Proteinase-activated Receptor-2-mediated Activation of Stress-activated Protein Kinases and Inhibitory κB Kinases in NCTC 2544 Keratinocytes*

Received for publication, January 16, 2001, and in revised form, June 12, 2001
Published, JBC Papers in Press, June 18, 2001, and DOI 10.1074/jbc.M100377200

Toru Kanke§§, Scott R. Macfarlane§§, Michael J. Seatter‡, Emma Davenport‡, Andrew Paul‡, Roderick C. McKenzie‡, and Robin Plevin‡

From the §Department of Physiology and Pharmacology, Strathclyde Institute for Biomedical Sciences, University of Strathclyde, 27 Taylor Street, Glasgow G4 0NR and the ¶Department of Dermatology, University of Edinburgh, Lauriston Building, Royal Infirmary of Edinburgh, Lauriston Place, Edinburgh EH3 9YW, United Kingdom

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

In this study we examined the regulation of the stress-activated protein (SAP) kinases and inhibitory κB kinases (IKKs) through stimulation of the novel G-protein-coupled receptor proteinase-activated receptor-2 in the human keratinocyte cell line NCTC2544. Trypsin and the peptide SLIGKV stimulated a time-dependent increase in both c-Jun N-terminal kinase and p38 mitogen-activated protein kinase activity. Trypsin also stimulated NFκB-DNA binding and the activation of the upstream kinases IκKα and -β. Phorbol 12-myristate 13-acetate also strongly activated both SAP kinases and IKK isoforms, suggesting the potential for a protein kinase C-mediated regulatory mechanism underlying the effects of trypsin. Pre-incubation with selective protein kinase C (PKC) inhibitors GF109203X and Gö6983, or transfection of dominant negative (DN)-PKCα, abolished phorbol 12-myristate 13-acetate-mediated c-Jun N-terminal kinase activity, although it only partially inhibited the response to trypsin. In contrast, Gö6983 reduced trypsin-stimulated p38 mitogen-activated protein kinase activity to a greater extent than GF109203X, although DN-PKCα or PKCζ had no substantial effect. Additionally, inhibitors of PKC partially reduced trypsin-stimulated NFκB activity, whereas DN-PKCα but not DN-PKCζ substantially reduced trypsin-stimulated Flag-IKKβ activity. This study shows for the first time proteinase-activated receptor-2-mediated stimulation of both SAP kinase and IKK signaling and differing roles for PKC isoforms in the regulation of each pathway.

Proteinase-activated receptor-2 (PAR-2) is a recently described member of the seven-transmembrane, G-protein-linked receptor family (1, 2) exemplified by the thrombin receptor (PAR-1) (3). It is activated by serine protease-mediated cleavage of the receptor to generate a new N terminus, which then interacts with the second exofacial loop of the receptor (see review in Ref. 4). PAR-2 is strongly activated by trypsin, tryptase, and potentially other unidentified enzymes (5) and is unique among the PAR family, as the newly described PAR-3 and PAR-4 are also thrombin-sensitive (6, 7).

PAR-2 is strongly expressed in smooth muscle cells of the airways, vasculature, and intestine, and in cells of epithelial origin such as endothelial cells and enterocytes (8, 9). It is also highly expressed in keratinocytes (10), where it regulates a number of inflammatory linked responses such as the expression of interleukin-6 and -8 and granulocyte macrophage-colony stimulating factor (11, 12). However, at present there is little information available regarding the role of specific cellular signaling pathways in regulating PAR-2-mediated cellular events. Studies have been limited principally to effects upon the generation of inositol trisphosphate and mobilization of intracellular Ca2+, both events associated with a receptor coupled to phospholipase C activation (13, 14).

Two significant intracellular signaling pathways potentially involved in mediating inflammatory responses in a number of cell types are the mitogen-activated protein (MAP) kinases, and the nuclear factor κB (NFκB) pathway. The MAP kinases consist of the classical isoforms of MAP kinase (extracellular signal-regulated kinases) and the stress-activated protein (SAP) kinases c-Jun N-terminal kinase (JNK) and p38 MAP kinase (15). All three kinases have been implicated in the regulation of a number of processes in keratinocytes such as inulcin expression, protein kinase C (PKC)-mediated hyperproliferation, and UV-mediated apoptosis (16–18). Previous studies have shown that, in rat aortic smooth muscle cells, trypsin stimulates p42/44 MAP kinase but does not activate JNK and p38 MAP kinase (19). However, different coupling mechanisms may exist in different target cells, particularly in keratinocytes where PAR-2 is highly expressed.

