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The role of intracellular Ca^{2+} in the regulation of proteinase-activated receptor-2 mediated nuclear factor kappa B signalling in keratinocytes

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1 In this study, we examined the role of Ca^{2+} in linking proteinase-activated receptor-2 (PAR2) to the nuclear factor kappa B (NFκB) pathway in a skin epithelial cell line NCTC2544 stably expressing PAR2 (clone G).

2 In clone G, PAR2-mediated NFκB luciferase reporter activity and NFκB DNA-binding activity was reduced by preincubation with BAPTA-AM but not BAPTA. Trypsin stimulation of inhibitory kappa B kinases, IKKα and IKKβ, was also inhibited following pretreatment with BAPTA-AM.

3 BAPTA/AM also prevented PAR2-mediated IKKα activation in cultured primary human keratinocytes.

4 The effect of BAPTA-AM was also selective for the IKK/NFκB signalling axis; PAR2 coupling to ERK, or p38 MAP kinase was unaffected.

5 Pharmacological inhibition of the Ca^{2+}-dependent regulatory protein calcineurin did not inhibit trypsin-stimulated IKK activity or NFκB-DNA binding; however, inhibition of Ca^{2+}-dependent protein kinase C isoforms or $InsP_3$ formation using GF109203X or the phospholipase C inhibitor U73122, respectively, reduced both IKK activity and NFκB-DNA binding.

6 Mutation of PAR2 within the C-terminal to produce a mutant receptor, which does not couple to Ca^{2+} signalling, but is able to activate ERK, abrogated NFκB-DNA binding and IKK activity stimulated by trypsin.

7 These results suggest a predominant role for the $InsP_3/Ca^{2+}$ axis in the regulation of IKK signalling and NFκB transcriptional activation.
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Abbreviations: CaM, calmodulin; DAG, diacylglycerol; ERK, extracellular signal regulated kinase; IKK, inhibitory kappa B kinase; $InsP_3$, inositol-1,4,5-trisphosphate; NFκB, nuclear factor kappa B; PAR, proteinase-activated receptor; PKC, protein kinase-C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; TNF, tumour necrosis factor

Introduction

Proteinase-activated receptor-2 (PAR2) is a member of the G-protein coupled receptor (GPCR) subfamily typified by the thrombin receptor, PAR1. The receptor is activated by serine proteinases, most notably trypsin and tryptase (Macfarlane *et al*., 2001), and extensive study of PAR2 has revealed the receptor to be of potential importance in the functional control of the blood vessels (Damiano *et al*., 1999; Sobey *et al*., 1999), lungs (Cocks & Sobey, 1998; Lan *et al*., 2000) and gastrointestinal tract (Corvera *et al*., 1997; Vergnolle, 2000). A number of recent studies have implicated a role for PAR2 in several disease states including inflammatory pain, colitis and chronic arthritis (Vergnolle *et al*., 1999; Steinhoff *et al*., 2000; Ferrell *et al*., 2003).

PAR2 has been identified in three of the main layers of keratinocytes that constitute the epidermis of the skin, with levels of PAR2 expression increasing with increased level of cellular differentiation. Again, the receptor is strongly implicated in inflammatory responses in this tissue (Wakita *et al*., 1997), being linked with disease states such as type IV dermatitis (Kawagoe *et al*., 2002), and also in the control of skin pigmentation (Seiberg *et al*., 2000). In the case of keratinocyte proliferation and differentiation, PAR2 has been shown to have an inhibitory influence (Derian *et al*., 1997); however, little is known about the intracellular signalling events that participate in PAR2-mediated events in this or other tissues.

The transcription factor NFκB is a major regulator of inflammation-related gene expression (Ghosh *et al*., 1998), and has recently been identified as an important factor in the development of normal epidermal structure (Hu *et al*., 1999) and in the switch between proliferation and differentiation in keratinocytes (Seitz *et al*., 2000). NFκB is regulated by two

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major kinases known as the inhibitory kappa B kinases or IKKs. IKKα has been shown to play a particularly important role in the control of epidermal differentiation (Hu *et al*., 2001), while IKKβ has been implicated in TNF-mediated inflammatory events in the skin (Pasparakis *et al*., 2002).

Since PAR2 is associated with increases in intracellular calcium (Santulli *et al*., 1995; Bohm *et al*., 1996) and increased activation of NFκB and IKK signalling in keratinocyte cell lines (Kanke *et al*., 2001), we have examined the potential involvement of Ca^{2+} in PAR2-dependent NFκB signalling. This includes a possible role for a number of intermediates including CAM kinase II and calcineurin (PP2B), both of which have been implicated as intermediates in IKK/NFκB signalling in other receptor cell systems (Howe *et al*., 2002; Alzuherri & Chang, 2003).

Using skin epithelial cells expressing human PAR2 (Kanke *et al*., 2001), we find a specific and selective role for intracellular Ca^{2+} derived from $InsP_3$ generation in PAR2 coupling to IKK/NFκB signalling. While Ca^{2+}-dependent PKCs are likely to be involved, no such role is apparent for either Cam kinase II or calcineurin. In contrast, we find that PAR2-mediated activation of two of the major MAP kinases, ERK and p38 MAP kinase, is not regulated by Ca^{2+}-dependent pathways.

Methods

Cell culture

Human skin epithelial cells NCTC2544 were maintained in M199 medium with Earl's salt supplement, 10% ($v\,v^{-1}$) foetal calf serum, 100 units penicillin ml^{-1} and 100 μg streptomycin ml^{-1} in a humidified atmosphere containing 5% CO_2 at 37°C. NCTC2544 cells stably expressing human PAR2 (clone G) (Kanke *et al*., 2001) were maintained in complete M199 medium containing 400 $\mu g\,ml^{-1}$ of Geneticin for selection pressure and passaged using Versene. A clone expressing both PAR2 and an NFκB reporter plasmid (see below) were grown in M199 supplemented with 400 $\mu g\,ml^{-1}$ Geneticin and 5 $\mu g\,ml^{-1}$ Blasticidin S. Primary human keratinocytes were cultured from neonatal foreskins obtained from the Royal Hospital for Sick Children (Edinburgh) as outlined previously (Kondo *et al*., 1993). Cells were grown in supplemented serum-free media and used at passages 3–5.

NFκB reporter activity assay

As transient cotransfection of PAR2 and NFκB luciferase gene reduced the transfection efficiency of PAR2, a stable clone G cell line expressing both PAR2 and the NFκB reporter plasmid coupled to 3× luciferase gene was established**.** These cells were grown on 96-well culture plates and were rendered quiescent in serum-free M199 overnight before being treated with appropriate agonists for 6 h and assayed for luciferase activity using the Steady Glo® kit (Promega, U.K.) as according to the manufacturer's instructions.

