*, 1997). A recent study has proposed the involvement of a novel protein called Arf nucleotide site opener/cytohesin-2 in linking the adenosine A_{2A} receptor to MAP kinase independently of G-protein (Gsandtner *et al.*, 2005). Other novel proteins that have been found to directly associate with various GPCRs to transmit signals include small G proteins such as Arf and RhoA (Mitchell *et al.*, 1998), Na^+/H^+ exchanger regulatory factor (Hall *et al.*, 1999), Homer (Xiao *et al.*, 2000) and Shank proteins (Tu *et al.*, 1999), all of which have been shown to mediate heterotrimeric G protein-independent signalling. In fact, along with NFκB activation, PAR-2-induced ERK and p38 MAPK phosphorylation in clone G and NHEK have been observed to be only partially dependent on $G\alpha_{q/11}$ and independent of $G\alpha_i$ and $G\alpha_{12/13}$ (unpublished data, see Chapter 4). Therefore PAR-2 might utilise other novel upstream components in addition to G proteins to effect its diverse signal transduction and this might represent a new area of investigation for GPCR signalling.

There is emerging evidence supporting the notion of GPCRs forming dimers or oligomers in a fashion similar to the non-GPCR families such as the growth-hormone receptor family as opposed to the classical assumption that GPCRs exist as monomeric component (reviewed in Hansen & Sheikh, 2004). It is now widely accepted that dimerisation/oligomerisation may have important implications in ligand binding, signal transduction and receptor trafficking. Some of the fascinating examples of GPCR

dimerisation include $GABA_B$ receptor, κ and δ opioid receptor, angiotensin and bradykinin receptor (reviewed in Hansen & Sheikh, 2004). $GABA_B$ receptor has been shown to require dimerisation of two receptor subtypes, R1 and R2, to confer receptor function (White *et al.*, 1998). On the other hand, a heterodimer can be formed from two fully functional κ and δ opioid receptors, and the resultant heterodimeric receptor exhibits distinct signalling profile compared to individual receptor (Jordan & Devi, 1999). Alternatively, heterodimerisation also seems possible between receptors of different subtypes. For example, bradykinin receptor has been found to physically associate with angiotensin receptor to form stable heterodimer that enhances the signal transduction of the latter receptor (AbdAlla *et al.*, 2000).

Although previous findings have indicated intermolecular signalling between PARs (see Section 1.2.2.6), there has been no direct evidence supporting the formation of PARs dimers. Only until recently some novel findings have suggested that PAR-1 and PAR-4 are able to form heterodimers in human platelets and fibroblasts, resulting in accelerated receptor cleavage and initiation of PAR-4 signalling (Leger *et al.*, 2006). As PARs have been found to be present in the same cell/tissue system in several instances, it is likely that these receptors might physically associate with each other to effect distinct receptor pharmacology and/or function, urging more intensive investigation into the aspect of dimerisation/oligomerisation, which signifies a novel mechanism of cross-talk between GPCRs.

PARs represent a novel group of cell-surface receptors by which the proteases utilise to mediate a wide range of physiological processes and inflammatory responses (see Section 1.2.1.5 and 1.2.2.8). The list of ligands for PARs is still growing, so is the biological significance of these receptors (see Sections 1.2.2.7, 1.2.1.5 and 1.2.2.8). PAR-2 has been strongly implicated in several pathological conditions including psoriasis and arthritis, thus representing a potential therapeutic target. However, unlike PAR-1 in which the antagonists of the receptor have entered clinical trails as potential therapeutic tools, no potent PAR-2 antagonist has been reported so far (Chackalamannil

& Xia, 2006). The effort to synthesise potent agonists and antagonists for PARs has proved difficult and there is a great deal of analysis to be performed in order to rule out ligand cross-reactivity and non-specific agonism/antagonism (see Chapter 1.2.2.2).

In conclusion, this study has shown that PAR-2 is able to signal to a diverse array of signalling molecules in keratinocytes. In addition, the regulation of NFκB mediated by PAR-2 has been delineated to consist of $G\alpha_{q/11}$, Ca^{2+}, PKC and IKKβ. Nevertheless, there are still missing links between PAR-2 and its downstream effectors that remain undefined. It appears that the classical linear signalling for a GPCR such as PAR-2 seems to be much more complex than initially anticipated and might involve both G protein–dependent and independent mechanisms, urging more intensive investigation in this area.

REFERENCES

AbdAlla, S., Lother, H. and Quitterer, U. (2000) AT(1)-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature*. **407**: 94-98.

Aguirre, V., Uchida, T., Yenush, L., Davis, R. and White, M.F. (2000) The c-Jun NH2-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *Journal of Biological Chemistry*. **275**: 9047-9054.

Ahn, H.S., Foster, C., Boykow, G., Stamford, A., Manna, M. and Graziano, M. (2000) Inhibition of cellular action of thrombin by N3-cyclopropyl-7-{ 4-(1-methylethyl)phenyl methyl}-7H-pyrrolo-3,2-f quinazoline-1,3-diamine (SCH 79797), a nonpeptide thrombin receptor antagonist. *Biochemical Pharmacology*. **60**: 1425-1434.

Ahn, S., Maudsley, S., Luttrell, L.M., Lefkowitz, R.J. and Daaka, Y. (1999) Src-mediated tyrosine phosphorylation of dynamin is required for beta(2)-adrenergic receptor internalization and mitogen-activated protein kinase signaling. *Journal of Biological Chemistry*. **274**: 1185-1188.

Akers, I.A., Parsons, M., Hill, M.R., Hollenberg, M.D., Sanjar, S., Laurent, G.J. and McAnulty, R.J. (2000) Mast cell tryptase stimulates human lung fibroblast proliferation via protease-activated receptor-2. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. **278**: L193-L201.

Al-Ani, B., Saifeddine, M. and Hollenberg, M.D. (1995) Detection of functional receptors for the proteinase-activated-receptor-2-activating polypeptide, SLIGRL-NH_2, in rat vascular and gastric smooth-muscle. *Canadian Journal of Physiology and Pharmacology*. **73**: 1203-1207.

Al-Ani, B., Wijesuriya, S.J. and Hollenberg, M.D. (2002a) Proteinase-activated receptor 2: Differential activation of the receptor by tethered ligand and soluble peptide analogs. *Journal of Pharmacology and Experimental Therapeutics*. **302**: 1046-1054.

Al-Ani, B., Hansen, K.K. and Hollenberg, M.D. (2004) Proteinase-activated receptor-2: Key role of amino-terminal dipeptide residues of the tethered ligand for receptor activation. *Molecular Pharmacology*. **65**: 149-156.

Al-Ani, B., Saifeddine, M., Kawabata, A. and Hollenberg, M.D. (1999a) Proteinase activated receptor 2: role of extracellular loop 2 for ligand-mediated activation. *British Journal of Pharmacology*. **128**: 1105-1113.

Al-Ani, B., Saifeddine, M., Wijesuriya, S.J. and Hollenberg, M.D. (2002b) Modified proteinase-activated receptor-1 and-2 derived peptides inhibit proteinase-activated receptor-2 activation by trypsin. *Journal of Pharmacology and Experimental Therapeutics*. **300**: 702-708.

Al-Ani, B., Saifeddine, M., Kawabata, A., Renaux, B., Mokashi, S. and Hollenberg, M.D. (1999b) Proteinase-activated receptor 2 (PAR(2)): Development of a ligand-binding assay correlating with activation of PAR(2) by PAR(1)- and PAR(2)-derived peptide ligands. *Journal of Pharmacology and Experimental Therapeutics*. **290**: 753-760.

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T. and Saltiel, A.R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein-kinase kinase *in vitro* and *in vivo*. *Journal of Biological Chemistry*. **270**: 27489-27494.

Alm, A.K., Gagnemo-Persson, R., Sorsa, T. and Sundelin, J. (2000) Extrapancreatic trypsin-2 cleaves proteinase-activated receptor-2. *Biochemical and Biophysical Research Communications*. **275**: 77-83.

Amadesi, S., Nie, J.J., Vergnolle, N., Cottrell, G.S., Grady, E.F., Trevisani, M., Manni, C., Geppetti, P., McRoberts, J.A., Ennes, H., Davis, B., Mayer, E.A. and Bunnet, N.W. (2004) Protease-activated receptor 2 sensitizes the capsaicin receptor transient receptor potential vanilloid receptor 1 to induce hyperalgesia. *Journal of Neuroscience*. **24**: 4300-4312.

Andrade-Gordon, P., Addo, M.F., Darrow, A.L., Derian, C.K., Eckardt, A.J., Hoekstra, W.J., Maryanoff, B. E., Oksenberg, D., Reynolds, E.,Santulli, R. J., Scarborough, R. M. and Zhang, H. C. (1999) A potent peptide-mimetic antagonist for the thrombin receptor (PAR1). *Circulation*. **100**: 471-471.

Andrade-Gordon, P., Derian, C.K., Maryanoff, B.E., Zhang, H.C., Addo, M.F., Cheung, W.M., Damiano, B. P.,D'Andrea, M. R.,Darrow, A. L.,De Garavilla, L.,Eckardt, A. J., Giardino, E. C.,Haertlein, B. J. and McComsey, D. F. (2001) Administration of a potent antagonist of protease-activated receptor-1 (PAR-1) attenuates vascular restenosis following balloon angioplasty in rats. *Journal of Pharmacology and Experimental Therapeutics*. **298**: 34-42.

Anrather, J., Csizmadia, V., Soares, M.P. and Winkler, H. (1999) Regulation of NF-kappa B RelA phosphorylation and transcriptional activity by p21(ras) and protein kinase C zeta in primary endothelial cells. *Journal of Biological Chemistry*. **274**: 13594-13603.

Aschnoune, K., Strassheim, D., Mitra, S., Kim, J.Y. and Abraham, E. (2005) Involvement of PKC alpha/beta in TLR4 and TLR2 dependent activation of NF-kappa B. *Cellular Signalling*. **17**: 385-394.

Asfaha, S., Brussee, V., Chapman, K., Zochodne, D.W. and Vergnolle, N. (2002) Proteinase-activated receptor-1 agonists attenuate nociception in response to noxious stimuli. *British Journal of Pharmacology*. **135**: 1101-1106.

Asokananthan, N., Graham, P.T., Stewart, D.J., Bakker, A.J., Eidne, K.A., Thompson, P.J. and Stewart, G.A. (2002a) House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: The cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *Journal of Immunology*. **169**: 4572-4578.

Asokananthan, N., Graham, P.T., Fink, J., Knight, D.A., Bakker, A.J., McWilliam, A.S., Thompson, P. J. and Stewart, G. A (2002b) Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E-2 release from human respiratory epithelial cells. *Journal of Immunology*. **168**: 3577-3585.

Atkinson, P.J., Young, K.W., Ennion, S.J., Kew, J.N.C., Nahorski, S.R. and Challiss, R.A.J. (2006) Altered expression of G(q)/(11)alpha protein shapes mGlu1 and mGlu5 receptor-mediated single cell inositol 1,4,5-trisphosphate and Ca^{2+} signaling. *Molecular Pharmacology*. **69**: 174-184.

Babiarz-Magee, L., Chen, N., Seiberg, M. and Lin, C.B. (2004) The expression and activation of protease-activated receptor-2 correlate with skin color. *Pigment Cell Research*. **17**: 241-251.

Bae, J.S., Jang, M.K., Hong, S.H., An, W.G., Choi, Y.H., Do Kim, H. and Cheong, J.H. (2003) Phosphorylation of NF-kappa B by calmodulin-dependent kinase IV activates anti-apoptotic gene expression. *Biochemical and Biophysical Research Communications*. **305**: 1094-1098.

Bahou, W.F., Nierman, W.C., Durkin, A.S., Potter, C.L. and Demetrick, D.J. (1993) Chromosomal assignment of the human thrombin receptor gene - Localization to region Q13 of chromosome-5. *Blood*. **82**: 1532-1537.

Baldwin, A.S. (2001) The transcription factor NF-kappa B and human disease. *Journal of Clinical Investigation*. **107**: 3-6.

Baldwin, J.M. (1993) The probable arrangement of the helices in G protein-coupled receptors. *EMBO Journal*. **12**: 1693-1703.

Barnes, P.J. and Larin, M. (1997) Mechanisms of disease - Nuclear factor-kappa B - A pivotal transcription factor in chronic inflammatory diseases. *New England Journal of Medicine*. **336**: 1066-1071.

Barnes, W.G., Reiter, E., Violin, J.D., Ren, X.R., Milligan, G. and Lefkowitz, R.J. (2005) beta-arrestin 1 and G(alpha q/11) coordinately activate RhoA and stress fiber formation following receptor stimulation. *Journal of Biological Chemistry*. **280**: 8041-8050.

Bar-Sagi, D. and Hall, A. (2000) Ras and Rho GTPases: A family reunion. *Cell*. **103**: 227-238.

Barshavit, R. and Wilner, G.D. (1986) Mediation of cellular events by thrombin. *International Review of Experimental Pathology*. **29**: 213-241.

Baugh, R.J., Dickinson, C.D., Ruf, W. and Krishnaswamy, S. (2000) Exosite interactions determine the affinity of factor X for the extrinsic Xase complex. *Journal of Biological Chemistry*. **275**: 28826-28833.

Beg, A.A., Sha, W.C., Bronson, R.T., Ghosh, S. and Baltimore, D. (1995) Embryonic lethality and liver degeneration in mice kacking the RelA component of NF-kappa-B. *Nature*. **376**: 167-170.

Beg, A.A., Ruben, S.M., Scheinman, R.I., Haskill, S., Rosen, C.A. and Baldwin, A.S. (1992) I-Kappa-B interacts with the nuclear-localization sequences of the subunits of NF-kappa-B - a mechanism for cytoplasmic retention. *Genes & Development*. **6**: 1899-1913.

Belham, C.L., Tate, R.J., Scott, P.H., Pemberton, A.D., Miller, H.R.P., Wadsworth, R.M., Gould, G. W. and Plevin, R. (1996) Trypsin stimulates proteinase-activated receptor-2-dependent and -independent activation of mitogen-activated protein kinases. *Biochemical Journal*. **320**: 939-946.

Bell, S., Degitz, K., Quirling, M., Jilg, N., Page, S. and Brand, K. (2003) Involvement of NF-kappa B signalling in skin physiology and disease. *Cellular Signalling*. **15**: 1-7.

Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W.M., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., and Anderson, D.W. (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proceedings of the National Academy of Sciences of the United States of America*. **98**: 13681-13686.

Beraud, C. (1999) A new role of PI 3-kinase in activating NF-kappa B. *M S-Medecine Sciences*. **15**: 747-749.

Berman, D.M., Kozasa, T. and Gilman, A.G. (1996) The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. *Journal of Biological Chemistry*. **271**: 27209-27212.

Bernstein, L.S., Ramineni, S., Hague, C., Cladman, W., Chidiac, P., Levey, A.I. and Hepler, J.R. (2004) RGS2 binds directly and selectively to the M1 muscarinic acetylcholine receptor third intracellular loop to modulate G(q/11 alpha) signaling. *Journal of Biological Chemistry*. **279**: 21248-21256.

Berridge, M.J., Downes, C.P. and Hanley, M.R. (1982) Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochemical Journal*. **206**: 587-595.

Bird, T.A., Schooley, K., Dower, S.K., Hagen, H. and Virca, G.D. (1997) Activation of nuclear transcription factor NF-kappa B by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit. *Journal of Biological Chemistry*. **272**: 32606-32612.

Blackhart, B., Emilsson, K., Nguyen, D., Teng, W., Martelli, A., Nystedt, S., Sundelin, J. and Scarborough, R. (1996) Ligand cross-reactivity within the protease-activated receptor family. *Journal of Biological Chemistry*. **271**: 16466-16471.

Blackhart, B.D., Ruslim-Litrus, L., Lu, C.C., Alves, V.L., Teng, W., Scarborough, R.M., Reynolds, E. E. and Oksenberg, D. (2000) Extracellular mutations of protease-activated receptor-1 result in differential activation by thrombin and thrombin receptor agonist peptide. *Molecular Pharmacology*. **58**: 1178-1187.

Böhm, S.K., Khitin, L.M., Grady, E.F., Aponte, G., Payan, D.G. and Bunnett, N.W. (1996a) Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. *Journal of Biological Chemistry*. **271**: 22003-22016.

Böhm, S.K., Kong, W.Y., Bromme, D., Smeekens, S.P., Anderson, D.C., Connolly, A., Kahn, M., Nelken, N. A.,Coughlin, S. R.,Payan, D. G. and Bunnett, N. W.(1996b) Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. *Biochemical Journal*. **314**: 1009-1016.