The NFκB family of transcription factors is well recognized as being involved in the regulation of a number of pro-inflammatory genes (20). In cytokine-stimulated cells, NFκB is regulated by the activation of isoforms of inhibitory κB kinase (IKK) that regulate phosphorylation of inhibitory κB (21–23). IKK itself is regulated by a number of upstream regulatory kinases, including NFκB-inducing kinase (NIK) and other potential intermediates such as protein kinase B and PKC (24–26). Although some studies have demonstrated activation of NFκB in response to thrombin, bradykinin, and other G-protein-coupled receptor agonists including trypsin (27–31), very little informa-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

¶ To whom correspondence should be addressed. Tel.: 44-141-548-1140; Fax: 44-141-552-2562; E-mail: r.plevin@strath.ac.uk.

The abbreviations used are: PAR, proteinase-activated receptor; NFκB, nuclear factor κB; SAP, stress-activated protein; IKK, inhibitory κB kinase; TNF-α, tumor necrosis factor α; PMA, phorbol 12-myristate 13-acetate; MAP, mitogen-activated protein; MAPKAP, mitogen-activated protein kinase-activated protein kinase; HA, hemagglutinin; AP-1, activator protein-1; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; PCR, polymerase chain reaction; RT, reverse transcription; DN, dominant negative; IP, inositol phosphate; NIK, NFκB-inducing kinase.
tion is available regarding the role and regulation of IKK isoforms in NF-κB signaling following activation of a G-protein coupled receptors.

Since PAR-2 activation displays many features similar to cytokines regarding the activation of inflammatory mediator release, we sought to determine if the signaling events mediated by PAR-2 were similar. Thus, we examined SAP kinase and NFκB activation, pathways that are known to be strongly activated by cytokines (15, 20). Here we show for the first time in a transfected epithelial cell line (32) strong activation of both SAP kinase and IKK/NFκB signaling pathways following PAR-2 activation, an effect mimicked by activation of protein kinase C. Using isoform-selective protein kinase C inhibitors (33, 34) and pleotropic dominant negative mutants of PKC (35), we demonstrate the involvement of PKC-dependent and -independent mechanisms in the regulation of SAP kinase activity. We also present evidence supporting an important role for typical PKC isoforms in mediating PAR-2-stimulated IKK/NFκB signaling.

EXPERIMENTAL PROCEDURES

Materials—All chemicals and reagents were obtained from appropriate commercial sources. Escherichia coli expression plasmids for GST-MAPKAP kinase-2 and GST-C-Jun (5–89) were kind gifts of J. Woodgett (Ontario Cancer Institute, Toronto, Canada) and C. J. Marshall (Chester Beatty Laboratories, London, United Kingdom (UK)). The plasmids encoding wild type and DN-IKKα and IKKβ were kind gifts from D. Goeddel (Tulalik Inc.,) whereas those encoding dominant-inhibitory PKC mutants PKCα (T/A), and PKCβ (T/A), were from P. J. Parker (Imperial Cancer Research Fund, London, United Kingdom). HA-JNK1, FLAG p38 MAP kinase, and GST-ATF-2 were kind gifts from Jonathan Blank (University of Leicester, Leicester, UK). Other materials included: oligonucleotide primers (Genesysy, UK); mammalian expression vectors pRe/RSV and pcDNA3 (Invitrogen, Netherlands); Lipofectin, LipofectAMINE, and PLUS reagent (Life Technologies, UK); mammalian expression vectors encoding wild type and DN-IKK (Ontario Cancer Institute, Toronto, Canada) and C. J. Marshall (Ches-ter Beatty Laboratories, London, United Kingdom). Human PAR-2 cDNA was cloned by PCR amplification from a human endothelial cells (TL-706), was obtained from CLONTECH Ltd. (UK). We also present evidence supporting an important role for typical PKC isoforms in mediating PAR-2-stimulated IKK/NFκB signaling.