EMSA

Cells were grown on six-well plates or 10 cm^2 dishes, exposed to vehicle or agents and reactions terminated by washing cells twice with ice-cold PBS. Cells were then removed by scraping and transferred to Eppendorf tubes. Nuclear extracts were prepared as described previously (Schreiber *et al*., 1989), and the protein content of the recovered samples was then determined by means of Bradford assay. Nuclear extracts (5 μg) were incubated in binding buffer (10 mM Tris–HCl pH 7.5, 4% ($v\,v^{-1}$) glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 50 $\mu g\,ml^{-1}$ poly(dI-dC).poly(dI-dC)) for 15 min prior to addition of 1 μl (50,000 c.p.m.) of ^{32}P-labelled double-stranded NFκB consensus oligonucleotide (Promega, U.K.) for 20–30 min. Following incubation, 1 μl of gel loading buffer (10×; 250 mM Tris–HCl pH 7.5, 0.2% ($w\,v^{-1}$) bromophenol blue, 40%($v\,v^{-1}$) glycerol) was added to samples and protein/DNA complexes resolved by nondenaturing electrophoresis on 5% ($w\,v^{-1}$) acrylamide slab gels. Gels were initially pre-run in (0.5×) Tris-borate-EDTA buffer (TBE) for 30 min at 100 V and subsequent to loading of samples electrophoresis maintained at 100 V for 45–60 min. Gels were dried and NFκB-probe complexes visualised by autoradiography.

IKK assay

Cells grown on six-well plates were stimulated with agonists for the defined period and then washed twice in ice-cold PBS. Monolayers were scraped into 300 μl solubilisation buffer (20 mM Tris–HCl (pH 7.6), 1 mM EDTA, 0.5 mM EGTA, 10% glycerol ($v\,v^{-1}$), 0.1% Brij 35, 150 mM NaCl, 1% Triton X-100 ($w\,v^{-1}$), 20 mM NaF, 20 mM β-glycerophosphate, 0.5 mM Na_3VO_4, 1 mM PMSF, 0.5 $mg\,ml^{-1}$ leupeptin, 0.5 $mg\,ml^{-1}$ aprotinin) and left on ice for 30 min. Following centrifugation, precleared samples were analysed for protein content and equivalent amounts (100–150 μg) were incubated with either IKKα or IKKβ polyclonal antibody coupled to Protein G sepharose beads, and mixed for 2 h at 4°C.

Immunoprecipitates were recovered by centrifugation and washed twice in the solubilisation buffer and once in kinase buffer (25 mM HEPES (pH 7.6), 20 mM $MgCl_2$, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM DTT). Precipitates were then resuspended in 25 μl kinase buffer, and the kinase reaction was initiated by addition of [γ-^{32}P] ATP (5 μCi), 25 μM ATP and 2 μg GST-IκBN (1–70, N-terminal truncated IκB). Samples were incubated in a final volume of 30 μl, with shaking, at 30°C for 30 min before termination of the kinase reaction by the addition of 4× Laemmli sample buffer. Samples were then resolved on 11% ($w\,v^{-1}$) acrylamide SDS–PAGE and the phosphorylated protein was identified by autoradiography.

Transient transfection

NCTC2544 clones expressing PAR2 were grown to 60–70% confluency on six-well plates and transiently cotransfected with various plasmids using the LIPOFECTAMINE PLUS® transfection system (Invitrogen, U.K.), according to the manufacturer's protocols (Seatter *et al*., 2004). Briefly, 2 μg of WT-PAR2 or δ34–43 PAR2 DNA was mixed with 6 μl of PLUS reagent and then 4 μl or LIPOFECTAMINE in a total volume of 200 μl. This mixture was then added to 800 μl of media free of serum and antibiotics on the cells to give a total volume of 1 ml. After a 4.5 h incubation period with the DNA mixture, cells were transferred into complete M199 medium for a further 30 h. Cells were then rendered quiescent by serum deprivation for 18 h before stimulation. For IKK activity,

at least two 3 cm^2 wells were used per point and for NFκB DNA-binding experiments, 9 cm^2 dishes were employed and a volume of 6 ml was used in the transfection.

Statistical analysis

Where experimental data are shown as a gel, this represents one of at least three experiments. Luciferase experiments were performed at least 4 times and data represent the mean ± s.e.m. Statistical analysis was performed by one-way ANOVA with Dunnet's post-test ($*P<0.05$).

Results

Intracellular Ca^{2+} chelation inhibits PAR2-mediated activation of the NFκB pathway

In preliminary studies, we found that removal of extracellular Ca^{2+} from the medium reduced trypsin-stimulated NFκB reporter activity (results not shown), suggesting a role for Ca^{2+} in the coupling of PAR2 to the NFκB signalling system. In order to assess this in more detail, cells were preincubated with BAPTA-AM, cell-permeable intracellular Ca^{2+} chelator or BAPTA, which chelates only extracellular Ca^{2+}, in order to better chelate intracellular and extracellular Ca^{2+}, respectively. In clone G cells, preincubation with 50 μM BAPTA-AM reduced the trypsin-stimulated rise in intracellular Ca^{2+} by approximately 95%, while incubation with 1 mM BAPTA was without effect.

Figure 1 shows the effect of BAPTA-AM pretreatment upon trypsin (50 nM)- or PMA (100 nM)-stimulated NFκB reporter activity and NFκB-DNA binding. Trypsin stimulated a 10–15-fold increase in luciferase activity, while the response to PMA was approximately double this response, giving a 20–30-fold increase over several experiments. Following pretreatment with BAPTA-AM, both the trypsin- and PMA-stimulated activity was significantly decreased, and in the case of trypsin reduced back to basal values. In contrast, no significant inhibition of trypsin- or PMA-stimulated reporter activity, nor NFκB-DNA binding, was observed following pretreatment with BAPTA (Figure 2). A similar effect was observed using the PAR2 activating peptide SLIGKV, suggesting effects upon trypsin responses related to PAR2 activation (results not shown).

We further investigated the effects of BAPTA-AM upon IKK signalling in response to PAR2 activation and PMA (Figure 3). The activity of both IKKα and IKKβ was strongly stimulated in response to trypsin or PMA. However, as with NFκB, preincubation of cells with BAPTA-AM substantially reduced the activity of either kinase to near basal levels. In a number of additional experiments, we found that BAPTA was without effect (shown in Figure 3c for IKKα), indicating a role for intracellular Ca^{2+} in the regulation of IKK activity. Further control experiments employed TNFα (see Figure 3 panel b) and it was found that BAPTA-AM was not effective against this agonist, suggesting a degree of selectivity.