Boire, A., Covic, L., Agarwal, A., Jacques, S., Sherifl, S. and Kuliopulos, A. (2005) PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell*. **120**: 303-313.

Bono, F., Lamarche, I. and Herbert, J.M. (1997) Induction of vascular smooth muscle cell growth by selective activation of the proteinase activated receptor-2 (PAR-2). *Biochemical and Biophysical Research Communications*. **241**: 762-764.

Boulton, T.G., Yancopoulos, G.D., Gregory, J.S., Slaughter, C., Moomaw, C., Hsu, J. and Cobb, M.H. (1990) An insulin-stimulated protein-kinase similar to yeast kinases involved in cell-cycle control. *Science*. **249**: 64-67.

Brach, M.A., Devos, S., Arnold, C., Gruss, H.J., Mertelsmann, R. and Herrmann, F. (1992) Leukotriene B-4 transcriptionally activates interleukin-6 expression involving NK-Chi-B and NF-Il6. *European Journal of Immunology*. **22**: 2705-2711.

Brass, L.F., Manning, D.R., Williams, A.G., Woolkalis, M.J. and Poncz, M. (1991) Receptor and G-Protein-mediated responses to thrombin in Hel Cells. *Journal of Biological Chemistry*. 266: 958-965.

Bretschneider, E., Kaufmann, R., Braun, M., Wittpoth, M., Glusa, E., Nowak, G. and Schror, K. (1999) Evidence for proteinase-activated receptor-2 (PAR-2)-mediated

mitogenesis in coronary artery smooth muscle cells. *British Journal of Pharmacology*. **126**: 1735-1740.

Buddenkotte, J., Stroh, C., Engels, I.H., Moormann, C., Shpacovitch, V.M., Seeliger, S., Vergnolle, N.,Vestweber, D., Luger, T. A.,Schulze-Osthoff, K. and Steinhoff, M. (2005) Agonists of proteinase-activated receptor-2 stimulate upregulation of intercellular cell adhesion molecule-1 in primary human keratinocytes via activation of NF-kappa B. *Journal of Investigative Dermatology*. **124**: 38-45.

Buresi, M.C., Buret, A.G., Hollenberg, M.D. and MacNaughton, W.K. (2002) Activation of proteinase-activated receptor 1 stimulates epithelial chloride secretion through a unique MAP kinase- and cyclo-oxygenase-dependent pathway. *FASEB Journal*. **16**: 1515-1525.

Bushell, T.J., Plevin, R., Cobb, S. and Irving, A.J. (2006) Characterization of proteinase-activated receptor 2 signalling and expression in rat hippocampal neurons and astrocytes. *Neuropharmacology*. **50**: 714-725.

Cabrera-Vera, T.M., Vanhauwe, J., Thomas, T.O., Medkova, M., Preininger, A., Mazzoni, M.R. and Hamm, H.E. (2003) Insights into G protein structure, function, and regulation. *Endocrine Reviews*. **24**: 765-781.

Camerer, E., Huang, W. and Coughlin, S.R. (2000) Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proceedings of the National Academy of Sciences of the United States of America*. **97**: 5255-5260.

Carbajal, J.M., Gratrix, M.L., Yu, C.H. and Schaeffer, R.C. (2000) ROCK mediates thrombin's endothelial barrier dysfunction. *American Journal of Physiology-Cell Physiology*. **279**: C195-C204.

Chackalamannil, S. and Xia, Y. (2006) Thrombin receptor (PAR-1) antagonists as novel antithrombotic agents. *Expert Opinion on Therapeutic Patents*. **16**: 493-505.

Chambers, L.S., Black, J.L., Poronnik, P. and Johnson, P.R.A. (2001) Functional effects of protease-activated receptor-2 stimulation on human airway smooth muscle. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. **281**: L1369-L1378.

Chang, M.S., Chen, B.C., Yu, M.T., Sheu, J.R., Chen, T.F. and Lin, C.H. (2005) Phorbol 12-myristate 13-acetate upregulates cyclooxygenase-2 expression in human pulmonary epithelial cells via Ras, Raf-1, ERK, and NF-kappa B, but not p38 MAPK, pathways. *Cellular Signalling*. **17**: 299-310.

Chen, B.C., Yu, C.C., Lei, H.C., Chang, M.S., Hsu, M.J., Huang, C.L. Chen, M. C., Sheu, J. R., Chen, T. F., Chen, T. L., Inoue, H. and Lin, C. H. (2004) Bradykinin B2 receptor mediates NF-kappa B activation and cyclooxygenase-2 expression via the

Ras/Raf-1/ERK pathway in human airway epithelial cells. *Journal of Immunology*. **173**: 5219-5228.

Chen, J., Ishii, M., Wang, L., Ishii, K. and Coughlin, S. (1994) Thrombin receptor activation. Confirmation of the intramolecular tethered liganding hypothesis and discovery of an alternative intermolecular liganding mode. *Journal of Biological. Chemistry*. **269**: 16041-16045.

Chen, Z., Gibson, T.B., Robinson, F., Silvestro, L., Pearson, G., Xu, B.E., Wright, A., Vanderbilt, C. and Cobb, M. H. (2001) MAP kinases. *Chemical Reviews*. **101**: 2449-2476.

Cheung, W.M., Andrade-Gordon, P., Derian, C.K. and Damiano, B.P. (1998) Receptor-activating peptides distinguish thrombin receptor (PAR-1) and protease activated receptor 2 (PAR-2) mediated hemodynamic responses in vivo. *Canadian Journal of Physiology and Pharmacology*. **76**: 16-25.

Chokki, M., Eguchi, H., Hamamura, I., Mitsuhashi, H. and Kamimura, T. (2005) Human airway trypsin-like protease induces amphiregulin release through a mechanism involving protease-activated receptor-2-mediated ERK activation and TNF alpha-converting enzyme activity in airway epithelial cells. *FEBS Journal*. **272**: 6387-6399.

Chouinard, N., Valerie, K., Rouabhia, M. and Huot, J. (2002) UVB-mediated activation of p38 mitogen-activated protein kinase enhances resistance of normal human keratinocytes to apoptosis by stabilizing cytoplasmic p53. *Biochemical Journal*. **365**: 133-145.

Cicala, C., Spina, D., Keir, S.D., Severino, B., Meli, R., Page, C.P. and Cirino, G. (2001) Protective effect of a PAR2-activating peptide on histamine-induced bronchoconstriction in guinea-pig. *British Journal of Pharmacology*. **132**: 1229-1234.

Cicala, C., Pinto, A., Bucci, M., Sorrentino, R., Walker, B., Harriot, P., Cruchley, A., Kapas, S., Howells, G. L. and Cirino, G. (1999) Protease-activated receptor-2 involvement in hypotension in normal and endotoxemic rats in vivo. *Circulation*. **99**: 2590-2597.

Claudio, E., Brown, K., Park, S., Wang, H.S. and Siebenlist, U. (2002) BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. *Nature Immunology*. **3**: 958-965.

Cocks, T.M. and Moffatt, J.D. (2001) Protease-activated receptor-2 (PAR2) in the airways. *Pulmonary Pharmacology & Therapeutics*. **14**: 183-191.

Cocks, T.M., Sozzi, V., Moffatt, J.D. and Selemidis, S. (1999a) Protease-activated receptors mediate apamin-sensitive relaxation of mouse and guinea pig gastrointestinal smooth muscle. *Gastroenterology*. **116**: 586-592.

Cocks, T.M., Fong, B., Chow, J.M., Anderson, G.P., Frauman, A.G., Goldie, R.G., Henry, P. J., Carr, M. J., Hamilton, J. R. and Moffatt, J. D. (1999b) A protective role for protease-activated receptors in the airways. *Nature*. **398**: 156-160.

Colquhoun, D. (2006) Agonist-activated ion channels. *British Journal of Pharmacology*. **147**: S17-S26.

Compton, S.J., Sandhu, S., Wijesuriya, S.J. and Hollenberg, M.D. (2002a) Glycosylation of human proteinase-activated receptor-2 (hPAR(2)) - role in cell surface expression and signalling. *Biochemical Journal*. **368**: 495-505.

Compton, S.J., McGuire, J.J., Saifeddine, M. and Hollenberg, M.D. (2002b) Restricted ability of human mast cell tryptase to activate proteinase-activated receptor-2 in rat aorta. *Canadian Journal of Physiology and Pharmacology*. **80**: 987-992.

Compton, S.J., Cairns, J.A., Palmer, K.J., Al-Ani, B., Hollenberg, M.D. and Walls, A.F. (2000) A polymorphic protease-activated receptor 2 (PAR2) displaying reduced sensitivity to trypsin and differential responses to PAR agonists. *Journal of Biological Chemistry*. **275**: 39207-39212.

Connolly, A.J., Ishihara, H., Kahn, M.L., Farese, R.V. and Coughlin, S.R. (1996) Role of the thrombin receptor in development and evidence for a second receptor. *Nature*. **381**: 516-519.

Coope, H.J., Atkinson, P.G.P., Huhse, B., Belich, M., Janzen, J., Holman, M.J., Klaus, G. G. B., Johnston, L. H. and Ley, S. C. (2002) CD40 regulates the processing of NF-kappa B2 p100 to p52. *EMBO Journal*. **21**: 5375-5385.

Corvera, C.U., Dery, O., McConalogue, K., Khitin, L., Bohm, S.K., Kong, W., Raymond, W., Caughey, G. H. and Bunnett, N. W. (1997) Mast cell tryptase regulates colonic myocytes through proteinase-activated receptor-2 (PAR-2). *Gastroenterology*. **112**: A1140-A1140.

Corvera, C.U., Dery, O., McConalogue, K., Gamp, P., Thoma, M., Al-Ani, B., Caughey, G. H. , Hollenberg, M. D. and Bunnett, N. W (1999) Thrombin and mast cell tryptase regulate guinea-pig myenteric neurons through proteinase-activated receptors-1 and-2. *Journal of Physiology-London*. **517**: 741-756.

Cottrell, G.S., Amadesi, S., Grady, E.F. and Bunnett, N.W. (2004) Trypsin IV, a novel agonist of protease-activated receptors 2 and 4. *Journal of Biological Chemistry*. **279**: 13532-13539.

Covic, L., Gresser, A.L. and Kuliopulos, A. (2000) Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. *Biochemistry*. **39**: 5458-5467.

Covic, L., Gresser, A.L., Talavera, J., Swift, S. and Kuliopulos, A. (2002) Activation and inhibition of G protein-coupled receptors by cell-penetrating membrane-tethered peptides. *Proceedings of the National Academy of Sciences of the United States of America*. **99**: 643-648.

Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P. R. and Lee, J.C. (1995) SB-203580 is a specific inhibitor of a Map Kinase homolog which is stimulated by cellular stresses and Interleukin-1. *FEBS Letters*. **364**: 229-233.

Cuffe, J.E., Bertog, M., Velazquez-Rocha, S., Dery, O., Bunnett, N. and Korbmacher, C. (2002) Basolateral PAR-2 receptors mediate KCl secretion and inhibition of Na^+ absorption in the mouse distal colon. *Journal of Physiology-London*. **539**: 209-222.

Dadke, D., Fryer, B.H., Golemis, E.A. and Field, J. (2003) Activation of p21-activated kinase 1-nuclear factor kappa B signaling by Kaposi's sarcoma-associated herpes virus G protein-coupled receptor during cellular transformation. *Cancer Research*. **63**: 8837-8847.

Dai, R.J., Frejtag, W., He, B., Zhang, Y. and Mivechi, N.F. (2000) c-Jun NH2-terminal kinase targeting and phosphorylation of heat shock factor-1 suppress its transcriptional activity. *Journal of Biological Chemistry*. **275**: 18210-18218.

Damiano, B.P., D'Andrea, M.R., de Garavilla, L., Cheung, W.M. and Andrade-Gordon, P. (1999a) Increased expression of protease activated receptor-2 (PAR-2) in balloon-injured rat carotid artery. *Thrombosis and Haemostasis*. **81**: 808-814.

Damiano, B.P., Cheung, W.M., Santulli, R.J., Fung-Leung, W.P., Ngo, K., Ye, R.D., Darrow, A. L., Derian, C. K., De Garavilla, L. and Andrade-Gordon, P.(1999b) Cardiovascular responses mediated by protease-activated receptor-2 (PAR-2) and thrombin receptor (PAR-1) are distinguished in mice deficient in PAR-2 or PAR-1. *Journal of Pharmacology and Experimental Therapeutics*. **288**: 671-678.

Danahay, H., Withey, L., Poll, C.T., Van De Graaf, S.F.J. and Bridges, R.J. (2001) Protease-activated receptor-2-mediated inhibition of ion transport in human bronchial epithelial cells. *American Journal of Physiology-Cell Physiology*. **280**: C1455-C1464.

D'Andrea, M.R., Derian, C.K., Leturcq, D., Baker, S.M., Brunmark, A., Ling, P., Darrow, A. L., Santulli, R. J., Brass, L. F. and Andrade-Gordon, P. (1998) Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues. *Journal of Histochemistry & Cytochemistry*. **46**: 157-164.

Darmoul, D., Gratio, V., Devaud, H., Peiretti, F. and Laburthe, M. (2004) Activation of proteinase-activated receptor 1 promotes human colon cancer cell proliferation through epidermal growth factor receptor transactivation. *Molecular Cancer Research*. **2**: 514-522.

Daub, H., Weiss, F.U., Wallasch, C. and Ullrich, A. (1996) Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature*. **379**: 557-560.

Davare, M.A., Avdonin, V., Hall, D.D., Peden, E.M., Burette, A., Weinberg, R.J., Horne, M. C., Hoshi, T. and Hell, J. W. (2001) A beta(2) adrenergic receptor signaling complex assembled with the Ca^{2+} channel Ca(v)1.2. *Science*. **293**: 98-101.

de Garavilla, L., Vergnolle, N., Young, S.H., Ennes, H., Steinhoff, M., Ossovskaya, V.S., D'Andrea, M. R., Mayer, E. A., Wallace, J. L., Hollenberg, M. D., Andrade-Gordon, P. and Bunnett, N. W. (2001) Agonists of proteinase-activated receptor 1 induce plasma extravasation by a neurogenic mechanism. *British Journal of Pharmacology*. **133**: 975-987.

DeFea, K.A., Zalevsky, J., Thoma, M.S., Dery, O., Mullins, R.D. and Bunnett, N.W. (2000) beta-Arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *Journal of Cell Biology*. **148**: 1267-1281.

Dery, O., Thoma, M.S., Wong, H., Grady, E.F. and Bunnett, N.W. (1999) Trafficking of proteinase-activated receptor-2 and beta- arrestin-1 tagged with green fluorescent protein - beta- arrestin-dependent endocytosis of a proteinase receptor. *Journal of Biological Chemistry*. **274**: 18524-18535.

Devin, A., Cook, A., Lin, Y., Rodriguez, Y., Kelliher, M. and Liu, Z.G. (2000) The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity*. **12**: 419-429.

Dhawan, P. and Richmond, A. (2002) A novel NF-kappa B-inducing kinase-MAPK signaling pathway up-regulates NF-kappa B activity in melanoma cells. *Journal of Biological Chemistry*. **277**: 7920-7928.

Dlugosz, A.A., Mischak, H., Mushinski, J.F. and Yuspa, S.H. (1992) Transcripts encoding Protein Kinase-C-Alpha, Kinase-C-Delta, Kinase-C, Kinase-C-Zeta, and Kinase-C-Eta are expressed in basal and differentiating mouse keratinocytes *in vitro* and exhibit quantitative ahanges in neoplastic cells. *Molecular Carcinogenesis*. **5**: 286-292.

Doyle, S.L., Jefferies, C.A. and O'Neill, L.A. (2005) Bruton's tyrosine kinase is involved in p65-mediated transactivation and phosphorylation of p65 on serine 536 during NF kappa B activation by lipopolysaccharide. *Journal of Biological Chemistry*. **280**: 23496-23501.

Eason, M.G., Kurose, H., Holt, B.D., Raymond, J.R. and Liggett, S.B. (1992) Simultaneous aoupling of alpha-2-adrenergic receptors to 2 G-Proteins with opposing effects - Subtype-selective coupling of alpha-2c10, Alpha-2c4, and Alpha-2c2 adrenergic-receptors to G(i) and G(s). *Journal of Biological Chemistry*. **267**: 15795-15801.

Eckert, R.L. and Welter, J.F. (1996) Transcription factor regulation of epidermal keratinocyte gene expression. *Molecular Biology Reports*. **23**: 59-70.

Eckert, R.L., Crish, J.F., Banks, E.B. and Welter, J.F. (1997) The epidermis: Genes on - Genes off. *Journal of Investigative Dermatology*. **109**: 501-509.