Materials—All chemicals and reagents were obtained from appropriate commercial sources. Escherichia coli expression plasmids for GST-MAPKAP kinase-2 and GST-C-Jun (5–89) were kind gifts of J. Woodgett (Ontario Cancer Institute, Toronto, Canada) and C. J. Marshall (Ches-ter Beatty Laboratories, London, United Kingdom (UK)). The plasmids encoding wild type and DN-IKKα and IKKβ were kind gifts from D. Goeddel (Tulalik Inc.,) whereas those encoding dominant-inhibitory PKC mutants PKCα (T/A), and PKCβ (T/A), were from P. J. Parker (Imperial Cancer Research Fund, London, United Kingdom). HA-JNK1, FLAG p38 MAP kinase, and GST-ATF-2 were kind gifts from Jonathan Blank (University of Leicester, Leicester, UK). Other materials included: oligonucleotide primers (Genesysy, UK); mammalian expression vectors pRe/RSV and pcDNA3 (Invitrogen, Netherlands); Lipofectin, LipofectAMINE, and PLUS reagent (Life Technologies, UK); mammalian expression vectors encoding wild type and DN-IKK (Ontario Cancer Institute, Toronto, Canada) and C. J. Marshall (Ches-ter Beatty Laboratories, London, United Kingdom). Human PAR-2 cDNA was cloned by PCR amplification from a human endothelial cells (TL-706), was obtained from CLONTECH Ltd. (UK).

PCR Cloning of the Human PAR-2 cDNA and Cellular Expression— Human PAR-2 cDNA was cloned by PCR amplification from a human umbilical vein endothelial cell cDNA library. PCR primer design was based on the published human PAR-2 sequence (2). The primer sequences were as follows: forward primer 5′ ACCAAGCTTTCGCTGTCGCTCAGT-3′; reverse primer, 5′CTCTAGACTCATCCATTTCCCTTCTGAGG-3′. The design also incorporated unique HindIII and XbaI sites in the forward and reverse primers respectively, to allow directional cloning. The PCR product was subcloned into a similarly digested pRe/RSV vector using T4 DNA ligase to generate the pRe/RSV-PAR-2 expression plasmid. Transient transfections of human keratinocytes NCTC2544 was then transfected with pRe/RSV-PAR-2 using Lipofectin, and clonal cell lines were isolated in 800 µg/ml Geneticin. Several clones were tested for PAR-2 expression using PCR and assay of [3H]inositol phosphate ([H]IP) accumulation (see below). One of these clones (G) was used in Transfection Procedures— NCTC2544 clones expressing PAR-2 were grown to 60–70% confluence on six-well plates and transiently co-transfected with various plasmids using the LipofectAMINE Plus™ transfection system, according to the manufacturer’s protocol. For PAR-2 kinase assays, 200 ng of the plasmid encoding HA-JNK1 was transfected together with 600 ng of DN-PKCa (T/A), PKCβ (T/A), or blank vector pcDNA3. For each transfection, the total amount of DNA was adjusted to 800 ng/well with blank vector. A similar procedure was carried out for FLAG-p38 MAP kinase except that 100 ng of the construct was employed. For epitope-IKK assays, wild type Myc-IKKα and FLAG-IKKβ were transfected at 900 and 100 ng/well, respectively, in the presence of either 300 ng of DN-PKCc (above) or blank vector. For the NFκB lucerase assays, 250 ng of a plasmid containing 3× NFκB DNA enhancer, linked to the firefly lucerase reporter gene luc, or blank vector were transfected together, or in conjunction with 500 ng of DN-IKKα and/or β, to give 1.25 µg/well (37). After a 6-h incubation period with the DNA mixture in media free of serum and antibiotics, cells were transferred into complete M199 medium for another 30 h. Cells were then renounced quiescent by serum deprivation for 24 h before stimulation.

Measurement of Inositol Phosphate Accumulation—Trypsin-stimulated [3H]inositol phosphate accumulation was measured in cells pre-labeled overnight with [3H]myoinositol as outlined previously (38). JNK and p38 MAP Kinase Assays—For assay of JNK, pre-cleared supernatants were added to a 20-µl slurry of GST-c-Jun (5–89)/GSH-Sepharose beads and mixed for 3 h at 4°C, whereas for p38 MAP kinase, full-length GST-MAPKAP kinase-2 was used. The precipitates were resuspended in 25 µl of kinase buffer and the kinase reaction started by the addition of [γ-32P]ATP (1–2 µCi, 25 µM) and incubated for 20 min at 30°C. The reaction was terminated by addition of 10 µl of 4× Laemmli sample buffer. Samples were boiled for 5 min and resolved by SDS-polyacrylamide gel electrophoresis (11% (w/v) gel). Gels were dried and subjected to autoradiography overnight (39).