Since trypsin may interact with other PARs, we sought to confirm that a similar effect of intracellular Ca^{2+} chelation was obtained with the selective PAR2 activating peptide 2fluoryl-LIGKV-OH (Ferrell *et al*., 2003; Kawabata *et al*., 2004). Indeed, pretreatment with BAPTA-AM but not BAPTA substantially reduced reporter activity, in response

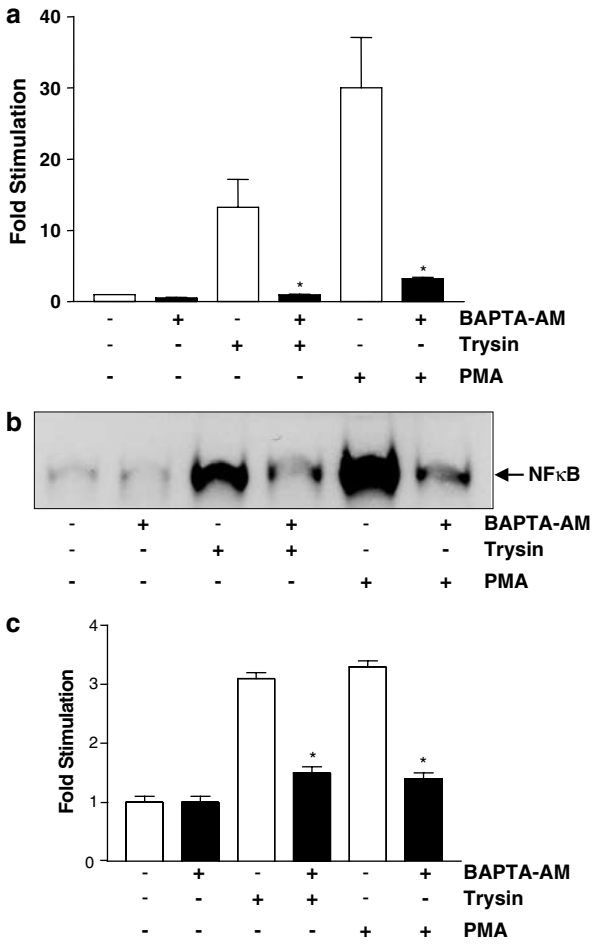

Figure 1 Effect of BAPTA-AM on trypsin-stimulated NFκB reporter and NFκB-DNA-binding activities in clone G cells. Cells were pretreated with 50 μM BAPTA-AM for 30 min prior to addition of 50 nM trypsin or 100 nM PMA for 6 h (a) or 1 h (b) NFκB reporter (a) or NFκB-DNA-binding activity (b) was assessed as described in Methods. In panel c, NFκB-DNA-binding activity blots were quantified and each value represents the mean ± s.e.m. Statistical analysis was performed by one-way ANOVA with Dunnet's post-test ($*P<0.05$).

to 2-fluoryl-LIGKV (Figure 4a). Furthermore, stimulation of both IKKα activation and NFκB-DNA binding was also substantially reduced following pretreatment with BAPTA-AM but not with BAPTA (Figure 4b and d). Taken together, these results suggest strongly that the effects of trypsin upon NFκB signalling are likely to be mediated *via* PAR2.

Since the NCTC2544 cell line represents only a model of PAR2 activation in skin, we sought to determine whether intracellular Ca^{2+} also played a role in the regulation of IKK in cultured human primary keratinocytes. Trypsin stimulation of PAR2 resulted in a modest, three-fold increase in IKK activity (Figure 5). However, similar to observations in clone G cells, incubation with BAPTA-AM but not BAPTA also substantially reduced IKK activity.

PAR2 coupling to MAP kinase is Ca^{2+} independent

In order to determine whether intracellular Ca^{2+} was required for other PAR2-dependent pathways, we analysed

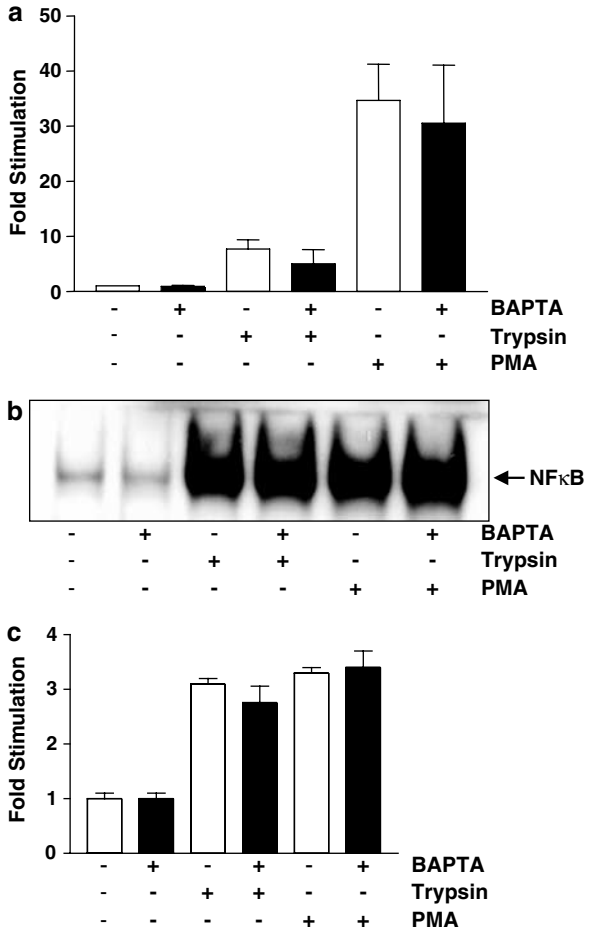

Figure 2 Effect of BAPTA on trypsin-stimulated NFκB reporter and NFκB-DNA-binding activities in clone G cells. Cells were pretreated with 1 mM BAPTA for 30 min prior to addition of 50 nM trypsin or 100 nM PMA for 6 h (a) or 1 h (b). NFκB reporter (a) or NFκB-DNA-binding activity (b) was assessed as described in Methods. In panel c, NFκB-DNA-binding activity blots were quantified and each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with Dunnet's post-test ($*P<0.05$).