Eliopoulos, A.G., Caamano, J.H., Flavell, J., Reynolds, G.M., Murray, P.G., Poyet, J.L. and Young, L.S. (2003) Epstein-Barr virus-encoded latent infection membrane protein 1 regulates the processing of p100 NF-kappa B2 to p52 via an IKK gamma/NEMO-independent signalling pathway. *Oncogene*. **22**: 7557-7569.

Emi, M., Nakamura, Y., Ogawa, M., Yamamoto, T., Nishide, T., Mori, T. and Matsubara, K. (1986) Cloning, characterization and nucleotide-sequences of 2 cDNAs encoding human pancreatic trypsinogens. *Gene*. **41**: 305-310.

Emilsson, K., Wahlestedt, C., Sun, M.K., Nystedt, S., Owman, C. and Sundelin, J. (1997) Vascular effects of proteinase-activated receptor 2 agonist peptide. *Journal of Vascular Research*. **34**: 267-272.

English, D. (1996) Phosphatidic acid: A lipid messenger involved in intracellular and extracellular signalling. *Cellular Signalling*. **8**: 341-347.

Even-Ram, S., Uziely, B., Cohen, P., Grisaru-Granovsky, S., Maoz, M., Ginzburg, Y., Reich, R., Vlodavsky, I. and Bar-Shavit, R. (1998) Thrombin receptor overexpression in malignant and physiological invasion processes. *Nature Medicine*. **4**: 909-914.

Fan, M.Y., Goodwin, M., Vu, T., Brantley-Finley, C., Gaarde, W.A. and Chambers, T.C. (2000) Vinblastine-induced phosphorylation of Bcl-2 and Bcl-X-L is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade. *Journal of Biological Chemistry*. **275**: 29980-29985.

Farmer, P. and Pugin, J. (2000) beta-Adrenergic agonists exert their "anti-inflammatory" effects in monocytic cells through the I kappa B/NF-kappa B pathway. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. **279**: L675-L682.

Faruqi, T.R., Weiss, E.J., Shapiro, M.J., Huang, W. and Coughlin, S.R. (2000) Structure-function analysis of protease-activated receptor 4 tethered ligand peptides - Determinants of specificity and utility in assays of receptor function. *Journal of Biological Chemistry*. **275**: 19728-19734.

Faure, M., Voynoyasenetskaya, T.A. and Bourne, H.R. (1994) cAMP and beta/gamma-subunits of heterotrimeric G-Proteins stimulate the mitogen-activated protein-kinase pathway in Cos-7 cells. *Journal of Biological Chemistry*. **269**: 7851-7854.

Feng, D.M., Veber, D.F., Connolly, T.M., Condra, C., Tang, M.J. and Nutt, R.F. (1995) Development of a potent thrombin receptor-ligand. *Journal of Medicinal Chemistry*. **38**: 4125-4130.

Ferrell, W.R., Lockhart, J.C., Kelso, E.B., Dunning, L., Plevin, R., Meek, S.E., Smith, A. J. H., Hunter, G. D., McLean, J. S., McGarry, F., Ramage, R., Jiang, L., Kanke, T. and Kawagoe, J. (2003) Essential role for proteinase-activated receptor-2 in arthritis. *Journal of Clinical Investigation*. **111**: 35-41.

Fiorucci, S., Distrutti, E., Federici, B., Palazzetti, B., Baldoni, M., Morelli, A. and Cirino, G. (2003) PAR-2 modulates pepsinogen secretion from gastric-isolated chief cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. **285**: G611-G620.

Fisher, G.J., Tavakkol, A., Leach, K., Burns, D., Basta, P., Loomis, C., Griffiths, C. E. M., Cooper, K. D., Reynolds, N. J., Elder, J. T., Livneh, E. and Voorhees, J. J. (1993) Differential expression of Protein-Kinase-C isoenzymes in normal and psoriatic adult human skin - reduced expression of Protein Kinase-C-beta in psoriasis. *Journal of Investigative Dermatology*. **101**: 553-559.

Folgueira, L., Algeciras, A., MacMorran, W.S., Bren, G.D. and Paya, C.V. (1996) The Ras-Raf pathway is activated in human immunodeficiency virus-infected monocytes and participates in the activation of NF-kappa B. *Journal of Virology*. **70**: 2332-2338.

Fox, M.T., Harriott, P., Walker, B. and Stone, S.R. (1997) Identification of potential activators of proteinase-activated receptor-2. *FEBS Letters*. **417**: 267-269.

Fyfe, M., Bergstrom, M., Aspengren, S. and Peterson, A. (2005) PAR-2 activation in intestinal epithelial cells potentiates interleukin-1 beta-induced chemokine secretion via MAP kinase signaling pathways. *Cytokine*. **31**: 358-367.

Gallois, C., Habib, A., Tao, J.C., Moulin, S., Maclouf, J., Mallat, A. and Lotersztajn, S. (1998) Role of NF-kappa B in the antiproliferative effect of endothelin-1 and tumor necrosis factor-alpha in human hepatic stellate cells - Involvement of cyclooxygenase-2. *Journal of Biological Chemistry*. **273**: 23183-23190.

Ge, L., Ly, Y., Hollenberg, M. and DeFea, K. (2003) A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. *Journal of Biological Chemistry*. **278**: 34418-34426.

Ghosh, S. and Karin, M. (2002) Missing pieces in the NF-kappa B puzzle. *Cell*. **109**: S81-S96.

Ghosh, S., May, M.J. and Kopp, E.B. (1998) NF-kappa B and Rel proteins: Evolutionarily conserved mediators of immune responses. *Annual Review of Immunology*. **16**: 225-260.

Gilmore, T.D., Koedood, M., Piffat, K.A. and White, D.W. (1996) Rel/NF-kappa B/I kappa B proteins and cancer. *Oncogene*. **13**: 1367-1378.

Green, B.T., Bunnett, N.W., Kulkarni-Narla, A., Steinhoff, M. and Brown, D.R. (2000) Intestinal type 2 proteinase-activated receptors: Expression in opioid-sensitive secretomotor neural circuits that mediate epithelial ion transport. *Journal of Pharmacology and Experimental Therapeutics*. **295**: 410-416.

Greenberg, D.L., Mize, G.J. and Takayama, T.K. (2003) Protease-activated receptor mediated RhoA signaling and cytoskeletal reorganization in LNCaP cells. *Biochemistry*. **42**: 702-709.

Greenfeder, S.A., Varnell, T., Powers, G., Lombardgillooly, K., Shuster, D., McIntyre, K.W., Ryan, D.E., Levin, W., Madison, V. and Ju., G. (1995) Insertion of a structural domain of Interleukin (Il)-1-beta confers agonist activity to the Il-1 receptor antagonist - implications for Il-1 bioactivity. *Journal of Biological Chemistry*. **270**: 22460-22466.

Grewal, S.S., Horgan, A.M., York, R.D., Withers, G.S., Banker, G.A. and Stork, P.J.S. (2000) Neuronal calcium activates a Rap1 and B-Raf signaling pathway via the cyclic adenosine monophosphate-dependent protein kinase. *Journal of Biological Chemistry*. **275**: 3722-3728.

Grotzinger, J. (2002) Molecular mechanisms of cytokine receptor activation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. **1592**: 215-223.

Gsandtner, I., Charalambous, C., Stefan, E., Ogris, E., Freissmuth, M. and Zezula, J. (2005) Heterotrimeric G protein-independent signaling of a G protein-coupled receptor. *Journal of Biological Chemistry*. **280**: 31898-31905.

Gschwendt, M., Muller, H.J., Kielbassa, K., Zang, R., Kittstein, W., Rincke, G. and Marks, F. (1994) Rottlerin, a novel protein-kinase inhibitor. *Biochemical and Biophysical Research Communications*. **199**: 93-98.

Hall, R.A., Premont, R.T. and Lefkowitz, R.J. (1999) Heptahelical receptor signaling: Beyond the G protein paradigm. *Journal of Cell Biology*. **145**: 927-932.

Hamilton, J.R., Chow, J.M. and Cocks, T.M. (1998) Protease-activated receptor-2 turnover can be stimulated independently of receptor activation. *Naunyn-Schmiedebergs Archives of Pharmacology*. **358**: P37204.

Hamilton, J.R., Frauman, A.G. and Cocks, T.M. (2001a) Increased expression of protease-activated receptor-2 (PAR2) and PAR4 in human coronary artery by

inflammatory stimuli unveils endothelium-dependent relaxations to PAR2 and PAR4 agonists. *Circulation Research*. **89**: 92-98.

Hamilton, J.R., Moffatt, J.D., Frauman, A.G. and Cocks, T.M. (2001b) Protease-activated receptor (PAR) 1 but not PAR2 or PAR4 mediates endothelium-dependent relaxation to thrombin and trypsin in human pulmonary arteries. *Journal of Cardiovascular Pharmacology*. **38**: 108-119.

Hamm, H.E. (1998) The many faces of G Protein signalling. *Journal of Biological Chemistry*. **273**: 669-672.

Hansen, J.L. and Sheikh, S.P. (2004) Functional consequences of 7TM receptor dimerization. *European Journal of Pharmaceutical Sciences*. **23**: 301-317.

Hansen, K.K., Saifeddine, M. and Hollenberg, M.D. (2004) Tethered ligand-derived peptides of proteinase-activated receptor 3 (PAR(3)) activate PAR(1) and PAR(2) in Jurkat T cells. *Immunology*. **112**: 183-190.

Hawes, B.E., Vanbiesen, T., Koch, W.J., Luttrell, L.M. and Lefkowitz, R.J. (1995) Distinct pathways of G(i)-mediated and G(q)-mediated mitogen-activated protein-kinase activation. *Journal of Biological Chemistry*. **270**: 17148-17153.

Hermans, E. (2003) Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacology & Therapeutics*. **99**: 25-44.

Heuss, C., Scanziani, M., Gahwiler, B.H. and Gerber, U. (1999) G-protein-independent signaling mediated by metabotropic glutamate receptors. *Nature Neuroscience*. **2**: 1070-1077.

Hildesheim, J., Awwad, R.T. and Fornace, A.J. (2004) p38 mitogen-activated protein kinase inhibitor protects the epidermis against the acute damaging effects of ultraviolet irradiation by blocking apoptosis and inflammatory responses. *Journal of Investigative Dermatology*. **122**: 497-502.

Hollenberg, M.D. and Saifeddine, M. (2001) Proteinase-activated receptor 4 (PAR4): activation and inhibition of rat platelet aggregation by PAR4-derived peptides. *Canadian Journal of Physiology and Pharmacology*. **79**: 439-442.

Hollenberg, M.D. and Compton, S.J. (2002) International Union of Pharmacology. XXVIII. Proteinase-activated receptors. *Pharmacological Reviews*. **54**: 203-217.

Hollenberg, M.D., Saifeddine, M. and AlAni, B. (1996) Proteinase-activated receptor-2 in rat aorta: Structural requirements for agonist activity of receptor-activating peptides. *Molecular Pharmacology*. **49**: 229-233.

Hollenberg, M.D., Saifeddine, M. and Zwiers, H. (2000) Proteinase-activated receptors (PARs): activation of PAR(1) and PAR(2) by a proteolytic fragment of the neuronal growth associated protein B-50/GAP-43. *Canadian Journal of Physiology and Pharmacology*. **78**: 81-85.

Hollenberg, M.D., Saifeddine, M., AlAni, B. and Kawabata, A. (1997) Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides. *Canadian Journal of Physiology and Pharmacology*. **75**: 832-841.

Hollenberg, M.D., Saifeddine, M., Al-Ani, B. and Gui, Y. (1999) Proteinase-activated receptor 4 (PAR(4)): action of PAR(4)-activating peptides in vascular and gastric tissue and lack of cross-reactivity with PAR(1) and PAR(2). *Canadian Journal of Physiology and Pharmacology*. **77**: 458-464.

Hollenberg, M.D., Saifeddine, M., Sandhu, S., Houle, S. and Vergnolle, N. (2004) Proteinase-activated receptor-4: Evaluation of tethered ligand-derived peptides as probes for receptor function and as inflammatory agonists *in vivo*. *British Journal of Pharmacology*. **143**: 443-454.

Hollinger, S. and Hepler, J.R. (2002) Cellular regulation of RGS proteins: Modulators and integrators of G protein signaling. *Pharmacological Reviews*. **54**: 527-559.

Horn, F., Bettler, E., Oliveira, L., Campagne, F., Cohen, F.E. and Vriend, G. (2003) GPCRDB information system for G protein-coupled receptors. *Nucleic Acids Research*. **31**: 294-297.

Hou, L., Kapas, S., Cruchley, A.T., Macey, M.G., Harriott, P., Chinni, C., Stone, S.R. and Howells, G.L. (1998) Immunolocalization of protease-activated receptor-2 in skin: receptor activation stimulates interleukin-8 secretion by keratinocytes in vitro. *Immunology*. **94**: 356-362.

Houle, S., Papez, M.D., Ferazzini, M., Hollenberg, M.D. and Vergnolle, N. (2005) Neutrophils and the kallikrein-kinin system in proteinase-activated receptor 4-mediated inflammation in rodents. *British Journal of Pharmacology*. **146**: 670-678.

Howell, D.C., Laurent, G.J. and Chambers, R.C. (2002) Role of thrombin and its major cellular receptor, protease-activated receptor-1, in pulmonary fibrosis. *Biochemical Society Transactions*. **30**: 211-216.

Howells, G.L., Macey, M.G., Chinni, C., Hou, L., Fox, M.T., Harriott, P. and Stone, S.R. (1997) Proteinase-activated receptor-2: Expression by human neutrophils. *Journal of Cell Science*. **110**: 881-887.

Hoxie, J.A., Ahuja, M., Belmonte, E., Pizarro, S., Parton, R. and Brass, L.F. (1993) Internalization and recycling of activated thrombin receptors. *Journal of Biological Chemistry*. **268**: 13756-13763.

Hsu, H.L., Xiong, J. and Goeddel, D.V. (1995) The TNF Receptor 1-Associated Protein TRADD signals cell-death and NF-kappa-B activation. *Cell*. **81**: 495-504.

Hu, Y., Baud, V., Oga, T., Kim, K.I., Yoshida, K. and Karin, M. (2001) IKKα controls formation of the epidermis independently of NFκB. *Nature*. **410**: 710-714.

Huang, C.F., De Sanctis, G.T., O'Brien, P.J., Mizgerd, J.P., Friend, D.S., Drazen, J.M., Brass, L. F. and Stevens, R. L. (2001) Evaluation of the substrate specificity of human mast cell tryptase beta I and demonstration of its importance in bacterial infections of the lung. *Journal of Biological Chemistry*. **276**: 26276-26284.

Huang, C.S., Ma, W.Y., Maxiner, A., Sun, Y. and Dong, Z.G. (1999) p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. *Journal of Biological Chemistry*. **274**: 12229-12235.

Hubbard, K.B. and Hepler, J.R. (2006) Cell signalling diversity of the Gq alpha family of heterotrimeric G proteins. *Cellular Signalling*. **18**: 135-150.

Hung, D.T., Wong, Y.H., Vu, T.K.H. and Coughlin, S.R. (1992a) The cloned platelet thrombin receptor couples to at least 2 distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. *Journal of Biological Chemistry*. **267**: 20831-20834.

Hung, D.T., Vu, T.K.H., Nelken, N.A. and Coughlin, S.R. (1992b) Thrombin-induced dvents in non-platelet cells are mediated by the unique proteolytic mechanism sstablished for the cloned platelet thrombin receptor. *Journal of Cell Biology*. **116**: 827-832.

Hur, E.M. and Kim, K.T. (2002) G protein-coupled receptor signalling and cross-talk - Achieving rapidity and specificity. *Cellular Signalling*. **14**: 397-405.

Hurley, J.H. (1999) Structure, mechanism, and regulation of mammalian adenylyl cyclase. *Journal of Biological Chemistry*. **274**: 7599-7602.

Hwa, J.J., Ghibaudi, L., Williams, P., Chintala, M., Zhang, R.M., Chatterjee, M. and Sybertz, E. (1996) Evidence for the presence of a proteinase-activated receptor distinct from the thrombin receptor in vascular endothelial cells. *Circulation Research*. **78**: 581-588.

Iaccarino, G., Rockman, H.A., Shotwell, K.F., Tomhave, E.D. and Koch, W.J. (1998) Myocardial overexpression of GRK3 in transgenic mice: evidence for in vivo selectivity

of GRKs. *American Journal of Physiology-Heart and Circulatory Physiology*. **44**: H1298-H1306.