For epitope-tagged JNK assays, agonist-stimulated cells were lysed in 400 µl of lysis buffer and supernatants incubated for 3 h at 4°C with 10 µl of protein G-Sepharose beads pre-coupled with anti-HA antibody (Y-11, Santa Cruz, 0.2 µg) for 1 h at 4°C. HA-JNK1 activity was detected in the beads buffer in 30 µl of kinase buffer containing 100 µCi of [γ-32P]ATP, and 2 µg of affinity-purified GST-c-Jun (5–89) as a substrate for 20 min at 30°C. A similar protocol was employed for FLAG-p38 MAP kinase activity except that 1 µg of anti-FLAG antibody (anti-Oct A; Santa Cruz) was employed in the immunoprecipitation step and 1 µg/tube GST-ATF-2 was used in the kinase assay.

MAPKAP Kinase-2 Assay—MAPKAP kinase-2 activity was assessed in cell extracts by in vitro assays on cell line using GS peptide (KKLNRTLSVSA) as substrate as described previously (39).

Electrophoretic Mobility Shift Assay—Following termination by washing in ice-cold PBS, cells were harvested and pelleted and crude nuclear extracts made as described previously (40).

NFκB-BDNA binding was assessed by electrophoretic mobility shift assay, according to the kit manufacturer’s instructions (Promega).

IKK Assay—Cell lysates were incubated in 300 µl of solubilization buffer (20 µx Trit-CHR (pH 7.6), 1 µx EDTA, 0.5 µx EGTA, 10% glycerol (v/v), 0.1% Brij 35, 150 µM NaCl, 1% Triton X-100 (w/v), 20 µM NaF, 20 µM β-glycerophosphate, 0.5 µM Na3VO4, 1 µM phenylmethylsulfonyl fluoride, 0.5 µM leupeptin, 0.5 µM aprotinin) on ice for 30 min. Pre-cleared samples were then added to 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 solubilization buffer and once in kinase buffer (25 µx HEPES (pH 7.6), 20 µx MgCl2, 5 µx β-glycerophosphate, 0.1 µM Na3VO4, 2 µx dithiothreitol). Precipitates were then resuspended in 25 µl of kinase buffer and the kinase reaction initiated by addition of [γ-32P]ATP (5 µCi, 25 µM ATP, and 2 µg of GST-1κB (1–97, N-terminal truncated IκB) was then incubated for 30 min with shaking, at 30°C for 30 min before termination of the kinase reaction by addition of 4× Laemmli sample buffer. Samples were then resolved on 11% (w/v) acrylamide SDS-polyacrylamide gel electrophoresis and the phosphorylated protein identified by autoradiography. For Flag-IKKβ assays the same procedure was followed except 1 µg/tube anti-FLAG antibody was used in the immunoprecipitation step.
RESULTS

In preliminary experiments we found that in NCTC2544 cells expressing PAR-2, trypsin, and the PAR-2-activating hexapeptide (SLIGKV) stimulated strong activation of \[^{3}H\]IP accumulation (-fold stimulation; 50 nM trypsin = 12.6 ± 2.1), which was not observed in wild type NCTC2544 cells or in cells transfected with empty vector. This response was comparable with the stimulation observed by 50 μM UTP, a P2Y2 receptor agonist (-fold stimulation: 8.75 ± 0.76), suggesting a level of PAR-2 expression comparable with other endogenous receptors. Trypsin and SLIGKV also stimulated p42/44 MAP kinase activation, but only in cells expressing hPAR-2. The level of MAP kinase activity was also comparable to stimulation with UTP. Furthermore, a comparison of PAR-2 mRNA levels in clone G and human primary keratinocytes by RT-PCR indicated that the level of receptor mRNA expression was similar (Fig. 1). This suggests that, in clone G cells, the level of PAR-2 expression is not substantially greater than might be expected for endogenous expression in other cell types.