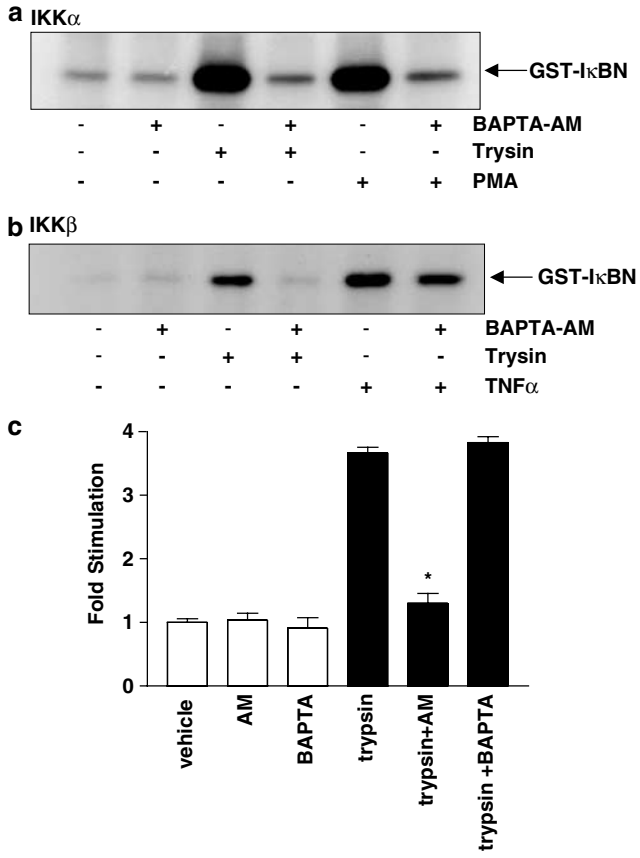

Figure 3 Effect of Ca^{2+} chelators BAPTA-AM and BAPTA upon trypsin stimulated IKKα and IKKβ activities in clone G cells. Cells were pretreated with 50 μM BAPTA-AM or 1 mM BAPTA for 30 min and then stimulated for a further 30 min with either 50 nM trypsin, 100 nM PMA or 20 ng ml^{-1} TNFα. Samples were assayed for either IKKα or IKKβ activity as outlined in the Methods section. In panel c, IKKα blots from trypsin stimulations ± BAPTA and BATA-AM were quantified. Each value represents the mean ± s.e.m. Statistical analysis was performed by one-way ANOVA with Dunnet's post-test ($*P<0.05$). Similar results were obtained for the quantification of IKKβ (results not shown).

the effects of Ca^{2+} removal upon MAP kinase activation (Figure 6). In preliminary studies, we found that trypsin-stimulated activation of ERK and p38 MAP kinase reached a peak at 15 and 30 min, respectively, and these time points were chosen to assess the effect of either BAPTA-AM or BAPTA. Preincubation with either chelating agent had no discernable effect upon trypsin-stimulated ERK (panel a) or p38 MAP kinase activation (panel b). In preliminary results, we also found that either agent was ineffective at reducing PMA-stimulated ERK and p38 MAP kinase activity (not shown), strongly suggesting the effect of intracellular Ca^{2+} depletion effects on IKK/NFκB signalling selectively.

Inhibition of Ca^{2+}-dependent protein kinase C abrogates NFκB but not MAP kinase signalling

Since a number of Ca^{2+}-dependent protein intermediates may regulate PAR2-mediated NFκB signalling, we assessed these using a number of specific inhibitors (Figure 7). Preincubation of cells with either the calcineurin inhibitor FK-506 (10 μM) (Raufman *et al*., 1996) or the Ca^{2+}/CaM inhibitor KN-62 (10 μM) (Praskova *et al*., 2002; Meffert *et al*., 2003) did not significantly reduce trypsin stimulation of IKK activity or NFκB-DNAbinding. In fact, treatment of cells with KN-62 alone increased basal IKK activity to a level similar for trypsin stimulation. By contrast, preincubation with the PKC inhibitor GF109203X (10 μM), which inhibits Ca^{2+}-dependent PKC isoforms (Toullec *et al*., 1991; Hofmann, 1997), virtually abrogated both IKK and NFκB-DNA binding following PAR2 stimulation.

The effect of FK-506, KN-62 and GF109203X upon MAP kinase signalling was also assessed. Once again, preincubation with FK-506 or KN-62 did not reduce trypsin-stimulated ERK (Figure 8) or p38 MAP kinase activation (not shown). KN-62 also resulted in a marked increase in basal cellular phospho-ERK content. However, pretreatment with GF109203X did not effect trypsin-stimulated ERK, suggesting that NFκB and MAP kinase pathways are differentially regulated in this cell type.