Ikawa, K., Nishioka, T., Yu, Z., Sugawara, Y., Kawagoe, J., Takizawa, T., Primo, V., Nikolic, B., Kuroishi, T., Sasano, T., Shimauchi, H., Takada, H., Endo, Y. and Sugawara, S. (2005) Involvement of neutrophil recruitment and protease-activated receptor 2 activation in the induction of IL-18 in mice. *Journal of Leukocyte Biology*. **78**: 1118-1126.

Ishihara, H., Connolly, A.J., Zeng, D.W., Kahn, M.L., Zheng, Y.W., Timmons, C., Tram, T. and Coughlin, S.R. (1997) Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature*. **386**: 502-506.

Ishii, K., Gerszten, R., Zheng, Y.W., Welsh, J.B., Turck, C.W. and Coughlin, S.R. (1995) Determinants of thrombin receptor cleavage - receptor domains involved, specificity, and role of the P3 aspartate. *Journal of Biological Chemistry*. **270**: 16435-16440.

Jalink, K., Vancorven, E.J., Hengeveld, T., Morii, N., Narumiya, S. and Moolenaar, W.H. (1994) Inhibition of lysophosphatidate-induced and thrombin-induced neurite retraction and neuronal cell rounding by ADP-ribosylation of the small GTP-binding protein-Rho. *Journal of Cell Biology*. **126**: 801-810.

Jeong, S.J., Pise-Masison, C.A., Radonovich, M.F., Park, H.U. and Brady, J.N. (2005) A novel NF-kappa B pathway involving IKK beta and p65/Re1A ser-536 phosphorylation results in p53 inhibition in the absence of NF-kappa B transcriptional activity. *Journal of Biological Chemistry*. **280**: 10326-10332.

Ji, T.H., Grossmann, M. and Ji, I.H. (1998) G protein-coupled receptors I. Diversity of receptor-ligand interactions. *Journal of Biological Chemistry*. **273**: 17299-17302.

Jiang, B.B., Brecher, P. and Cohen, R.A. (2001) Persistent activation of nuclear factor-kappa B by interleukin-1 beta and subsequent inducible NO synthase expression requires extracellular signal-regulated kinase. *Arteriosclerosis Thrombosis and Vascular Biology*. **21**: 1915-1920.

Jiang, X., Takahashi, N., Matsui, N., Tetsuka, T. and Okamoto, T. (2003) The NF-kappa B activation in lymphotoxin beta receptor signaling depends on the phosphorylation of p65 at serine 536. *Journal of Biological Chemistry*. **278**: 919-926.

Jin, G., Hayashi, T., Kawagoe, J., Takizawa, T., Nagata, T., Nagano, I., Syoji. M. and Abe, K. (2005) Deficiency of PAR-2 gene increases acute focal ischemic brain injury. *Journal of Cerebral Blood Flow and Metabolism*. **25**: 302-313.

Johansen, C., Kragballe, K., Westergaard, M., Henningsen, J., Kristiansen, K. and Iversen, L. (2005) The mitogen-activated protein kinases p38 and ERK1/2 are increased in lesional psoriatic skin. *British Journal of Dermatology*. **152**: 37-42.

Johansson, U., Lawson, C., Dabare, M., Syndercombe-Court, D., Newland, A.C., Howells, G.L. and Macey, M.G. (2005) Human peripheral blood monocytes express protease receptor-2 and respond to receptor activation by production of IL-6, IL-8, and IL-1 beta. *Journal of Leukocyte Biology*. **78**: 967-975.

Johnson, K.L. and Ingram, G.C. (2005) Sending the right signals: regulating receptor kinase activity. *Current Opinion in Plant Biology. Cell biology - edited by Patricia C Zambryski and Karl J Oparka*. **8**: 648-656.

Jordan, B.A. and Devi, L.A. (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature*. **399**: 697-700.

Kahan, C., Seuwen, K., Meloche, S. and Pouyssegur, J. (1992) Coordinate, biphasic activation of p44 mitogen-activated protein-kinase and S6 kinase by growth-factors in hamster fibroblasts - evidence for thrombin-induced signals different from phosphoinositide turnover and adenylyl cyclase inhibition. *Journal of Biological Chemistry*. **267**: 13369-13375.

Kahn, M.L., Hammes, S.R., Botka, C. and Coughlin, S.R. (1998) Gene and locus structure and chromosomal localization of the protease-activated receptor gene family. *Journal of Biological Chemistry*. **273**: 23290-23296.

Kahn, M.L., Nakanishi-Matsui, M., Shapiro, M.J., Ishihara, H. and Coughlin, S.R. (1999) Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *Journal of Clinical Investigation*. **103**: 879-887.

Kanda, Y., Mizuno, K., Kuroki, Y. and Watanabe, Y. (2001) Thrombin-induced p38 mitogen-activated protein kinase activation is mediated by epidermal growth factor receptor transactivation pathway. *British Journal of Pharmacology*. **132**: 1657-1664.

Kang, O.H., Jeong, H.J., Kim, D.K., Choi, S.C., Kim, T.H., Nah, Y.H., Kim, H.M. and Lee, Y.M. (2003) Trypsin induces tumour necrosis factor-alpha secretion from a human leukemic mast cell line. *Cell Biochemistry and Function*. **21**: 161-167.

Kanke, T., Macfarlane, S.R., Seatter, M.J., Davenport, E., Paul, A., McKenzie, R.C. and Plevin, R. (2001) Proteinase-activated receptor-2-mediated activation of stress-activated protein kinases and inhibitory kappa B kinases in NCTC 2544 keratinocytes. *Journal of Biological Chemistry*. **276**: 31657-31666.

Karin, M., Liu, Z.-g. and Zandi, E. (1997) AP-1 function and regulation. *Current Opinion in Cell Biology*. **9**: 240-246.

Kato, T., Delhase, M., Hoffmann, A. and Karin, M. (2003) CK2 is a C-terminal I kappa B kinase responsible for NF-kappa B activation during the UV response. *Molecular Cell*. **12**: 829-839.

Kaufman, C.K. and Fuchs, E. (2000) It's got you covered: NF-kappa B in the epidermis. *Journal of Cell Biology*. **149**: 999-1004.

Kaufmann, R., Schulze, B., Krause, G., Mayr, L.M., Settmacher, U. and Henklein, P. (2005) Proteinase-activated receptors (PARs) - the PAR(3) neo-N-terminal peptide TFRGAP interacts with PAR(1). *Regulatory Peptides*. **125**: 61-66.

Kaufmann, R., Patt, S., Zieger, M., Kraft, R., Tausch, S., Henklein, P. and Nowak, G. (2000) The two-receptor system PAR-1/PAR-4 mediates alpha-thrombin-induced Ca^{2+} (i) mobilization in human astrocytoma cells. *Journal of Cancer Research and Clinical Oncology*. **126**: 91-94.

Kawabata, A., Kinoshita, M., Kuroda, R. and Kakehi, K. (2002) Capsazepine partially inhibits neurally mediated gastric mucus secretion following activation of protease-activated receptor 2. *Clinical and Experimental Pharmacology and Physiology*. **29**: 360-361.

Kawabata, A., Kuroda, R., Minami, T., Kataoka, K. and Taneda, M. (1998) Increased vascular permeability by a specific agonist of protease-activated receptor-2 in rat hindpaw. *British Journal of Pharmacology*. **125**: 419-422.

Kawabata, A., Saifeddine, M., Al-Ani, B., Leblond, L. and Hollenberg, M.D. (1999) Evaluation of proteinase-activated receptor-1 (PAR(1)) agonists and antagonists using a cultured cell receptor desensitization assay: Activation of PAR(2) by PAR(1)-targeted ligands. *Journal of Pharmacology and Experimental Therapeutics*. **288**: 358-370.

Kawabata, A., Nishikawa, H., Kuroda, R., Kawai, K. and Hollenberg, M.D. (2000a) Proteinase-activated receptor-2 (PAR-2): regulation of salivary and pancreatic exocrine secretion in vivo in rats and mice. *British Journal of Pharmacology*. **129**: 1808-1814.

Kawabata, A., Kuroda, R., Kuroki, N., Nishikawa, H. and Kawai, K. (2000b) Dual modulation by thrombin of the motility of rat oesophageal muscularis mucosae via two distinct protease-activated receptors (PARs): a novel role for PAR-4 as opposed to PAR-1. *British Journal of Pharmacology*. **131**: 578-584.

Kawabata, A., Morimoto, N., Nishikawa, H., Kuroda, R., Oda, Y. and Kakehi, K. (2000c) Activation of protease-activated receptor-2 (PAR-2) triggers mucin secretion in the rat sublingual gland. *Biochemical and Biophysical Research Communications*. **270**: 298-302.

Kawabata, A., Kuroda, R., Nagata, N., Kawao, N., Masuko, T., Nishikawa, H. and Kawai, K. (2001a) In vivo evidence that protease-activated receptors 1 and 2 modulate gastrointestinal transit in the mouse. *British Journal of Pharmacology*. **133**: 1213-1218.

Kawabata, A., Kinoshita, M., Nishikawa, H., Kuroda, R., Nishida, M., Araki, H., Arizono, N., Oda, Y. and Kakehi, K. (2001b) The protease-activated receptor-2 agonist induces gastric mucus secretion and mucosal cytoprotection. *Journal of Clinical Investigation*. **107**: 1443-1450.

Kawabata, A., Kanke, T., Yonezawa, D., Ishiki, T., Saka, M., Kabeya, M., Sekiguchi, F., Kubo, S., Kuroda, R., Iwaki, M., Katsura, K. and Plevin, R. (2004) Potent and metabolically stable agonists for protease-activated receptor-2: Evaluation of activity in multiple assay systems in vitro and in vivo. *Journal of Pharmacology and Experimental Therapeutics*. **309**: 1098-1107.

Kawagoe, J., Takizawa, T., Matsumoto, J., Tamiya, M., Meek, S.E., Smith, A.J.H., Hunter, G. D., Plevin, R., Saito, N., Kanke, T., Fujii, M. and Wada, Y. (2002) Effect of protease-activated receptor-2 deficiency on allergic dermatitis in the mouse ear. *Japanese Journal of Pharmacology*. **88**: 77-84.

Kawao, N., Sakaguchi, Y., Tagome, A., Kuroda, R., Nishida, S., Irimajiri, K., Nishikawa, H., Kawai, K., Hollenberg, M. D. and Kawabata, A. (2002) Protease-activated receptor-2 (PAR-2) in the rat gastric mucosa: immunolocalization and facilitation of pepsin/pepsinogen secretion. *British Journal of Pharmacology*. **135**: 1292-1296.

Kawao, N., Nagataki, M., Nagasawa, K., Kubo, S., Cushing, K., Wada, T., Sekiguchi, F., Ichida, S., Hollenberg, M. D., MacNaughton, W. K., Nishikawa, H. and Kawabata, A. (2005) Signal transduction for proteinase-activated receptor-2-triggered prostaglandin E-2 formation in human lung epithelial cells. *Journal of Pharmacology and Experimental Therapeutics*. **315**: 576-589.

Kelso, E.B., Lockhart, J.C., Hembrough, T., Dunning, L., Plevin, R., Hollenberg, M.D., Sommerhoff, C. P., McLean, J. S. and Ferrell, W. R. (2006) Therapeutic promise of proteinase-activated receptor-2 antagonism in joint inflammation. *Journal of Pharmacology and Experimental Therapeutics*. **316**: 1017-1024.

Kenakin, T. (1995a) Agonist-receptor efficacy-2. Agonist trafficking of receptor signals. *Trends in Pharmacological Sciences*. **16**: 232-238.

Kenakin, T. (1995b) Agonist-receptor efficacy-1. Mechanisms of efficacy and receptor promiscuity. *Trends in Pharmacological Sciences*. **16**: 188-192.

Klarenbach, S.W., Chipiuk, A., Nelson, R.C., Hollenberg, M.D. and Murray, A.G. (2003) Differential actions of PAR2 and PAR1 in stimulating human endothelial cell

exocytosis and permeability: The role of Rho-GTPases. *Circulation Research.* **92**: 272-278.

Knight, D.A., Lim, S., Scaffidi, A.K., Roche, N., Chung, K.F., Stewart, G.A. and Thompson, P.J. (2001) Protease-activated receptors in human airways: Upregulation of PAR-2 in respiratory epithelium from patients with asthma. *Journal of Allergy and Clinical Immunology*. **108**: 797-803.

Koch, W.J., Hawes, B.E., Allen, L.F. and Lefkowitz, R.J. (1994) Direct dvidence that G(i)-coupled receptor stimulation of mitogen-activated protein-kinase is mediated by G(beta/gamma) and activation of p21(Ras). *Proceedings of the National Academy of Sciences of the United States of America*. **91**: 12706-12710.

Kohno, M. and Pouyssegur, J. (2003) Pharmacological inhibitors of the ERK signalling pathway: application as anticancer drug. *Prog. Cell Cycle*. **5**: 219-224.

Kolakowski, L.F. (1994) Gcrdb - a G-protein-coupled receptor database. *Receptors & Channels*. **2**: 1-7.

Kong, W.Y., McConalogue, K., Khitin, L.M., Hollenberg, M.D., Payan, D.G., Bohm, S.K. and Bunnett, N.W. (1997) Luminal trypsin may regulate enterocytes through proteinase- activated receptor 2. *Proceedings of the National Academy of Sciences of the United States of America*. **94**: 8884-8889.

Koo, B.H., Chung, K.H., Hwang, K.C. and Kim, D.S. (2002) Factor Xa induces mitogenesis of coronary artery smooth muscle cell via activation of PAR-2. *FEBS Letters*. **523**: 85-89.

Koshikawa, N., Nagashima, Y., Miyagi, Y., Mizushima, H., Yanoma, S., Yasumitsu, H. and Miyazaki, K. (1997) Expression of trypsin in vascular endothelial cells. *FEBS Letters*. **409**: 442-448.

Koshikawa, N., Hasegawa, S., Nagashima, Y., Mitsuhashi, K., Tsubota, Y., Miyata, S., Miyagi, Y., Yasumitsu, H. and Miyazaki, K. (1998) Expression of trypsin by epithelial cells of various tissues, leukocytes, and neurons in human and mouse. *American Journal of Pathology*. **153**: 937-944.

Kozasa, T., Jiang, X.J., Hart, M.J., Sternweis, P.M., Singer, W.D., Gilman, A.G., Bollag, G. and Sternweis, P. C. (1998) p115 RhoGEF, a GTPase activating protein for G alpha(12) and G alpha(13). *Science*. **280**: 2109-2111.

Kramer, R.M., Roberts, E.F., Um, S.L., BorschHaubold, A.G., Watson, S.P., Fisher, M.J. and Jakubowski, J.A. (1996) p38 Mitogen-activated protein kinase phosphorylates cytosolic phospholipase A(2) (cPLA(2)) in thrombin-stimulated platelets - Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA(2). *Journal of Biological Chemistry*. **271**: 27723-27729.

Kravchenko, V.V., Mathison, J.C., Schwamborn, K., Mercurio, F. and Ulevitch, R.J. (2003) IKKi/IKK epsilon plays a key role in integrating signals induced by pro-inflammatory stimuli. *Journal of Biological Chemistry*. **278**: 26612-26619.

Kravchenko, V.V., Pan, Z.X., Han, J.H., Herbert, J.M., Ulevitch, R.J. and Ye, R.D. (1995) Platelet-activating-factor induces NF-kappa-B activation through a G-protein-coupled pathway. *Journal of Biological Chemistry*. **270**: 14928-14934.

Kunsch, C., Ruben, S.M. and Rosen, C.A. (1992) Selection of optimal kappa-B/Rel DNA-binding motifs - Interaction of both subunits of NF-kappa-B with DNA is required for transcriptional activation. *Molecular and Cellular Biology*. **12**: 4412-4421.

Kyriakis, J.M. and Avruch, J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiological Reviews*. **81**: 807-869.

Lallena, M.J., Diaz-Meco, M.T., Bren, G., Paya, C.V. and Moscat, J. (1999) Activation of I kappa B kinase beta by protein kinase C isoforms. *Molecular and Cellular Biology*. **19**: 2180-2188.

Lan, R.S., Knight, D.A., Stewart, G.A. and Henry, P.J. (2001) Role of PGE(2) in protease-activated receptor-1,-2 and-4 mediated relaxation in the mouse isolated trachea. *British Journal of Pharmacology*. **132**: 93-100.

Laniyonu, A.A. and Hollenberg, M.D. (1995) Vascular actions of thrombin receptor-derived polypeptides - Structure-activity profiles for contractile and relaxant effects in rat aorta. *British Journal of Pharmacology*. **114**: 1680-1686.