However, in contrast to the moderate activation of p42/44 MAP kinase, trypsin strongly stimulated both the known SAP kinases JNK and p38 MAP kinase (Figs. 2 and 3). The activation of both kinases was rapid in onset and reached a peak between 25 and 30 min before returning to basal values by 90 min. Maximum activation was 22.3 ± 6.6- and 24.9 ± 9.3-fold for JNK and p38 MAP kinase, respectively, a level of stimulation comparable with TNF-α (-20-fold). The PAR-2 peptide mimetic SLIGKV also stimulated both JNK and p38 MAP kinase (Figs. 2 and 3B). However, the degree of activation of both kinases was less than that observed for trypsin and the duration of activation was much shorter. This was not due to any nonspecific effect of trypsin, since no activation of SAP kinase activity was observed in response to trypsin in nontransfected cells (Fig. 2C). In additional experiments, trypsin was also found to stimulate strongly MAPKAP kinase-2 activity (Fig. 3D) but not in vector control cells.

In addition to activation of SAP kinase signaling, trypsin (30 nM) or SLIGKV (200 μM) stimulated a strong increase in NFκB

NfκB Reporter Activity Assay—NCTC2544 cells grown on six-well culture plates were transfected as described previously and were quiesced in serum-free M199 overnight. Cells were treated with appropriate agonists for 5 h and assayed as outlined previously (37).

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Expression of PAR-2 mRNA in clone G and primary keratinocyte cells. The level of PAR-2 mRNA expression was determined by RT-PCR from total cellular RNA (as outlined under “Experimental Procedures”) isolated from clone G cells (G) and primary human keratinocytes (P). Primers for actin were used as a positive control. Sizes were approximately determined by comparison to a 1-kilobase pair DNA ladder. This experiment is representative of two others, which used cells obtained from different individuals.

![Figure 2](http://www.jbc.org/)

**Fig. 2.** PAR-2-mediated JNK activation in NCTC2544 keratinocytes. Clone G cells expressing PAR-2 (panels A and B) or vector controls (panel C) were stimulated with either 30 nM trypsin (panels A and C) or 200 μM SLIGKV (panel B) for the times indicated (in min) and assayed for JNK activity as outlined under “Experimental Procedures.”

![Figure 3](http://www.jbc.org/)

**Fig. 3.** PAR-2-mediated p38 MAP kinase activation NCTC2544 keratinocytes. Clone G cells expressing PAR-2 (panels A–C) or vector controls (panel D) were stimulated with either 30 nM trypsin (panels A, C, and D) or 200 μM SLIGKV (panel B) for the times indicated (in min) and assayed for p38 MAP kinase activity (panels A–C) as outlined under “Experimental Procedures.” In panel C, blots of trypsin-stimulated p38 MAP kinase activity were quantified by scanning densitometry. In panel D, samples from trypsin-stimulated clone G cells (■) or vector controls (○) were assayed for MAPKAP kinase-2 activity as outlined under “Experimental Procedures.” Each value represents the mean ± S.E. of at least four individual experiments.
 NF-κB DNA-binding complex consisted of p50 and p65 as a result of NF-κB activation. This was observed in NCTC2544 cells as early as 10 min (Fig. 4, panel C) and JNK activity (Fig. 4, panel D) as outlined under “Experimental Procedures.” This autoradiogram is representative of at least three others. In panel E, autoradiographs of trypsin-stimulated IKK isoform activity were quantified by scanning densitometry (panel C; ∧, IKKα; ♦, IKKβ). Each value represents mean ± S.E. of at least four individual experiments.

**Table 1**

| Stimulation | + Luciferase reporter | + DN-IKKα | + DN-IKKβ | + DN-IKKα and DN-IKKβ |
|-------------|----------------------|----------|-----------|-----------------------|
| Control     | 1                    | 0.4 ± 0.3| 0.2 ± 0.15| 0.1 ± 0.1             |
| Trypsin     | 3.9 ± 0.5            | 2.3 ± 1.3| 1.0 ± 0.6 | 1.0 ± 0.68            |
| TNF-α       | 7.9 ± 0.6            | 6.0 ± 0.3| 0.4 ± 0.3 | 0.1 ± 0.1             |

DNA binding in NCTC2544 cells. This was observed as early as 30 min and was maximal by 60 min (Fig. 4, A and B). Super-shift analysis using anti-NFκB antibodies indicated that the stimulated DNA-binding complex consisted of p50 and p65 (data not shown). Activation of NFκB DNA binding was also accompanied by stimulation of NFκB luciferase reporter activity (Table 1) and the loss in expression of both α and β isoforms of IκB. Furthermore, we observed strong activation of both isoforms of IKK by trypsin (Fig. 4, C and D). An increase in activity of both IKKα and IKKβ was observed as early as 10 min, with maximum stimulation by 30 min (3.7 ± 0.2- and 5.4 ± 0.1-fold, respectively; n = 3, Fig. 4E), which was again comparable to that observed with the cytokine TNF-α.