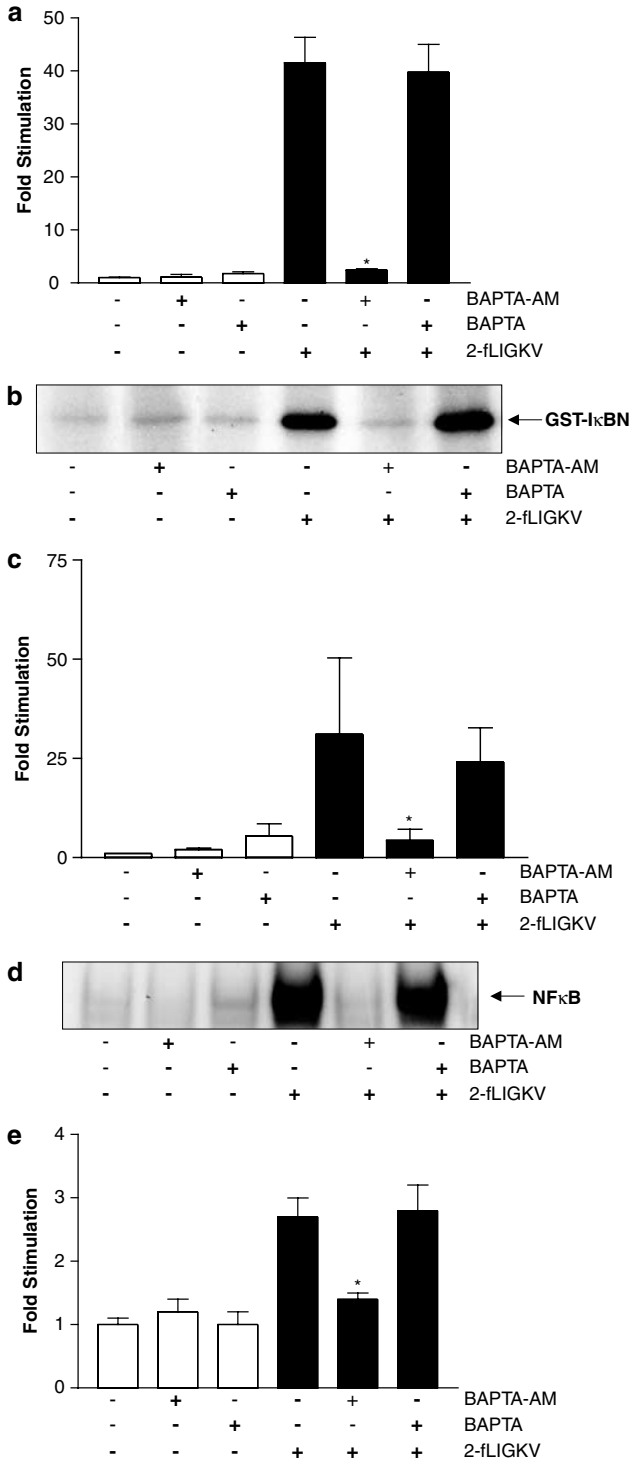

Figure 4 The effect of Ca^{2+} chelators BAPTA-AM and BAPTA upon PAR2 activating peptide stimulation of NFκB reporter activity and NFκB-DNA binding in clone G cells. Cells were pretreated with 50 μM BAPTA-AM or 1 mM BAPTA for 30 min then stimulated for a further 60 min with 100 μM 2-fluoryl-LIGKV-OH. Samples were assayed for reporter activity (a), IKKα kinase activity (b and c) and NFκB-DNA binding activity (d and e) as outlined in the Methods section. Each blot is representative of at least two others. In panels c and e blots were quantified, each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with Dunnet's post-test (*$P<0.05$).

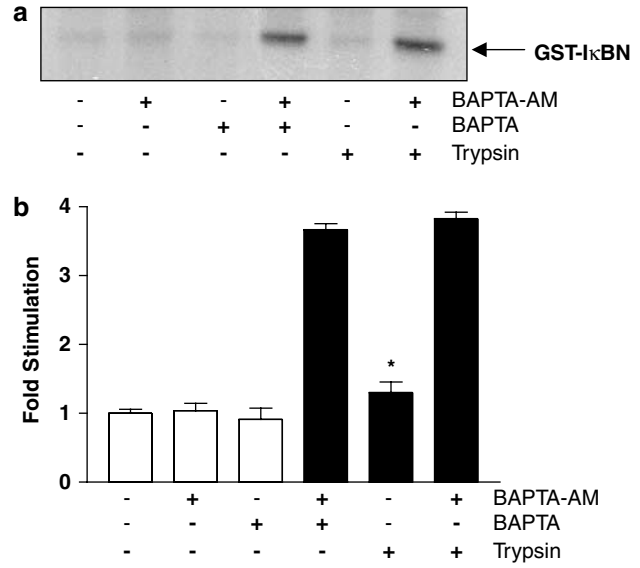

Figure 5 Effect of Ca^{2+} chelators BAPTA-AM and BAPTA upon trypsin stimulated IKKα in primary cultures of human keratinocytes. Cells were pretreated with 50 μM BAPTA-AM or 1 mM BAPTA for 30 min and then stimulated for a further 30 min with 50 nM trypsin (a). Samples were assayed for IKKα as outlined in the Methods section. Each blot is representative of at least three others. In panel b, the trypsin-stimulated IKKα blots were quantified; each value represents the mean ± s.e.m.

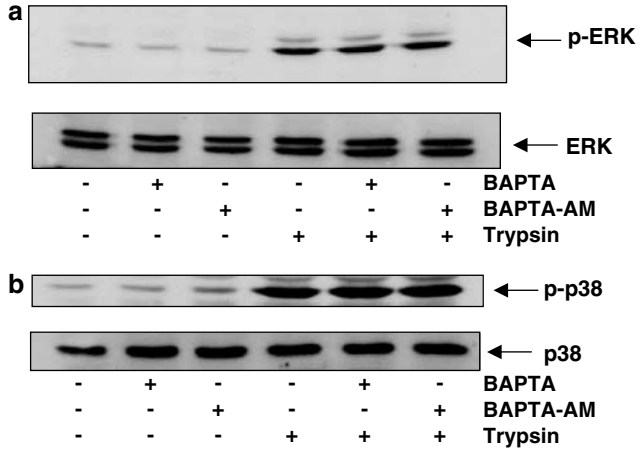

Figure 6 Effect of Ca^{2+} chelators BAPTA-AM and BAPTA upon trypsin-stimulated ERK and p38 MAP kinase activation. Cells were pretreated with 50 μM BAPTA-AM or 1 mM BAPTA for 30 min then stimulated with a further 30 min with 50 nM trypsin. Samples were assayed for either phospho ERK (a), phospho p38 MAP kinase (b), as outlined in the Methods section. Each blot is representative of at least two others.

Disruption of PAR2 coupling to PLC abrogates IKK and NFκB activation

Since the studies utilising pharmacological inhibitors pointed to a specific role in PAR2-mediated NFκB signalling for PKC isoforms known to be dependent upon the increase in intracellular Ca^{2+} and formation of DAG *via* PLC-mediated hydrolysis of $PtdInsP_2$, we assessed the effect of the specific