Leger, A.J., Jacques, S.L., Badar, J., Kaneider, N.C., Derian, C.K., Andrade-Gordon, P., Covic, L. and Kuliopulos, A. (2006) Blocking the protease-activated receptor 1-4 heterodimer in platelet-mediated thrombosis. *Circulation*. **113**: 1244-1254.

Lerner, D.J., Chen, M., Tram, T. and Coughlin, S.R. (1996) Agonist recognition by proteinase-activated receptor 2 and thrombin receptor - Importance of extracellular loop interactions for receptor function. *Journal of Biological Chemistry*. **271**: 13943-13947.

Li, N.X. and Karin, M. (1998) Ionizing radiation and short wavelength UV activate NF-kappa B through two distinct mechanisms. *Proceedings of the National Academy of Sciences of the United States of America*. **95**: 13012-13017.

Li, Y.G., Kang, J., Friedman, J., Tarassishin, L., Ye, J.J., Kovalenko, A., Wallach, D. and Horwitz, M. S. (1999) Identification of a cell protein (FIP-3) as a modulator of NF-kappa B activity and as a target of an adenovirus inhibitor of tumor necrosis factor alpha-induced apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*. **96**: 1042-1047.

Lindner, J.R., Kahn, M.L., Coughlin, S.R., Sambrano, G.R., Schauble, E., Bernstein, D., Foy, D., Hafezi-Moghadam, A. and Ley, K. (2000) Delayed onset of inflammation in protease-activated receptor-2- deficient mice. *Journal of Immunology*. **165**: 6504-6510.

Liu, G.X., Shi, J.Y., Yang, L., Cao, L.X., Park, S.M., Cui, J.M. and Marx, S.O. (2004) Assembly of a Ca^{2+}-dependent BK channel signaling complex by binding to beta 2 adrenergic receptor. *EMBO Journal*. **23**: 2196-2205.

Liu, W.S. and Heckman, C.A. (1998) The sevenfold way of PKC regulation. *Cellular Signalling*. **10**: 529-542.

Lourbakos, A., Potempa, J., Travis, J., D'Andrea, M.R., Andrade-Gordon, P., Santulli, R., Mackie, E. J. and Pike, R. N. (2001) Arginine-specific protease from Porphyromonas gingivalis activates protease-activated receptors on human oral epithelial cells and induces interleukin-6 secretion. *Infection and Immunity*. **69**: 5121-5130.

Luo, W.B., Wang, Y.F., Hanck, T., Stricker, R. and Reiser, G. (2006) Jab1, a novel protease-activated receptor-2 (PAR-2)-interacting protein, is involved in PAR-2-induced activation of activator protein-1. *Journal of Biological Chemistry*. **281**: 7927-7936.

Luttrell, L.M. (2002) Activation and targeting of mitogen-activated protein kinases by G-protein-coupled receptors. *Canadian Journal of Physiology and Pharmacology*. **80**: 375-382.

Luttrell, L.M. (2005) Composition and function of G protein-coupled receptor signalsomes controlling mitogen-activated protein kinase activity. *Journal of Molecular Neuroscience*. **26**: 253-263.

Luttrell, L.M., Roudabush, F.L., Choy, E.W., Miller, W.E., Field, M.E., Pierce, K.L. and Lefkowitz, R.J. (2001) Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proceedings of the National Academy of Sciences of the United States of America*. **98**: 2449-2454.

Ma, Y.J., Zhang, B., Qian, R.Z., Lu, C., Zhao, F.D. and Yin, L.H. (2006) Tryptase activates PKB in inflammatory reaction in ECV304 cells. *Biochimica Et Biophysica Acta-Molecular Cell Research*. **1763**: 313-321.

Macfarlane, S.R., Seatter, M.J., Kanke, T., Hunter, G.D. and Plevin, R. (2001) Proteinase-activated receptors. *Pharmacological Reviews*. **53**: 245-282.

Macfarlane, S.R., Sloss, C.M., Cameron, P., Kanke, T., McKenzie, R.C. and Plevin, R. (2005) The role of intracellular Ca^{2+} in the regulation of proteinase-activated receptor-2 mediated nuclear factor kappa B signalling in keratinocytes. *British Journal of Pharmacology*. **145**: 535-544.

Madamanchi, N.R., Li, S., Patterson, C. and Runge, M.S. (2001) Thrombin regulates vascular smooth muscle cell growth and heat shock proteins via the JAK-STAT pathway. *J. Biol. Chem.* **276**: 18915-18924.

Magazine, H.I., King, J.M. and Srivastava, K.D. (1996) Protease activated receptors modulate aortic vascular tone. *International Journal of Cardiology*. **53**: S75-S80.

Majumdar, M., Seasholtz, T.M., Buckmaster, C., Toksoz, D. and Brown, J.H. (1999) A Rho exchange factor mediates thrombin and G alpha(12)-induced cytoskeletal responses. *Journal of Biological Chemistry*. **274**: 26815-26821.

Malarkey, K., Belham, C.M., Paul, A., Graham, A., McLees, A., Scott, P.H. and Plevin, R. (1995) The regulation of tyrosine kinase dignaling pathways by growth-factor and G-protein-coupled receptors. *Biochemical Journal*. **309**: 361-375.

Malinin, N.L., Boldin, M.P., Kovalenko, A.V. and Wallach, D. (1997) MAP3K-related kinase involved in NF-kappa B induction by TNF, CD95 and IL-1. *Nature*. **385**: 540-544.

Marok, R., Winyard, P.G., Coumbe, A., Kus, M.L., Gaffney, K., Blades, S., Mapp, P. I., Morris, C. J., Blake, D. R., Kaltschmidt, C. and Baeuerle, P. A. (1996) Activation of the transcription factor nuclear factor-kappa B in human inflamed synovial tissue. *Arthritis and Rheumatism*. **39**: 583-591.

Maryanoff, B.E., Santulli, R.J., McComsey, D.F., Hoekstra, W.J., Hoey, K., Smith, C.E., Addo, M., Darrow, A. L. and Andrade-Gordon, P. (2001) Protease-activated receptor-2 (PAR-2): Structure-function study of receptor activation by diverse peptides related to tethered- ligand epitopes. *Archives of Biochemistry and Biophysics*. **386**: 195-204.

Masamune, A., Kikuta, K., Satoh, M., Suzuki, N. and Shimosegawa, T. (2005) Protease-activated receptor-2-mediated proliferation and collagen production of rat pancreatic stellate cells. *Journal of Pharmacology and Experimental Therapeutics*. **312**: 651-658.

Mattioli, I., Sebald, A., Bucher, C., Charles, R.P., Nakano, H., Doi, T., Kracht, M. and Schmitz, M. L. (2004) Transient and selective NF-kappa B p65 serine 536 phosphorylation induced by T cell costimulation is mediated by I kappa B kinase beta and controls the kinetics of p65 nuclear import. *Journal of Immunology*. **172**: 6336-6344.

Maudsley, S., Pierce, K.L., Zamah, A.M., Miller, W.E., Ahn, S., Daaka, Y., Lefkowitz, R. J. and Luttrell, L. M. (2000) The β_2-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with the epidermal growth factor receptor. *Journal of Biological Chemistry*. **275**: 9572-9580.

Maytin, E.V., Ubeda, M., Lin, J.C. and Habener, J.F. (2001) Stress-inducible transcription factor CHOP/gadd153 induces apoptosis in mammalian cells via p38 kinase-dependent and -independent mechanisms. *Experimental Cell Research*. **267**: 193-204.

McGuire, J.J., Saifeddine, M., Triggle, C.R., Sun, K. and Hollenberg, M.D. (2004) 2-Furoyl-LIGRLO-amide: A potent and selective proteinase- activated receptor 2 agonist. *Journal of Pharmacology and Experimental Therapeutics*. **309**: 1124-1131.

McLaughlin, J.N., Shen, L.X., Holinstat, M., Brooks, J.D., DiBenedetto, E. and Hamm, H.E. (2005) Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. *Journal of Biological Chemistry*. **280**: 25048-25059.

McNamara, C.A., Sarembock, I.J., Gimple, L.W., Fenton, J.W., Coughlin, S.R. and Owens, G.K. (1993) Thrombin stimulates proliferation of cultured rat aortic smooth-muscle cells by a proteolytically activated receptor. *Journal of Clinical Investigation*. **91**: 94-98.

Mercurio, F., Zhu, H.Y., Murray, B.W., Shevchenko, A., Bennett, B.L., Li, J.W., Young, D. B., Barbosa, M. and Mann, M. (1997) IKK-1 and IKK-2: Cytokine-activated I kappa B kinases essential for NF-kappa B activation. *Science*. **278**: 860-866.

Mercurio, F., Murray, B.W., Shevchenko, A., Bennett, B.L., Young, D.B., Li, J.W., Pascual, G., Motiwala, A., Zhu, H. Y., Mann, M. and Manning, A. M. (1999) I kappa B kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. *Molecular and Cellular Biology*. **19**: 1526-1538.

Miike, S., McWilliam, A.S. and Kita, H. (2001) Trypsin induces activation and inflammatory mediator release from human eosinophils through protease-activated receptor-2. *Journal of Immunology*. **167**: 6615-6622.

Milia, A.F., Salis, M.B., Stacca, T., Pinna, A., Madeddu, P., Trevisani, M., Geppetti, P. and Emanueli, C. (2002) Protease-activated receptor-2 stimulates angiogenesis and accelerates hemodynamic recovery in a mouse model of hindlimb ischemia. *Circulation Research*. **91**: 346-352.

Milligan, G. and Kostenis, E. (2006) Heterotrimeric G-proteins: a short history. *British Journal of Pharmacology*. **147**: S46-S55.

Miotto, D., Hollenberg, M.D., Bunnett, N.W., Papi, A., Braccioni, F., Boschetto, P., Rea, F., Zuin, A., Geppetti, P., Saetta, M., Maestrelli, P., Fabbri, L. M. and Mapp, C. E. (2002) Expression of protease activated receptor-2 (PAR-2) in central airways of smokers and non-smokers. *Thorax*. **57**: 146-151.

Mirza, H., Yatsula, V. and Bahou, W.F. (1996) The proteinase activated receptor-2 (PAR-2) mediates mitogenic responses in human vascular endothelial cells - Molecular characterization and evidence for functional coupling to the thrombin receptor. *Journal of Clinical Investigation*. **97**: 1705-1714.

Mirza, H., Schmidt, V.A., Derian, C.K., Jesty, J. and Bahou, W.F. (1997) Mitogenic responses mediated through the proteinase-activated receptor-2 are induced by expressed forms of mast cell alpha- or beta-tryptases. *Blood*. **90**: 3914-3922.

Mitchell, R., McCulloch, D., Lutz, E., Johnson, M., MacKenzie, C., Fennell, M., Fink, G., Zhou, W. and Sealfon, S. C. (1998) Rhodopsin-family receptors associate with small G proteins to activate phospholipase D. *Nature*. **392**: 411-414.

Mochizuki, N., Ohba, Y., Kiyokawa, E., Kurata, T., Murakami, T., Ozaki, T., Kitabatake, A., Nagashima, K. and Matsuda, M. (1999) Activation of the ERK/MAPK pathway by an isoform of rap1GAP associated with G alpha(i). *Nature*. **400**: 891-894.

Moffatt, J.D. and Cocks, T.M. (1998) Endothelium-dependent and -independent responses to protease- activated receptor-2 (PAR-2) activation in mouse isolated renal arteries. *British Journal of Pharmacology*. **125**: 591-594.

Moffatt, J.D., Jeffrey, K.L. and Cocks, T.M. (2002) Protease-activated receptor-2 activating peptide SLIGRL inhibits bacterial lipopolysaccharide-induced recruitment of polymorphonuclear leukocytes into the airways of mice. *American Journal of Respiratory Cell and Molecular Biology*. **26**: 680-684.

Molino, M., Woolkalis, M.J., Reavey-Cantwell, J., Pratico, D., Andrade-Gordon, P., Barnathan, E.S. and Brass, L.F. (1997a) Endothelial cell thrombin receptors and PAR-2 - Two protease-activated receptors located in a single cellular environment. *Journal of Biological Chemistry*. **272**: 11133-11141.

Molino, M., Barnathan, E.S., Numerof, R., Clark, J., Dreyer, M., Cumashi, A., Hoxie, J. A., Schechter, N., Woolkalis, M. and Brass, L. F. (1997b) Interactions of mast cell tryptase with thrombin receptors and PAR-2. *Journal of Biological Chemistry*. **272**: 4043-4049.

Momota, F., Hirano, K., Hirano, M., Nishimura, J. and Kanaide, H. (2006) Involvement of G(i/0) in the PAR-4-induced NO production in endothelial cells. *Biochemical and Biophysical Research Communications*. **342**: 365-371.

Morris, A.J. and Scarlata, S. (1997) Regulation of effectors by G-protein alpha- and beta gamma-subunits - Recent insights from studies of the phospholipase C-beta isoenzymes. *Biochemical Pharmacology*. **54**: 429-435.

Mule, F. and Baffi, M.C. (2002) Mechanisms underlying the protease-activated receptor-2-evoked mechanical responses in rat colon. *Gastroenterology*. **122**: M1034.

Mutoh, H., Ishii, S., Izumi, T., Kato, S. and Shimizu, T. (1994) Platelet-activating-factor (PAF)positively auto-regulates the expression of human PAF receptor transcript-1 (leukocyte-type) through NF-kappa-B. *Biochemical and Biophysical Research Communications*. **205**: 1137-1142.

Muzio, M., Ni, J., Feng, P. and Dixit, V.M. (1997) IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science*. **278**: 1612-1615.

Nairn, A.C. and Picciotto, M.R. (1994) Calcium/calmodulin-dependent protein-kinases. *Seminars in Cancer Biology*. **5**: 295-303.

Nakanishi-Matsui, M., Zheng, Y.W., Sulciner, D.J., Weiss, E.J., Ludeman, M.J. and Coughlin, S.R. (2000) PAR3 is a cofactor for PAR4 activation by thrombin. *Nature*. **404**: 609-613.

Nanevicz, T., Ishii, M., Wang, L., Chen, M., Chen, J., Turck, C.W., Cohen, F. E. and Coughlin, S. R. (1995) Mechanisms of thrombin receptor agonist specificity - chimeric receptors and complementary mutations identify an agonist recognition site. *Journal of Biological Chemistry*. **270**: 21619-21625.

Napoli, C., De Nigris, F., Cicala, C., Wallace, J.L., Caliendo, G., Condorelli, M., Santagada, V. and Cirino, G. (2002) Protease-activated receptor-2 activation improves efficiency of experimental ischemic preconditioning. *American Journal of Physiology-Heart and Circulatory Physiology*. **282**: H2004-H2010.

Napoli, C., Cicala, C., Wallace, J.L., de Nigris, F., Santagada, V., Caliendo, C., Santagada, V. and Cirino, G. (2000) Protease-activated receptor-2 modulates myocardial ischemia- reperfusion injury in the rat heart. *Proceedings of the National Academy of Sciences of the United States of America*. **97**: 3678-3683.

Nathans, J. and Hogness, D.S. (1983) Isolation, sequence-analysis, and intron exon arrangement of the gene encoding bovine rhodopsin. *Cell*. **34**: 807-814.

Neurath, M.F., Pettersson, S., zumBuschenfelde, K.H.M. and Strober, W. (1996) Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nature Medicine*. **2**: 998-1004.

Nguyen, Q.D., Faivre, S., Bruyneel, E., Rivat, C., Seto, M., Endo, T., Mareel, M., Emami, S. and Gespach, C. (2002) RhoA- and RhoD-dependent regulatory switch of G alpha subunit signaling by PAR-1 receptors in cellular invasion. *FASEB Journal*. **16**: 565-576.

Nguyen, T.D., Moody, M.W., Steinhoff, M., Okolo, C., Koh, D.S. and Bunnett, N.W. (1999) Trypsin activates pancreatic duct epithelial cell ion channels through proteinase-activated receptor-2. *Journal of Clinical Investigation*. **103**: 261-269.

Nicklin, S.A. and Baker, A.H. (1999) Vascular Disease: Molecular Biology and Gene Therapy Protocols. *Methods in Molecular Medine*: *-edited by Baker, A.H., The Humana Press, Totowa, NJ.* pp.271-283.

Nishibori, M., Mori, S. and Takahashi, H.K. (2005) Physiology and pathophysiology of proteinase-activated receptors (PARs): PAR-2-mediated proliferation of colon cancer cell. *Journal of Pharmacological Sciences*. **97**: 25-30.

Nishikawa, H., Kawai, K., Nishimura, S., Tanaka, S., Araki, H., Al-Ani, B., Hollenberg, M. D., Kuroda, R. and Kawabata, A. (2002) Suppression by protease-activated receptor-2 activation of gastric acid secretion in rats. *European Journal of Pharmacology*. **447**: 87-90.