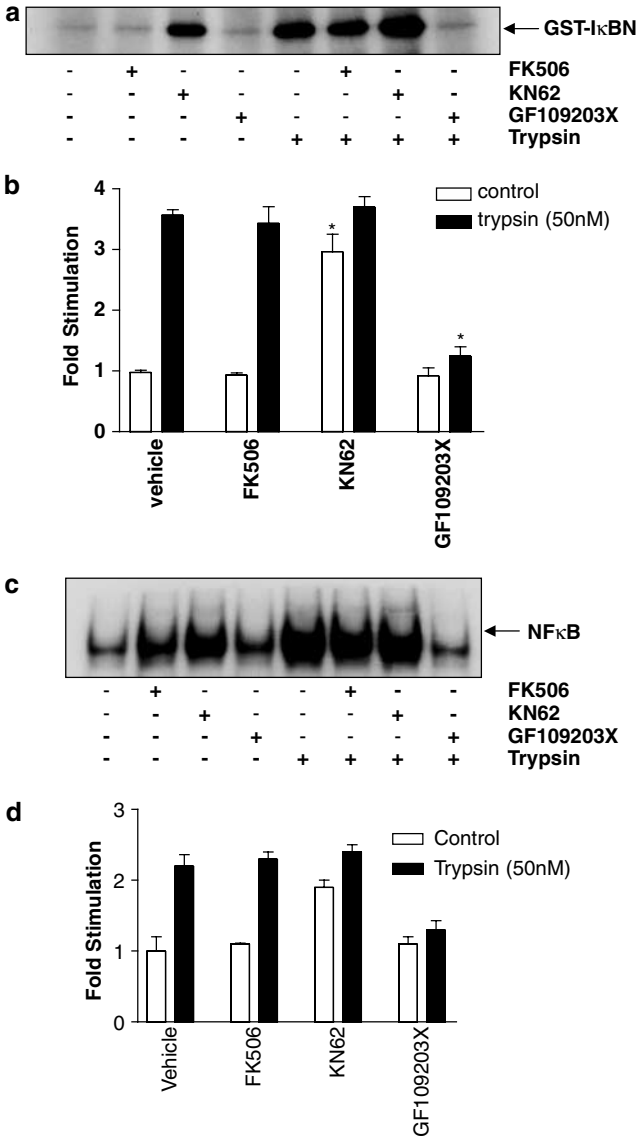

Figure 7 Effects of FK-506, KN-62 and GF109203X on trypsin-stimulated IKK and NFκB-DNA binding in clone G cells. Cells were pretreated with either the calcineurin inhibitor FK-506 (10 μM), the calmodulin inhibitor KN-62 (10 μM) or the PKC inhibitor GF109203X (10 μM), for 30 min, prior to stimulation with trypsin (50 nM) for either 30 min (a) or 1 h (c). Samples were assessed for IKKα activity (a) or NFκB DNA-binding activity (c) as described in Materials. Each gel is representative of at least three others. In panels b and d, respectively, IKKα activity and NFκB-DNA binding activity blots were quantified and each value represents the mean ± s.e.m. Statistical analysis was performed by one-way ANOVA with Dunnet's post-test (*$P<0.05$).

PLC inhibitor U73122 upon PAR2-mediated NFκB signalling (Figure 9). At concentrations of 1–30 μM, known to abolish agonist-stimulated Ca^{2+} formation (Schechter *et al*., 1998; Fahlman *et al*., 2002), U73122 was found to have a concentration-dependent effect on the activities of IKKα and IKKβ (Figure 9a). A similar effect was observed on PAR2-mediated NFκB-DNA binding, with complete inhibition of trypsin-stimulated binding by 10 μM U73122. In contrast, U73122 was without significant effect upon PMA-stimulated NFκB-DNA binding (Figure 9c).

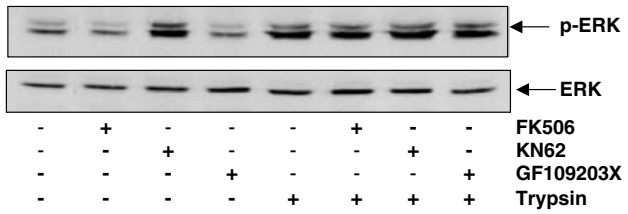

Figure 8 Effects of FK-506, KN-62 and GF109203X on trypsin-stimulated ERK activation in clone G cells. Cells were pretreated with either the calcineurin inhibitor FK-506 (10 μM), the calmodulin inhibitor KN-62 (10 μM) or the PKC inhibitor, GF109203X (10 μM), for 30 min, prior to stimulation with trypsin (50 nM) for 30 min. Samples were assessed for phospho-ERK (a) total ERK content (b) as described in Methods. Each gel is representative of at least two others.

In order to confirm that PLC is directly involved in PAR2-mediated activation of IKK and NFκB, we utilised a novel PAR2 receptor mutant, which is unable to couple to inositol phosphate formation and intracellular Ca^{2+} mobilisation. This receptor encodes a 9-amino-acid mutation in the C-terminus δ34–43 and has been previously characterised for this property (Seatter *et al*., 2004). Following transient transfection of WT-PAR2 and δ34–43 PAR2, trypsin-stimulated IKK activity and NFκB-DNA binding were assessed (Figure 10). In NCTC2544 expressing WT-PAR2, trypsin stimulated a concentration-dependent increase in IKKα activity, giving a maximum 3–4-fold increase with 100 nM of the enzyme. By contrast, in cells expressing δ34–43 PAR2, no increase in IKKα activity was observed at concentrations up to 100 nM of trypsin. A similar finding was obtained measuring NFκB-DNA binding activity (Figure 10c). In WT-PAR-transfected cells trypsin stimulated a robust increase in binding; however, in cells transfected with PAR2 δ34–43, binding activity was lost. Under both conditions, PMA strongly stimulated NFκB-DNA binding, suggesting that in cells expressing either wild-type or δ34–43 PAR2, the IKK/NFκB pathway was not generally impaired by the transfection procedure.

Discussion

The data presented in this paper further support our previous findings that PAR2 can activate the NFκB pathway in human keratinocyte cell lines and primary cultures (Kanke *et al*., 2001). They also support the hypothesis that PAR2 NFκB signalling is strongly reliant upon initiation of intracellular calcium mobilisation. However, our results indicate that influx of extracellular Ca^{2+} plays little role in the signalling of PAR2 to the NFκB pathway, and that the main source of calcium is the intracellular pool.

In order to manipulate both intra- and extracellular Ca^{2+}, we initially employed BAPTA-AM and BAPTA, respectively. Indeed, the use of these agents clearly demonstrated intracellular Ca^{2+} to have a significant effect upon NFκB signalling by virtue of reducing IKK activity. Removal of extracellular Ca^{2+} with BAPTA did not have a significant effect on trypsin-stimulated activities at any level of the NFκB pathway measured in either clone G cells or in primary cultures. These results contrast with a number of other studies; for example,

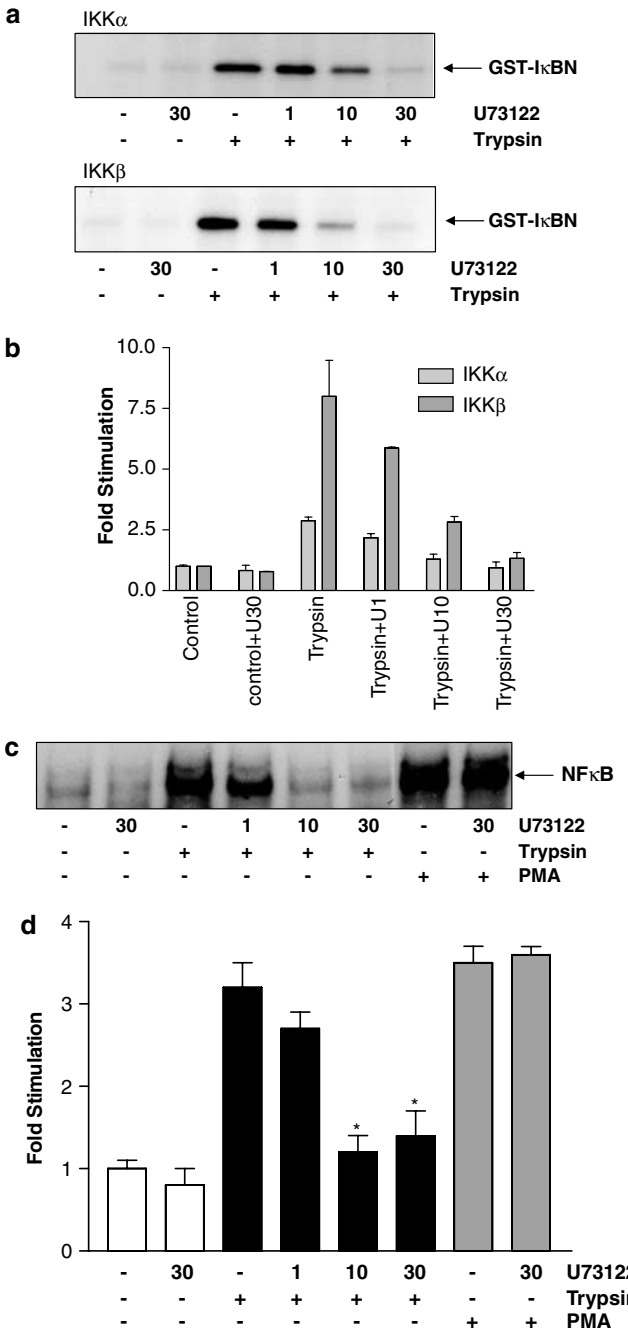

Figure 9 The effect of the PLC inhibitor U73122 on trypsin-stimulated IKK and NFκB-DNA binding in clone G cells. Cells were pretreated with U73122 (1–30 μM), prior to stimulation with trypsin (50 nM) for either 30 min (a) or 1 h (c). Samples were assessed for IKKα and IKKβ activity (a) or NFκB-DNA binding activity (c) as described in Methods. Each gel is representative of at least three experiments. In panels b and d, respectively, IKKα activity and NFκB-DNA binding activity blots were quantified and each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with Dunnet's post-test (*$P<0.05$).

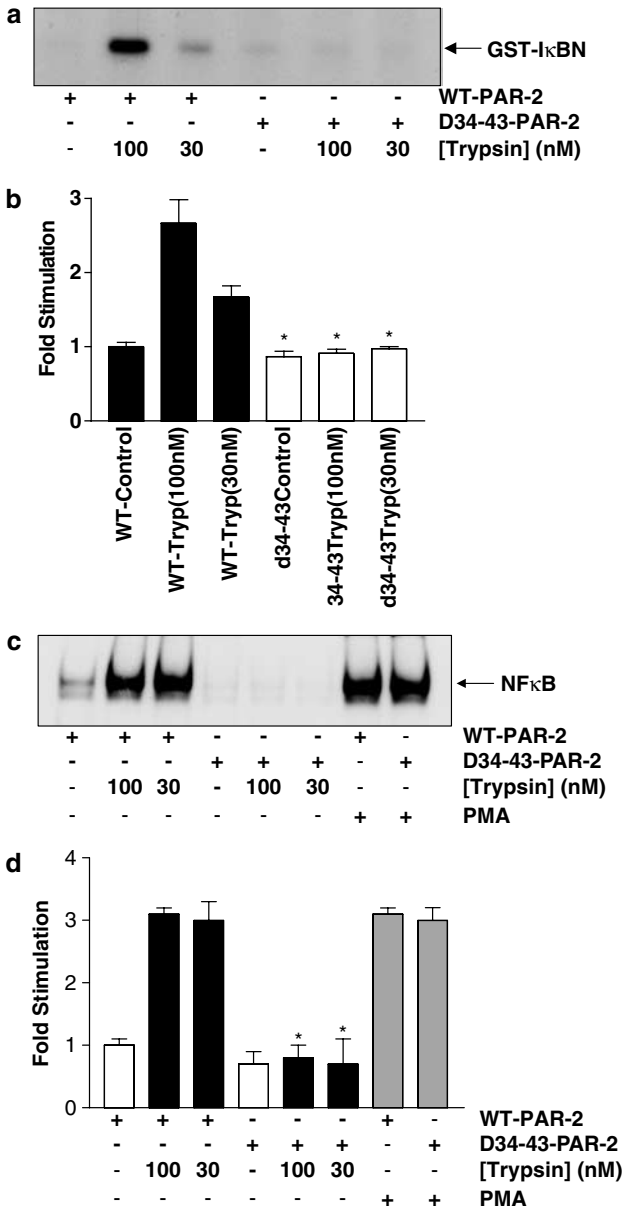

Figure 10 Effect of C-terminal deletion mutation upon PAR2-mediated IKK activation and NFκB-DNA binding. NCTC 2544 cells were transiently transfected with WT-PAR-2 or C-terminal mutant δ34–43. Cells were stimulated with 30 or 100 nM trypsin, for 30 min (a) or 1 h (c). Samples were assessed for IKKα activity (a) or NFκB-DNA-binding activity (c) as described in Methods. Each gel is representative of at least three experiments. In panels b and d, respectively, IKKα activity and NFκB-DNA-binding activity blots were quantified and each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with Dunnet's post-test (*$P<0.05$).

previous studies have shown that entry of Ca^{2+} *via* L-type Ca^{2+} channels can effect NFκB activity in the human mesangium (Hayashi *et al*., 2000). However, it is recognised that keratinocytes within the basal layer are normally subjected to reduced extracellular Ca^{2+} concentrations (Menon *et al*., 1985), and this may reflect the finding that in primary cultures of basal keratinocytes, PAR2-mediated activation of NFκB signalling was also found to be dependent upon intra- rather than extracellular Ca^{2+}. Thus, intracellular stores of Ca^{2+} may play a more predominant role in the regulation of NFκB signalling in other cell types. Indeed, a recent study has shown that in neoplastic cells intracellular Ca^{2+} chelation reduces NFκB-DNA binding, although IKK was not examined (Petranka *et al*., 2001).