Nishina, H., Wada, T. and Katada, T. (2004) Physiological roles of SAPK/JNK signalling pathway. *Journal of Biochemistry (Tokyo)*. **136**: 123-126.

Noorbakhsh, F., Vergnolle, N., McArthur, J.C., Silva, C., Vodjgani, M., Andrade-Gordon, P., Hollenberg, M. D. and Power, C. (2005) Proteinase-activated receptor-2 induction by neuroinflammation prevents neuronal death during HIV infection. *Journal of Immunology*. **174**: 7320-7329.

Nose, T., Shimohigashi, Y., Ohno, M., Costa, T., Shimizu, N. and Ogino, Y. (1993) Enhancement of thrombin receptor activation by thrombin receptor-derived heptapeptide with para-fluorophenylalanine in place of phenylalanine. *Biochemical and Biophysical Research Communications*. **193**: 694-699.

Nyaruhucha, C.N.M., Kito, M. and Fukuoka, S.-I. (1997) Identification and expression of the cDNA-encoding human mesotrypsin(ogen), an isoform of trypsin with inhibitor resistance. *Journal of Biological Chemistry*. **272**: 10573-10578.

Nystedt, S., Ramakrishnan, V. and Sundelin, J. (1996) The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells - Comparison with the thrombin receptor. *Journal of Biological Chemistry*. **271**: 14910-14915.

Nystedt, S., Emilsson, I.E., Wahlestedt, C. and Sundelin, J. (1994) Molecular-cloning of a potential proteinase activated receptor. *Proceedings of the National Academy of Sciences of the United States of America*. **91**: 9208-9212.

Nystedt, S., Larsson, A.K., Aberg, H. and Sundelin, J. (1995a) The mouse proteinase-activated receptor-2 cDNA and gene - Molecular cloning and functional expression. *Journal of Biological Chemistry*. **270**: 5950-5955.

Nystedt, S., Emilsson, K., Larsson, A.K., Strombeck, B. and Sundelin, J. (1995b) Molecular-cloning and functional expression of the gene encoding the human proteinase-activated receptor-2. *European Journal of Biochemistry*. **232**: 84-89.

O'Brien, P.J., Prevost, N., Molino, M., Hollinger, M.K., Woolkalis, M.J., Woulfe, D.S. and Brass, L.F. (2000) Thrombin responses in human endothelial cells. Contributions from receptors other than PAR1 include the transactivationi of PAR2 by thrombin-cleaved PAR1. *Journal of Biological Chemistry*. **275**: 13502-13509.

Offermanns, S. and Simon, M.I. (1995) G-alpha(15) and G-alpha(16) couple a wide variety of receptors to phospholipase-C. *Journal of Biological Chemistry*. **270**: 15175-15180.

Offermanns, S., Toombs, C.F., Hu, Y.H. and Simon, M.I. (1997) Defective platelet activation in G alpha(q)-deficient mice. *Nature*. **389**: 183-186.

Oitzinger, W., Hofer-Warbinek, R., Schmid, J.A., Koshelnick, Y., Binder, B.R. and de Martin, R. (2001) Adenovirus-mediated expression of a mutant I kappa B kinase 2 inhibits the response of endothelial cells to inflammatory stimuli. *Blood*. **97**: 1611-1617.

Oldham, K.G. (1990). Polyphosphoinositide turnover. *Receptor-effector coupling – a practical approach - edited by Hulme, E.C., Oxford University Press.*. pp. 99-116

O'Mahony, A.M., Montano, M., Van Beneden, K., Chen, L.F. and Greene, W.C. (2004) Human T-cell lymphotropic virus type 1 tax induction of biologically active NF-kappa B requires I kappa B kinase-1-mediated phosphorylation of RelA/p65. *Journal of Biological Chemistry*. **279**: 18137-18145.

Oshiro, A., Otani, H., Yagi, Y., Fukuhara, S. and Inagaki, C. (2002) Protease-activated receptor-2-mediated Ca^{2+} signaling in guinea pig tracheal epithelial cells. *Life Sciences*. **71**: 547-558.

Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M. and Donner, D.B. (1999) NF-kappa B activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature*. **401**: 82-85.

Page, K., Strunk, V.S. and Hershenson, M.B. (2003) Cockroach proteases increase IL-8 expression in human bronchial epithelial cells via activation of protease-activated receptor (PAR)-2 and extracellular-signal-regulated kinase. *Journal of Allergy and Clinical Immunology*. **112**: 1112-1118.

Page, K., Hughes, V.S., Odoms, K.K., Dunsmore, K.E. and Hershenson, M.B. (2005) German cockroach proteases regulate interleukin-8 expression via nuclear factor for interleukin-6 in human bronchial epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*. **32**: 225-231.

Paing, M.M., Stutts, A.B., Kohout, T.A., Lefkowitz, R.J. and Trejo, J. (2002) beta-arrestins regulate protease-activated receptor-1 desensitization but not internalization or down-regulation. *Journal of Biological Chemistry*. **277: 1292-1300.**

Palanche, T., Ilien, B., Zoffmann, S., Reck, M.P., Bucher, B., Edelstein, S.J. and Galzi, J.L. (2001) The neurokinin A receptor activates calcium and cAMP responses through distinct conformational states. *Journal of Biological Chemistry*. **276**: 34853-34861.

Pan, Z.K., Zuraw, B.L., Lung, C.C., Prossnitz, E.R., Browning, D.D. and Ye, R.D. (1996) Bradykinin stimulates NF-kappa B activation and interleukin 1 beta gene expression in cultured human fibroblasts. *Journal of Clinical Investigation*. **98**: 2042-2049.

Parmentier, M., Libert, F. and Vassart, G. (1995) G-Protein-Coupled Orphan Receptors. *M S-Medecine Sciences*. **11**: 222-231.

Parmer, T.G., Ward, M.D. and Hait, W.N. (1997) Effects of rottlerin, an inhibitor of calmodulin-dependent protein kinase III, on cellular proliferation, viability, and cell cycle distribution in malignant glioma cells. *Cell Growth & Differentiation*. **8**: 327-334.

Parry, G.C.N. and Mackman, N. (1994) A set of inducible genes expressed by activated human monocytic and endothelial cells contain kappa-B-like sites that specifically bind C-Rel/p65 heterodimers. *Journal of Biological Chemistry*. **269**: 20823-20825.

Parry, G.C.N. and Mackman, N. (1997) Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappa B-mediated transcription. *Journal of Immunology*. **159**: 5450-5456.

Pearson, G., Robinson, F., Gibson, T.B., Xu, B.E., Karandikar, M., Berman, K. and Cobb, M.H. (2001) Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. *Endocrine Reviews*. **22**: 153-183.

Peters, R.T. and Maniatis, T. (2001) A new family of IKK-related kinases may function as I kappa B kinase kinases. *Biochimica et Biophysica Acta-Reviews on Cancer*. **1471**: M57-M62.

Peters, R.T., Liao, S.M. and Maniatis, T. (2000) IKK epsilon is part of a novel PMA-inducible I kappa B kinase complex. *Molecular Cell*. **5**: 513-522.

Pircher, T.J., Petersen, H., Gustafsson, J.A. and Haldosen, L.A. (1999) Extracellular signal-regulated kinase (ERK) interacts with signal transducer and activator of transcription (STAT) 5A. *Molecular Endocrinology*. **13**: 555-565.

Pohjanpelto, P. (1977) Proteases stimulate proliferation of human fibroblasts. *Journal of Cellular Physiology*. **91**: 387-392.

Pommier, B., Da Nascimento, S., Dumont, S., Bellier, B., Million, E., Garbay, C., Roques, B. P. and Noble, F. (1999) The cholecystokinin(B) receptor is coupled to two effector pathways through pertussis toxin-sensitive and -insensitive G proteins. *Journal of Neurochemistry*. **73**: 281-288.

Porter, C.M., Havens, M.A. and Clipstone, N.A. (2000) Identification of amino acid residues and protein kinases involved in the regulation of NFATc subcellular localization. *Journal of Biological Chemistry*. **275**: 3543-3551.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*. **402**: 884-888.

Rahman, A., True, A.L., Anwar, K.N., Ye, R.D., Voyno-Yasenetskaya, T.A. and Malik, A.B. (2002) G alpha(q) and G beta gamma regulate PAR-1 signaling of thrombin-induced NF-kappa B activation and ICAM-1 transcription in endothelial cells. *Circulation Research*. **91**: 398-405.

Raj, G.V., Sekula, J.A., Guo, R.S., Madden, J.F. and Daaka, Y. (2004) Lysophosphatidic acid promotes survival of androgen-insensitive prostate cancer PC3 cells via activation of NF-kappa B. *Prostate*. **61**: 105-113.

Rasmussen, U.B., Vouretcraviari, V., Jallat, S., Schlesinger, Y., Pages, G., Pavirani, A., Lecocq, J. P., Pouyssegur, J. and Vanobberghenschilling, E. (1991) cDNA cloning and expression of a hamster alpha-thrombin receptor coupled to Ca^{2+} mobilization. *FEBS Letters*. **288**: 123-128.

Rawlings, N.D., Tolle, D.P. and Barrett, A.J. (2004) MEROPS: the peptidase database. *Nucleic Acids Research*. **32**: D160-D164.

Reed, D.E., Barajas-Lopez, C., Cottrell, G., Velazquez-Rocha, S., Dery, O., Grady, E.F., Bunnett, N. W. and Vanner, S. J. (2003) Mast cell tryptase and proteinase-activated receptor 2 induce hyperexcitability of guinea-pig submucosal neurons. *Journal of Physiology-London*. **547**: 531-542.

Regnier, C.H., Song, H.Y., Gao, X., Goeddel, D.V., Cao, Z.D. and Rothe, M. (1997) Identification and characterization of an I kappa B kinase. *Cell*. **90**: 373-383.

Reiter, E. and Lefkowitz, R.J. (2006) GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. *Trends in Endocrinology and Metabolism*. **17**: 159-165.

Rennecke, J., Rehberger, P.A., Furstenberger, G., Johannes, F.J., Stohr, M., Marks, F. and Richter, K.H. (1999) Protein-kinase-C mu expression correlates with enhanced

keratinocyte proliferation in normal and neoplastic mouse epidermis and in cell culture. *International Journal of Cancer*. **80**: 98-103.

Rhee, S.G. and Bae, Y.S. (1997) Regulation of phosphoinositide-specific phospholipase C isozymes. *Journal of Biological Chemistry*. **272**: 15045-15048.

Ricciardolo, F.L.M., Steinhoff, M., Amadesi, S., Guerrini, R., Tognetto, M., Trevisani, M., Creminon, C., Bertrand, C., Bunnett, N. W., Fabbri, L. M., Salvadori, S. and Geppetti, P. (2000) Presence and bronchomotor activity of protease-activated receptor-2 in guinea pig airways. *American Journal of Respiratory and Critical Care Medicine*. **161**: 1672-1680.

Rickard, A. and McHowat, J. (2002) Phospholipid metabolite production in human urothelial cells after protease-activated receptor cleavage. *American Journal of Physiology-Renal Physiology*. **283**: F944-F951.

Riewald, M. and Ruf, W. (2001) Mechanistic coupling of protease signaling and initiation of coagulation by tissue factor. *Proceedings of the National Academy of Sciences of the United States of America*. **98**: 7742-7747.

Riobo, N.A. and Manning, D.R. (2005) Receptors coupled to heterotrimeric G proteins of the G(12) family. *Trends in Pharmacological Sciences*. **26**: 146-154.

Robin, J., Kharbanda, R., McLean, P., Campbell, R. and Vallance, P. (2003) Protease-activated receptor 2-mediated vasodilatation in humans in vivo - Role of nitric oxide and prostanoids. *Circulation*. **107**: 954-959.

Rocheville, M., Lange, D.C., Kumar, U., Patel, S.C., Patel, R.C. and Patel, Y.C. (2000) Receptors for dopamine and somatostatin: Formation of hetero-oligomers with enhanced functional activity. *Science*. **288**: 154-157.

Roosterman, D., Schmidlin, F. and Bunnett, N.W. (2003) Rab5a and rab11a mediate agonist-induced trafficking of protease-activated receptor 2. *American Journal of Physiology-Cell Physiology*. **284**: C1319-C1329.

Rothwarf, D.M., Zandi, E., Natoli, G. and Karin, M. (1998) IKK-gamma is an essential regulatory subunit of the I kappa B kinase complex. *Nature*. **395**: 297-300.

Roux, P.P. and Blenis, J. (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiology and Molecular Biology Reviews*. **68**: 320-344.

Roviezzo, F., Bucci, M., Brancaleone, V., Di Lorenzo, A., Geppetti, P., Farneti, S., Parente, L., Lungarella, G., Fiorucci, S. and Cirino, G. (2005) Proteinase-activated receptor-2 mediates arterial vasodilation in diabetes. *Arteriosclerosis Thrombrosis Vascular Biology*. **25**: 2349-2354.

Rumenapp, U., Asmus, M., Schablowski, H., Woznicki, M., Han, L., Jakobs, K.H., Fahimi-Vahid, M., Michalek, C., Wieland, T. and Schmidt, M. (2001) The M-3 muscarinic acetylcholine receptor expressed in HEK-293 cells signals to phospholipase D via G(12) but not G(q)-type G proteins - Regulators of G proteins as tools to dissect pertussis toxin-resistant G proteins in receptor-effector coupling. *Journal of Biological Chemistry*. **276**: 2474-2479.

Sabri, A., Short, J., Guo, J. and Steinberg, S.F. (2002) Protease-activated receptor-1-mediated DNA synthesis in cardiac fibroblast is via epidermal growth factor receptor transactivation: Distinct PAR-1 signalling pathways in cardiac fibroblasts and cardiomyocytes. *Circulation Research*. **91**: 532-539.

Sabri, A., Muske, G., Zhang, H.L., Pak, E., Darrow, A., Andrade-Gordon, P. and Steinberg, S.F. (2000) Signaling properties and functions of two distinct cardiomyocyte protease-activated receptors. *Circulation Research*. **86**: 1054-1061.

Sagan, S., Chassaing, G., Pradier, L. and Lavielle, S. (1996) Tachykinin peptides affect differently the second messenger pathways after binding to CHO-expressed human NK-1 receptors. *Journal of Pharmacology and Experimental Therapeutics*. **276**: 1039-1048.

Saifeddine, M., AlAni, B., Cheng, C.H., Wang, L. and Hollenberg, M.D. (1996) Rat proteinase-activated receptor-2 (PAR-2): cDNA sequence and activity of receptor-derived peptides in gastric and vascular tissue. *British Journal of Pharmacology*. **118**: 521-530.

Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T. and Toriumi, W. (1999) I kappa B kinases phosphorylate NF-kappa B p65 subunit on serine 536 in the transactivation domain. *Journal of Biological Chemistry*. **274**: 30353-30356.

Sakurai, H., Suzuki, S., Kawasaki, N., Nakano, H., Okazaki, T., Chino, A., Doi, T. and Saiki, I. (2003) Tumor necrosis factor-alpha-induced IKK phosphorylation of NF-kappa B p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. *Journal of Biological Chemistry*. **278**: 36916-36923.

Sambrano, G.R., Huang, W., Faruqi, T., Mahrus, S., Craik, C. and Coughlin, S.R. (2000) Cathepsin G activates protease-activated receptor-4 in human platelets. *Journal of Biological Chemistry*. **275**: 6819-6823.

Sanderson, M.P., Dempsey, P.J. and Dunbar, A.J. (2006) Control of ErbB signaling through metalloprotease mediated ectodomain shedding of EGF-like factors. *Growth Factors*. **24**: 121-136.

Santagada, V., Caliendo, G., Severino, B., Perissutti, E., Fiorino, F., Cicala, C., De Filippis, V. and Cirino, G. (2002) Minimal structural requirements for agonist activity of PAR-2 activating peptides. *Bioorganic & Medicinal Chemistry Letters*. **12**: 21-24.

Santulli, R.J., Derian, C.K., Darrow, A.L., Tomko, K.A., Eckardt, A.J., Seiberg, M., Scarborough, R. M. and Andrade-Gordon, P. (1995) Evidence for the presence of a protease-activated receptor distinct from the thrombin receptor in human keratinocytes. *Proceedings of the National Academy of Sciences of the United States of America*. **92**: 9151-9155.

Sanz, L., Sanchez, P., Lallena, M.J., Diaz-Meco, M.T. and Moscat, J. (1999) The interaction of p62 with RIP links the atypical PKCs to NF-kappa B activation. *EMBO Journal*. **18**: 3044-3053.

Sassone-Corsi, P., Mizzen, C.A., Cheung, P., Crosio, C., Monaco, L., Jacquot, S., Hanauer, A. and Allis, C. D. (1999) Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3. *Science*. **285**: 886-891.