In our study, both IKKα and IKKβ activation was substantially reduced by Ca^{2+} chelation or PLC inhibition, which is significant since IKKα and IKKβ have differential roles in the cellular function. While IKKβ is involved in NFκB-DNA, binding other studies have implicated IKKα in the regulation of a number of functions including processing of p105/100 (Senftleben *et al*., 2001), and phosphorylation of p65 NFκB (Sizemore *et al*., 1999) and histone (Anest *et al*., 2003; Yamamoto *et al*., 2003). Since PAR2 is able to regulate hyperproliferation and differentiation and also NFκB-dependent inflammatory cytokine release, it is possible that each isoform plays a different role in PAR2-mediated cellular effects. However, this is unlikely to be manifest at the level of DNA binding since both IKKα and IKKβ were inhibited, and clearly, more studies are required to assess the role of IKKα downstream of this event. Nevertheless, the fact that both isoforms are reduced by Ca^{2+} inhibition, a common site of mechanism of action possibly being further upstream in the pathway. This is consistent with previous studies from our laboratory, which show that both IKK isoforms are sensitive to PKC inhibition (Kanke *et al*., 2001). Since the IKKs lack any likely PKC concensus phosphorylation sites, the potential for an additional intermediate, which is Ca^{2+} sensitive and which regulates both kinases, becomes more likely. Taken together, these findings would argue for a lack of functional compartmentalisation for IKKα and IKKβ at least in PAR2-responsive systems.

Previous investigators have identified possible roles for a number of other Ca^{2+}-dependent intermediates in the regulation of NFκB activation, in particular both calcineurin and CaM kinases. However, the involvement of these Ca^{2+}-sensitive proteins has generally been attributed to an effect at the level of the IKK complex, although very few studies have examined this directly. In this present study, the role of CaMKII was investigated using the well-characterised inhibitor KN-62, and surprisingly, we found that inhibition of this kinase did not lead to an inhibitory effect on NFκB as had been previously reported in other cell types (Praskova *et al*., 2002; Meffert *et al*., 2003). Rather, the inhibition of CaMKII led to strong activation of NFκB-DNA binding and transcriptional activity (not shown); an effect that may represent a cell-specific response to disruption in Ca^{2+} signalling. Additionally, this increase in NFκB-DNA binding and transcription did not appear to be additive in the case of trypsin. Interestingly, this CaMKII inhibitor has been previously shown to inhibit the activation of ERK in keratinocytes (Praskova *et al*., 2002). However, when applied to clone G cells, this agent was found to increase the phosphorylation of the p42/44 MAP kinases, indicating that the actions of this inhibitor in the cell type used were inconsistent with the known function in primary keratinocytes. Given the limitations using this compound, a definitive role for CaMKII in the regulation of IKK or MAP kinase signalling in keratinocytes cannot be determined.

Similarly, there was no evidence for a role for the Ca^{2+}-dependent phosphatase calcineurin in the regulation of PAR2 coupling to NFκB. FK-506 was without significant effect upon either trypsin-stimulated IKK activation or NFκB-DNA binding activity. This contrasts with other studies that show strong inhibition of NFκB following FK-506 treatment or, alternatively, increased NFκB activation in cells overexpressing calcineurin (Alzuherri & Chang, 2003). However, at least one other study has shown no effect of FK-506 upon NFκB-DNA binding, despite inhibition of the expression of genes such as iNOS, known to be regulated by NFκB (Hamalainen *et al*., 2002). This suggests that cell and stimulus-specific differences are also likely to be a factor in the role of calcineurin in regulating NFκB activation. Indeed, recent evidence shows that calcineurin overexpression results in increased NFκB-DNA binding independently of IKK activation (Biswas *et al*., 2003). FK-506, or tacrolimus, has been implicated as an antipsoriatic agent (Michel *et al*., 1996), in conjunction with the role of an immunosuppressant used in organ transplantation. Nevertheless, from our studies these effects, at least in our model, are unlikely to be due to direct effects upon IKK signalling pathways but rather well-defined effects upon NFAT activation (Al-Daraji *et al*., 2002).

Thus, the results in this study clearly point to a central role for intracellular Ca^{2+}, generated through the formation of $InsP_3$, in the regulation of IKK/NFκB signalling. This was assessed in two ways. Firstly, we utilised the PLC inhibitor, U73122 to abrogate IKK signalling, a finding consistent with studies that show a link between $G_{q\alpha}$ and NFκB activation (Shi & Kehrl, 2001). Secondly, we utilised a novel PAR2 deletion mutant, which has been previously characterised (Seatter *et al*., 2004) and is unable to couple up $InsP_3$ formation and Ca^{2+} mobilisation. The mutation incorporates a putative palmitoylation site cytseine^{361}, within the C-terminus (Milligan *et al*., 1995), and we are currently assessing the functional role of this modification upon PAR2 function. Nevertheless, in cells expressing the mutated receptor, no IKK/NFκB signalling was observed in response to trypsin. This contrasted with WT-PAR2, which, when stimulated, strongly activated both parameters. In the case of the WT-PAR2, a small level of activation of NFκB was also observed in nonstimulated cells; however, this may be due to the fact that unphysiologically high levels of receptor expression may have resulted in ligand-independent activation of intracellular signalling pathways.

In conjunction with these results, the additional observation gained using the PKC inhibitor suggests that the effect of Ca^{2+} is unlikely to be direct or involve an unknown protein but rather is quite simply mediated *via* Ca^{2+}-dependent PKC isoforms. This has been confirmed in the current study and suggested previously using the expression of a DN-PKCα mutant, which completely abrogated trypsin-stimulated IKK activity (Kanke *et al*., 2001). However, our present study reveals that Ca^{2+}-dependent proteins, including certain PKC isoforms, are unlikely to regulate PAR2-mediated MAP kinase activation, showing that these pathways are distinctly regulated. In this regard, the present study supports previous work utilising the C-terminal PAR2 mutant, which, while being unable to couple to Ca^{2+} mobilisation, is still able to activate the major MAP kinases (Seatter *et al*., 2004). Our findings do, however, contrast with other studies implicating a role for intracellular Ca^{2+} in the regulation of ERK signalling in the hippocampus (Fahlman *et al*., 2002). Nevertheless, such differential coupling in keratinocytes may be important since both the MAP kinases and NFκB have clear roles in epidermal function/disease (Hu *et al*., 2001; Li *et al*., 2001). Thus, under conditions of aberrant Ca^{2+} signalling such as in psoriasis (Karvonen *et al*., 2000), certain PAR2-mediated, Ca^{2+}-independent, signalling events may predominate, thus leading to deleterious effects. We are currently studying these concepts in our laboratory.

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