Sawada, K., Nishibori, M., Nakaya, N., Wang, Z. and Saeki, K. (2000) Purification and characterization of a trypsin-like serine proteinase from rat brain slices that degrades laminin and type IV collagen and stimulates protease-activated receptor-2. *Journal of Neurochemistry*. **74**: 1731-1738.

Scarborough, R.M., Naughton, M.A., Teng, W., Hung, D.T., Rose, J., Vu, T.K.H., Wheaton, V. I., Turck, C. W. and Coughlin, S. R. (1992) Tethered ligand agonist peptides - structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *Journal of Biological Chemistry*. **267**: 13146-13149.

Scherer, D.C., Brockman, J.A., Chen, Z.J., Maniatis, T. and Ballard, D.W. (1995) Signal-induced degradation of I-kappa-B-alpha requires site-specific ubiquitination. *Proceedings of the National Academy of Sciences of the United States of America*. **92**: 11259-11263.

Schmidlin, F., Amadesi, S., Dabbagh, K., Lewis, D.E., Knott, P., Bunnett, N.W., Gater, P. R., Geppetti, P., Bertrand, C. and Stevens, M. E. (2002) Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. *Journal of Immunology*. **169**: 5315-5321.

Schmidlin, F., Amadesi, S., Vidil, R., Trevisani, M., Martinet, N., Caughey, G., Tognetto, M., Cavallesco, G., Mapp, C., Geppetti, P. and Bunnett, N. W. (2001) Expression and function of proteinase-activated receptor 2 in human bronchial smooth muscle. *American Journal of Respiratory and Critical Care Medicine*. **164**: 1276-1281.

Schmidt, V.A., Nierman, W.C., Maglott, D.R., Cupit, L.D., Moskowitz, K.A., Wainer, J.A. and Bahou, W.F. (1998) The human proteinase-activated receptor-3 (PAR-3) gene - Identification within a PAR gene cluster and characterization in vascular endothelial cells and platelets. *Journal of Biological Chemistry*. **273**: 15061-15068.

Schmitz, M.L., Silva, M.A.D. and Baeuerle, P.A. (1995) Transactivation Domain-2 (TA(2)) of p65 NF-kappa-B - Similarity to TA(1) and phorbol ester-stimulated activity and phosphorylation in intact cells. *Journal of Biological Chemistry*. **270**: 15576-15584.

Schoonbroodt, S., Ferreira, V., Best-Belpomme, M., Boelaert, J.R., Legrand-Poels, S., Korner, M. and Piette, J. (2000) Crucial role of the amino-terminal tyrosine residue 42 and the carboxyl-terminal PEST domain of I kappa B alpha in NF-kappa B activation by an oxidative stress. *Journal of Immunology*. **164**: 4292-4300.

Schultheiss, M., Neumcke, B. and Richter, H.P. (1997) Endogenous trypsin receptors in *Xenopus* oocytes: linkage to internal calcium stores. *Cellular and Molecular Life Sciences*. **53**: 842-849.

Schwabe, R.F. and Sakurai, H. (2005) IKK beta phosphorylates p65 at S468 in transactivaton domain 2. *FASEB Journal*. **19**.

Schwabe, R.F., Bennett, B.L., Manning, A.M. and Brenner, D.A. (2001) Differential role of I kappa B kinase 1 and 2 in primary rat hepatocytes. *Hepatology*. **33**: 81-90.

Schwabe, R.F., Bradham, C.A., Uehara, T., Hatano, E., Bennett, B.L., Schoonhoven, R. and Brenner, D.A. (2003) c-Jun-N-terminal kinase drives cyclin D1 expression and proliferation during liver regeneration. *Hepatology*. **37**: 824-832.

Schwarz, M. and Murphy, P.M. (2001) Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor constitutively activates NF-kappa B and induces proinflammatory cytokine via a C-terminal signaling and chemokine production determinant. *Journal of Immunology*. **167**: 505-513.

Scott, G., Leopardi, S., Parker, L., Babiarz, L., Seiberg, M. and Han, R.J. (2003) The proteinase-activated receptor-2 mediates phagocytosis in a Rho-dependent manner in human keratinocytes. *Journal of Investigative Dermatology*. **121**: 529-541.

Scott, G., Deng, A., Rodriguez-Burford, C., Seiberg, M., Han, R.J., Babiarz, L., Grizzle, W., Bell, W. and Pentland, A. (2001) Protease-activated receptor 2, a receptor involved in melanosome transfer, is upregulated in human skin by ultraviolet irradiation. *Journal of Investigative Dermatology*. **117**: 1412-1420.

Seatter, M.J., Drummond, R., Kanke, T., Macfarlane, S.R., Hollenberg, M.D. and Plevin, R. (2004) The role of the C-terminal tail in protease-activated receptor- 2-mediated Ca^{2+} signalling, proline-rich tyrosine kinase-2 activation, and mitogen-activated protein kinase activity. *Cellular Signalling*. **16**: 21-29.

Seeliger, S., Derian, C.K., Vergnolle, N., Bunnett, N.W., Nawroth, R., Schmelz, M., von der Weid, P. Y., Buddenkotte, J., Sunderkotter, C., Metze, D., Andrade-Gordon, P., Harms, E., Vestweber, D., Luger, T. A. and Steinhoff, M.(2003) Proinflammatory role of proteinase-activated receptor-2 in humans and mice during cutaneous inflammation in vivo. *FASEB Journal*. **17**: 1871-1885.

Sefton, B.M. and Rubin, H. (1970) Release from density dependent growth inhibition by proteolytic enzymes. *Nature*. **227**: 843-&.

Seiberg, M., Paine, C., Sharlow, E., Andrade-Gordon, P., Costanzo, M., Eisinger, M. and Shapiro, S.S. (2000) The protease-activated receptor 2 regulates pigmentation via keratinocyte-melanocyte interactions. *Experimental Cell Research*. **254**: 25-32.

Sen, R. and Baltimore, D. (1986) Multiple Nuclear factors interact with the immunoglobulin enhancer sequences. *Cell*. **46**: 705-716.

Senftleben, U., Cao, Y.X., Xiao, G.T., Greten, F.R., Krahn, G., Bonizzi, G., Chen, Y., Hu, Y. L., Fong, A., Sun, S. C. and Karin, M. (2001) Activation by IKK alpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science*. **293**: 1495-1499.

Seuwen, K., Kahan, C., Hartmann, T. and Pouyssegur, J. (1990) Strong and persistent activation of inositol lipid breakdown induces early mitogenic events but not Go to S phase progression in hamster fibroblasts. Comparison of thrombin and carbachol action in cells expressing M1 muscarinic acetylcholine receptors. *Journal of Biological Chemistry*. **265**: 22292-22299.

Sexl, V., Mancusi, G., Holler, C., Gloria-Maercker, E., Schutz, W. and Freissmuth, M. (1997) Stimulation of the mitogen-activated protein kinase via the A2A-Adenosine receptor in primary human endothelial cells. *Journal of Biological Chemistry*. **272**: 5792-5799.

Shahrestanifar, M., Fan, X.M. and Manning, D.R. (1999) Lysophosphatidic acid activates NF-kappa B in fibroblasts - A requirement for multiple inputs. *Journal of Biological Chemistry*. **274**: 3828-3833.

Shapiro, M.J., Weiss, E.J., Faruqi, T.R. and Coughlin, S.R. (2000) Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *Journal of Biological Chemistry*. **275**: 25216-25221.

Sharlow, E.R., Paine, C.S., Babiarz, L., Eisinger, M., Shapiro, S. and Seiberg, M. (2000) The protease-activated receptor-2 upregulates keratinocyte phagocytosis. *Journal of Cell Science*. **113**: 3093-3101.

Shaywitz, A.J. and Greenberg, M.E. (1999) CREB: A stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annual Review of Biochemistry*. **68**: 821-861.

Shenoy, S.K. and Lefkowitz, R.J. (2003) Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. *Biochemical Journal*. **375**: 503-515.

Shepard, L.W., Yang, M., Xie, P., Browning, D.D., Voyno-Yasenetskaya, T., Kozasa, T. and Ye, R.D. (2001) Constitutive activation of NF-kappa B and secretion of interleukin-8 induced by the G protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus involve G alpha(13) and RhoA. *Journal of Biological Chemistry*. **276**: 45979-45987.

Shi, C.S. and Kehrl, J.H. (2001) PYK2 links G(q)alpha and G(13)alpha signaling to NF-kappa B activation. *Journal of Biological Chemistry*. **276**: 31845-31850.

Shishodia, S. and Aggarwal, B.B. (2004) Nuclear factor-KB: a friend or a foe in cancer? *Biochemical Pharmacology*. **68**: 1071-1080.

Shpacovitch, V.M., Brzoska, T., Buddenkotte, J., Stroh, C., Sommerhoff, C.P., Ansel, J.C., Schulze-Osthoff, K., Bunnett, N. W., Luger, T. A. and Steinhoff, M. (2002) Agonists of proteinase-activated receptor 2 induce cytokine release and activation of nuclear transcription factor kappa B in human dermal microvascular endothelial cells. *Journal of Investigative Dermatology*. **118**: 380-385.

Siehler, S., Wang, Y.R., Fan, X.M., Windh, R.T. and Manning, D.R. (2001) Sphingosine 1-phosphate activates nuclear factor-kappa B through Edg receptors. *Journal of Biological Chemistry*. **276**: 48733-48739.

Smith-Swintosky, V.L., CheoIsaacs, C.T., Dandrea, M.R., Santulli, R.J., Darrow, A.L. and AndradeGordon, P. (1997) Protease-activated receptor-2 (PAR-2) is present in the rat hippocampus and is associated with neurodegeneration. *Journal of Neurochemistry*. **69**: 1890-1896.

Sobey, C.G. and Cocks, T.M. (1998) Activation of protease-activated receptor-2 (PAR-2) elicits nitric oxide-dependent dilatation of the basilar artery in vivo. *Stroke*. **29**: 1439-1444.

Sobey, C.G., Moffatt, J.D. and Cocks, T.M. (1999) Evidence for selective effects of chronic hypertension on cerebral artery vasodilatation to protease-activated receptor-2 activation. *Stroke*. **30**: 1933-1940.

Stalheim, L., Ding, Y., Gullapalli, A., Paing, M.M., Wolfe, B.L., Morris, D.R. and Trejo, J. (2005) Multiple independent functions of arrestins in the regulation of protease-activated receptor-2 signaling and trafficking. *Molecular Pharmacology*. **67**: 78-87.

Stanislawska, J. and Olszewski, W.L. (2005) RNA interference - significance and applications. *Archivum Immunologiae Et Therapiae Experimentalis*. **53**: 39-46.

Stark, G.R., Kerr, I.M., Williams, B.R.G., Silverman, R.H. and Schreiber, R.D. (1998) How cells respond to interferons. *Annual Review of Biochemistry*. **67**: 227-264.

Steinhoff, M., Corvera, C.U., Thoma, M.S., Kong, W., Caughey, G.H., Ansel, J.C. and Bunnett, N.W. (1999) Proteinase-activated receptor-2 in human skin: tissue distribution and activation of keratinocytes by mast cell tryptase. *Experimental Dermatology*. **8**: 282-294.

Steinhoff, M., Neisius, U., Ikoma, A., Fartasch, M., Heyer, G., Skov, P.S., Luger, T. A. and Schmelz, M. (2003) Proteinase-activated receptor-2 mediates itch: A novel pathway for pruritus in human skin. *Journal of Neuroscience*. **23**: 6176-6180.

Steinhoff, M., Vergnolle, N., Young, S.H., Tognetto, M., Amadesi, S., Ennes, H.S., Trevisani, M., Hollenberg, M. D., Wallace, J. L., Caughey, G. H., Mitchell, S. E., Williams, L. M., Geppetti, P., Mayer, E. A. and Bunnett, N. W. (2000) Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. *Nature Medicine*. **6**: 151-158.

Strader, C.D., Fong, T.M., Tota, M.R., Underwood, D. and Dixon, R.A.F. (1994) Structure and function of G-protein-coupled receptors. *Annual Review of Biochemistry*. **63**: 101-132.

Suh, B.C., Kim, J.S., Namgung, U., Han, S. and Kim, K.T. (2001) Selective inhibition of beta(2)-adrenergic receptor-mediated cAMP generation by activation of the P2Y(2) receptor in mouse pineal gland tumor cells. *Journal of Neurochemistry*. **77**: 1475-1485.

Sun, G., Stacey, M.A., Schmidt, M., Mori, L. and Mattoli, S. (2001) Interaction of mite allergens Der P3 and Der P9 with protease- activated receptor-2 expressed by lung epithelial cells. *Journal of Immunology*. **167**: 1014-1021.

Syeda, F., Grosjean, J., Houliston, R.A., Keogh, R.J., Carter, T.D., Paleolog, E. and Wheeler-Jones, C.P.D. (2006) Cyclooxygenase-2 induction and prostacyclin release by protease-activated receptors in endothelial cells require cooperation between mitogen-activated protein kinase and NF-kappa B pathways. *Journal of Biological Chemistry*. **281**: 11792-11804.

Takaesu, G., Surabhi, R.M., Park, K.J., Ninomiya-Tsuji, J., Matsumoto, K. and Gaynor, R.B. (2003) TAK1 is critical for I kappa B kinase-mediated activation of the NF-kappa B pathway. *Journal of Molecular Biology*. **326**: 105-115.

Takasaki, J., Saito, T., Taniguchi, M., Kawasaki, T., Moritani, Y., Hayashi, K. and Kobori, M. (2004) A novel G alpha(q/11)-selective inhibitor. *Journal of Biological Chemistry*. **279**: 47438-47445.

Takeuchi, T., Harris, J.L., Huang, W., Yan, K.W., Coughlin, S.R. and Craik, C.S. (2000) Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *Journal of Biological Chemistry*. **275**: 26333-26342.

Tan, Y., Rouse, J., Zhang, A.H., Cariati, S., Cohen, P. and Comb, M.J. (1996) FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO Journal*. **15**: 4629-4642.

Tancharoen, S., Sarker, K.P., Imamura, T., Biswas, K.K., Matsushita, K., Tatsuyama, S., Travis, J., Potempa, J., Torii, M. and Maruyama, I. (2005) Neuropeptide release from dental pulp cells by RgpB via proteinase-activated receptor-2 signaling. *Journal of Immunology*. **174**: 5796-5804.

Taniguchi, M., Nagai, K., Arao, N., Kawasaki, T., Saito, T., Moritani, Y., Takasaki, J., Hayashi, K., Fujita, S., Suzuki, K. and Tsukamoto, S. (2003) YM-254890, a novel platelet aggregation inhibitor produced by Chromobacterium sp QS3666. *Journal of Antibiotics*. **56**: 358-363.

Tantivejkul, K., Loberg, R.D., Mawocha, S.C., Day, L.L., St John, L., Pienta, B.A., Rubin, M. A. and Pienta, K. J. (2005) PAR1-mediated NF kappa B activation promotes survival of prostate cancer cells through a Bcl-xL-dependent mechanism. *Journal of Cellular Biochemistry*. **96**: 641-652.

Temkin, V., Kantor, B., Weg, V., Hartman, M.L. and Levi-Schaffer, F. (2002) Tryptase activates the mitogen-activated protein kinase/activator protein-1 pathway in human peripheral blood eosinophils, causing cytokine production and release. *Journal of Immunology*. **169**: 2662-2669.

Terrillon, S. and Bouvier, M. (2004) Roles of G-protein-coupled receptor dimerization - From ontogeny to signalling regulation. *EMBO Reports*. **5**: 30-34.

Thompson, J.E., Phillips, R.J., Erdjumentbromage, H., Tempst, P. and Ghosh, S. (1995) I-kappa-B-beta regulates the persistent response in a biphasic activation of NF-kappa-B. *Cell*. **80**: 573-582.

Thomson, S., Clayton, A.L., Hazzalin, C.A., Rose, S., Barratt, M.J. and Mahadevan, L.C. (1999) The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase. *EMBO Journal*. **18**: 4779-4793.

Tiruppathi, C., Yan, W.H., Sandoval, R., Naqvi, T., Pronin, A.N., Benovic, J.L. and Malik, A.B. (2000) G protein-coupled receptor kinase-5 regulates thrombin-activated signaling in endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America*. **97**: 7440-7445.

Todisco, A., Ramamoorthy, S., Pausawasdi, N. and Tacey, K. (1999) Carbachol activates I kappa B kinase in isolated canine gastric parietal cells. *Biochemical and Biophysical Research Communications*. **261**: 877-884.

Tojima, Y., Fujimoto, A., Delhase, M., Chen, Y., Hatakeyama, S., Nakayama, K., Kaneko, Y., Nimura, Y., Motoyama, N., Ikeda, K., Karin, M. and Nakanishi, M. (2000) NAK is an I kappa B kinase-activating kinase. *Nature*. **404**: 778-782.

Torii, S., Nakayama, K., Yamamoto, T. and Nishida, E. (2004) Regulatory mechanisms and function of ERK MAP kinases. *Journal of Biochemistry*. **136**: 557-561.

Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grandperret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D. and Kirilovsky, J. (1991) The bisindolylmaleimide GF-109203X is a potent and selective inhibitor of protein-kinase-C. *Journal of Biological Chemistry*. **266**: 15771-15781.

Tovey, S.C. and Willars, G.B. (2004) Single-cell imaging of intracellular Ca^{2+} and phospholipase C activity reveals that RGS 2, 3, and 4 differentially regulate signaling via the G alpha(q/11)-linked muscarinic M-3 receptor. *Molecular Pharmacology*. **66**: 1453-1464.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets - procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America*. **76**: 4350-4354.

Traenckner, E.B.M., Pahl, H.L., Henkel, T., Schmidt, K.N., Wilk, S. and Baeuerle, P.A. (1995) Phosphorylation of human I-kappa-B-alpha on Serine-32 and Serine-36 controls I-kappa-B-alpha proteolysis and NF-kappa-B activation in response to diverse stimuli. *EMBO Journal*. **14**: 2876-2883.

Tran, T. and Stewart, A.G. (2003) Protease-activated receptor (PAR)-independent growth and pro-inflammatory actions of thrombin on human cultured airway smooth muscle. *British Journal of Pharmacology*. **138**: 865-875.

Treisman, R. (1996) Regulation of transcription by MAP kinase cascades. *Current Opinion in Cell Biology*. **8**: 205-215.

Trejo, J., Connolly, A.J. and Coughlin, S.R. (1996) The cloned thrombin receptor is necessary and sufficient for activation of mitogen-activated protein kinase and mitogenesis in mouse lung fibroblasts - Loss of responses in fibroblasts from receptor knockout mice. *Journal of Biological Chemistry*. **271**: 21536-21541.

Trejo, J., Altschuler, Y., Fu, H.W., Mostov, K.E. and Coughlin, S.R. (2000) Protease-activated receptor-1 down-regulation - A mutant HeLa cell line suggests novel

requirements for PAR1 phosphorylation and recruitment to clathrin-coated pits. *Journal of Biological Chemistry*. **275**: 31255-31265.

Tu, J.C., Xiao, B., Naisbitt, S., Yuan, J.P., Petralia, R.S., Brakeman, P., Doan, A., Aakalu, V. K., Lanahan, A. A., Sheng, M. and Worley, P. F. (1999) Coupling of mGluR/Homer and PSD-95 complexes by the shank family of postsynaptic density proteins. *Neuron*. **23**: 583-592.

Ubl, J.J., Vohringer, C. and Reiser, G. (1998) Co-existence of two types of Ca^{2+} (i)-inducing protease-activated receptors (PAR-1 and PAR-2) in rat astrocytes and C6 glioma cells. *Neuroscience*. **86**: 597-609.

Uehara, A., Sugawara, S., Muramoto, K. and Takada, H. (2002) Activation of human oral epithelial cells by neutrophil proteinase 3 through protease-activated receptor-2. *Journal of Immunology*. **169**: 4594-4603.

Ui, H., Andoh, T., Lee, J.-B., Nojima, H. and Kuraishi, Y. (2006) Potent pruritogenic action of tryptase mediated by PAR-2 receptor and its involvement in anti-pruritic effect of nafamostat mesilate in mice. *European Journal of Pharmacology*. **530**: 172-178.

Vancorven, E.J., Hordijk, P.L., Medema, R.H., Bos, J.L. and Moolenaar, W.H. (1993) Pertussis toxin-sensitive activation of p21(Ras) by G-protein-coupled receptor agonists in fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*. **90**: 1257-1261.

Vancurova, I., Miskolci, V. and Davidson, D. (2001) NF-kappa B activation in tumor necrosis factor alpha-stimulated neutrophils is mediated by protein kinase C delta - Correlation to nuclear I kappa B alpha. *Journal of Biological Chemistry*. **276**: 19746-19752.

Vaughan, P.J., Pike, C.J., Cotman, C.W. and Cunningham, D.D. (1995) Thrombin receptor activation protects neurons and astrocytes from cell-death produced by environmental insults. *Journal of Neuroscience*. **15**: 5389-5401.

Vergnolle, N. (1999) Proteinase-activated receptor-2-activating peptides induce leukocyte rolling, adhesion, and extravasation in vivo. *Journal of Immunology*. **163**: 5064-5069.

Vergnolle, N., Hollenberg, M.D., Sharkey, K.A. and Wallace, J.L. (1999) Characterization of the inflammatory response to proteinase- activated receptor-2 (PAR(2))-activating peptides in the rat paw. *British Journal of Pharmacology*. **127**: 1083-1090.

Vergnolle, N., Derian, C.K., D'Andrea, M.R., Steinhoff, M. and Andrade-Gordon, P. (2002) Characterization of thrombin-induced leukocyte rolling and adherence: A

potential proinflammatory role for proteinase-activated receptor-4. *Journal of Immunology*. **169**: 1467-1473.

Vergnolle, N., Macnaughton, W.K., Al-Ani, B., Saifeddine, M., Wallace, J.L. and Hollenberg, M.D. (1998) Proteinase-activated receptor 2 (PAR(2))-activating peptides: Identification of a receptor distinct from PAR(2) that regulates intestinal transport. *Proceedings of the National Academy of Sciences of the United States of America*. **95**: 7766-7771.

Vergnolle, N., Bunnett, N.W., Sharkey, K.A., Brussee, V., Compton, S.J., Grady, E.F., Cirino, G., Gerard, N., Basbaum, A. I., Andrade-Gordon, P., Hollenberg, M. D. and Wallace, J. L. (2001) Proteinase-activated receptor-2 and hyperalgesia: A novel pain pathway. *Nature Medicine*. **7**: 821-826.

Viatour, P., Merville, M.P., Bours, V. and Chariot, A. (2005) Phosphorylation of NF-kappa B and I kappa B proteins: implications in cancer and inflammation. *Trends in Biochemical Sciences*. **30**: 43-52.

Vliagoftis, H., Hollenberg, M.D., Befus, A.D. and Moqbel, R. (2000) Airway epithelial cells release eosinophil survival promoting factors following stimulation of proteinase-activated receptor- 2 (PAR-2). *Journal of Allergy and Clinical Immunology*. **105**: 520.

Vossler, M.R., Yao, H., York, R.D., Pan, M.G., Rim, C.S. and Stork, P.J.S. (1997) cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell*. **89**: 73-82.

Vouret-Craviari, V., Boquet, P., Pouyssegur, J. and Van Obberghen-Schilling, E. (1998) Regulation of the actin cytoskeleton by thrombin in human endothelial cells: Role of Rho proteins in endothelial barrier function. *Molecular Biology of the Cell*. **9**: 2639-2653.

Vouret-Craviari, V., Vanobberghenschilling, E., Scimeca, J.C., Vanobberghen, E. and Pouyssegur, J. (1993) Differential activation of p44 MAPK (ERK1) by alpha-thrombin and thrombin-receptor peptide agonist. *Biochemical Journal*. **289**: 209-214.

Vu, T.K.H., Hung, D.T., Wheaton, V.I. and Coughlin, S.R. (1991) Molecular-cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*. **64**: 1057-1068.

Waite, M. (1999) The PLD superfamily: insights into catalysis. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. **1439**: 187-197.

Wakita, H., Furukawa, F. and Takigawa, M. (1997) Thrombin and trypsin induce granulocyte-macrophage colony-stimulating factor and interleukin-6 gene expression in cultured normal human keratinocytes. *Proceedings of the Association of American Physicians*. **109**: 190-207.

Wang, D., Westerheide, S.D., Hanson, J.L. and Baldwin, A.S. (2000) Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser(529) is controlled by casein kinase II. *Journal of Biological Chemistry*. **275**: 32592-32597.

Wang, H., Ubl, J.J., Stricker, R. and Reiser, G. (2002) Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways. *American Journal of Physiology-Cell Physiology*. **283**: C1351-C1364.

Wang, X.Z. and Ron, D. (1996) Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP kinase. *Science*. **272**: 1347-1349.

Wang, Z.Q., Zhang, B.C., Wang, M.F. and Carr, B.I. (2003) Persistent ERK phosphorylation negatively regulates cAMP response element-binding protein (CREB) activity via recruitment of CREB-binding protein to pp90(RSK). *Journal of Biological Chemistry*. **278**: 11138-11144.

Weinstein, J.R., Gold, S.J., Cunningham, D.D. and Gall, C.M. (1995) Cellular-localization of thrombin receptor messenger-RNA in rat-brain - expression by mesencephalic dopaminergic-neurons and codistribution with prothrombin messenger-RNA. *Journal of Neuroscience*. **15**: 2906-2919.

Werry, T.D., Sexton, P.M. and Christopoulos, A. (2005) 'Ins and outs' of seven-transmembrane receptor signalling to ERK. *Trends in Endocrinology and Metabolism*. **16**: 26-33.

White, J.H., Wise, A., Main, M.J., Green, A., Fraser, N.J., Disney, G.H., Barnes, A. A., Emson, P., Foord, S. M. and Marshall, F. H. (1998) Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature*. **396**: 679-682.

Whiteside, S.T., Epinat, J.C., Rice, N.R. and Israel, A. (1997) I kappa B epsilon, a novel member of the I kappa B family, controls RelA and cRel NF-kappa B activity. *EMBO Journal*. **16**: 1413-1426.

Whitmarsh, A.J. and Davis, R.J. (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *Journal of Molecular Medicine-Jmm*. **74**: 589-607.

Whitmarsh, A.J., Shore, P., Sharrocks, A.D. and Davis, R.J. (1995) Integration of MAP kinase signal-transduction pathways at the serum response element. *Science*. **269**: 403-407.

Wiegand, U., Corbach, S., Minn, A., Jie Kang and Muller-Hill, B. (1993) Cloning of the cDNA encoding human brain trypsinogen and characterization of its product. *Gene*. **136**: 167-175.

Woolkalis, M.J., DeMelfi, T.M., Brass, L.F. and Molino, M. (1996) Stimulation of proteinase-activated receptor-2 in human umbilical vein endothelial cells activates extracellular signal-regulated kinases, ERK-1 and ERK-2. *Molecular Biology of the Cell*. **7**: 2998-2998.

Woronicz, J.D., Gao, X., Cao, Z., Rothe, M. and Goeddel, D.V. (1997) I kappa B kinase-beta: NF-kappa B activation and complex formation with I kappa B kinase-alpha and NIK. *Science*. **278**: 866-869.

Xia, Z.G., Dickens, M., Raingeaud, J., Davis, R.J. and Greenberg, M.E. (1995) Opposing effects of ERK and JNK/p38 MAP kinases on apoptosis. *Science*. **270**: 1326-1331.

Xiao, B., Cheng Tu, J. and Worley, P.F. (2000) Homer: a link between neural activity and glutamate receptor function. *Current Opinion in Neurobiology*. **10**: 370-374.

Xiao, G.T., Harhaj, E.W. and Sun, S.C. (2001a) NF-kappa B-inducing kinase regulates the processing of NF-kappa B2 p100. *Molecular Cell*. **7**: 401-409.

Xiao, G.T., Cvijic, M.E., Fong, A., Harhaj, E.W., Uhlik, M.T., Waterfield, M. and Sun, S.C. (2001b) Retroviral oncoprotein Tax induces processing of NF-kappa B2/p100 in T cells: evidence for the involvement of IKK alpha. *EMBO Journal*. **20**: 6805-6815.

Xie, P., Browning, D.D., Hay, N., Mackman, N. and Ye, R.D. (2000) Activation of NF-kappa B by bradykinin through a G alpha(q)-and G beta/gamma-dependent pathway that involves phosphoinositide 3-kinase and Akt. *Journal of Biological Chemistry*. **275**: 24907-24914.

Xu, W.F., Andersen, H., Whitmore, T.E., Presnell, S.R., Yee, D.P., Ching, A., Yowe, D., Offermanns, S., Muallem, S. and Wilkie, T. M. (1998) Cloning and characterization of human protease-activated receptor 4. *Proceedings of the National Academy of Sciences of the United States of America*. **95**: 6642-6646.

Xu, X., Zeng, W.H., Popov, S., Berman, D.M., Davignon, I., Yu, K., Gilbert, T., Davie, E. W. and Foster, D. C. (1999) RGS proteins determine signaling specificity of G(q)-coupled receptors. *Journal of Biological Chemistry*. **274**: 3549-3556.

Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S.T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J. and Israel, A. (1998) Complementation cloning of NEMO, a component of the I kappa B kinase complex essential for NF-kappa B activation. *Cell*. **93**: 1231-1240.

Yang, F., Tang, E., Guan, K.L. and Wang, C.Y. (2003) IKK beta plays an essential role in the phosphorylation of RelA/p65 on serine 536 induced by lipopolysaccharide. *Journal of Immunology*. **170**: 5630-5635.

Ye, R.D. (2001) Regulation of nuclear factor kappa B activation by G-protein-coupled receptors. *Journal of Leukocyte Biology*. **70**: 839-848.

Yu, Z.B., Ahmad, S., Schwartz, J.L., Banville, D. and Shen, S.H. (1997) Protein-tyrosine phosphatase SHP2 is positively linked to proteinase-activated receptor 2-mediated mitogenic pathway. *Journal of Biological Chemistry*. **272**: 7519-7524.

Yun, Y.P., Joo, J.D., Lee, J.Y., Nam, H.Y., Kim, Y.H., Lee, K.H., Lim, C. S., Kim, H. J., Lim, Y. G. and Lim, Y. (2005) Induction of nuclear factor-kappa B activation through TAK1 and NIK by diesel exhaust particles in L2 cell lines. *Toxicology Letters*. **155**: 337-342.

Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M. and Karin, M. (1997) The I kappa B kinase complex (IKK) contains two kinase subunits, IKK alpha and IKK beta, necessary for I kappa B phosphorylation and NF-kappa B activation. *Cell*. **91**: 243-252.

Zeng, W.Z., Xu, X., Popov, S., Mukhopadhyay, S., Chidiac, P., Swistok, J., Danho, W., Yagaloff, K. A., Fisher, S. L., Ross, E. M., Muallem, S. and Wilkie, T. M. (1998) The N-terminal domain of RGS4 confers receptor-selective inhibition of G protein signaling. *Journal of Biological Chemistry*. **273**: 34687-34690.

Zhang, B.C., Perpetua, M., Fulmer, M. and Harbrecht, B.G. (2004) JNK signaling involved in the effects of cyclic AMP on IL-1 beta plus IFN gamma-induced inducible nitric oxide synthase expression in hepatocytes. *Cellular Signalling*. **16**: 837-846.

Zhang, J., Berenstein, E.H., Evans, R.L. and Siraganian, R.P. (1996) Transfection of Syk protein tyrosine kinase reconstitutes high affinity IgE receptor-mediated degranulation in a Syk-negative variant of rat basophilic leukemia RBL-2H3 cells. *Journal of Experimental Medicine*. **184**: 71-79.

Zhao, D.Z., Zhan, Y.N., Zeng, H.Y., Koon, H.W., Moyer, M.P. and Pothoulakis, C. (2005) Neurotensin stimulates interleukin-8 expression through modulation of I kappa B alpha phosphorylation and p65 transcriptional activity: Involvement of protein kinase C alpha. *Molecular Pharmacology*. **67**: 2025-2031.

Zhao, Q. and Lee, F.S. (1999) Mitogen-activated protein kinase ERK kinase kinases 2 and 3 activate nuclear factor-kappa B through I kappa B kinase-alpha and I kappa B kinase-beta. *Journal of Biological Chemistry*. **274**: 8355-8358.

Zhu, Y.M., Bradbury, D.A., Pang, L.H. and Knox, A.J. (2003) Transcriptional regulation of interleukin (IL)-8 by bradykinin in human airway smooth muscle cells involves prostanoid-dependent activation of AP-1 and nuclear factor (NF)-IL-6 and prostanoid-independent activation of NF-kappa B. *Journal of Biological Chemistry*. **278**: 29366-29375.

Zwick, E., Wallasch, C., Daub, H. and Ullrich, A. (1999) Distinct calcium-dependent pathways of epidermal growth factor receptor transactivation and PYK2 tyrosine phosphorylation in PC12 cells. *Journal of Biological Chemistry*. **274**: 20989-20996.