Further experiments found that PAR-2 coupling to the NFκB pathway was not a result of transfection conditions. In human basal keratinocytes, trypsin stimulated both NFκB-DNA binding activity and JNK activation with kinetics similar to that observed in the transfected cells (Fig. 5). The level of trypsin-stimulated NFκB-DNA binding was again comparable to that observed in response to TNF-α.

We also found the known activator of protein kinase C, PMA, was also a strong activator of both SAP kinases (Fig. 6). PMA strongly activated JNK and p38 MAP kinase activity with kinetics similar to that observed for trypsin. Maximal activation of both PAR-2 were stimulated with 30 nM trypsin for the times indicated (min) and assayed for NFκB DNA binding activity (panel A) and JNK activity (panel B) as indicated under “Experimental Procedures.” Autoradiograms were representative of two others. TNF-α (25 ng/ml; 60 min) was used as a positive control.
basal values within 60 min.

PMA also strongly stimulated NFκB-DNA binding activity (Fig. 7). Maximal DNA binding was obtained between 30 and 60 min and was accompanied by a strong increase in the activity of both IKKα and IKKβ isoforms with kinetics similar to those observed for trypsin and TNF-α (Fig. 7, A–D).

In order to investigate the role of protein kinase C isoforms in the regulation of PMA-mediated SAP kinase and NFκB signaling, selective inhibitors of PKC isoforms and DN-PKC mutants were utilized. Pre-treatment of cells with GF109203X, which inhibits PKC isoforms α, β, δ, and ε but which shows some preference for PKCα (33, 34), abolished PMA-stimulated JNK and p38 MAP kinase activity (Fig. 8, A–C). However, over a number of experiments, it was found that trypsin-stimulated JNK and p38 MAP kinase activities were both reduced by ~30%, respectively (Fig. 8, A–C). In addition, following transfection of the cells with DN-PKCa (35), PMA-stimulated activation of HA-JNK1 was reduced by 80%, whereas trypsin-stimulated JNK-1 activity was again only partially reduced (Fig. 9). This inhibition was ~20% and correlated well with the effects observed with the PKC inhibitor. Furthermore, transfection with DN-PKCi did not affect PMA- or trypsin-stimulated HA-JNK1 activity (Fig. 9), suggesting no input from this atypical isoform. Additional control experiments showed that transfection of clone G with either DN-PKCa or DN-PKCi did not affect JNK activity in response to TNF-α (data not shown).

However, pre-treatment with another isoform selective inhibitor of PKC Go6983, which, in addition to effects similar to

![Fig. 7. PMA-stimulated IKK activity and NFκB-DNA binding in NCTC2544 keratinocytes.](image)

![Fig. 8. The effect of GF109203X on PMA- and trypsin-stimulated JNK and p38 MAP kinase activity in NCTC2544 keratinocytes.](image)

![Fig. 9. The effect of DN-PKCa and PKCi on PMA- and trypsin-stimulated JNK activity.](image)
and C), strongly inhibited the p38 MAP kinase response to trypsin (Fig. 10, B and C). Trypsin-stimulated p38 MAP kinase activity was reduced by 75%, whereas JNK activity was reduced by 20%. However, results obtained using DN-PKCa and DN-PKζ indicated that trypsin-stimulated p38 MAP kinase activity was not under additional control from PKCζ (Fig. 11). Furthermore, although DN-PKCa abolished PMA-stimulated p38 MAP kinase activity (data not shown), it had only a marginal effect upon trypsin-stimulated FLAG-p38 MAP kinase activity (Fig. 11) even when the transfected ratio of DN-PKCa to p38 MAP kinase was increased from 3:1, the ratio used in the JNK assays, to 10:1. Similarly, DN-PKζ was without effect on trypsin-stimulated FLAG-p38 MAP kinase activity (Fig. 11).

Pre-treatment of cells with GF109203X abolished PMA-stimulated JNK and p38 MAP kinase activity in NCTC2544 keratinocytes. Cells were pretreated with vehicle or 10 μM Go6983 for 30 min and stimulated with PMA or trypsin for another 30 min. Samples were assayed for JNK (panel A) and p38 MAP kinase activity (panel B) as indicated under “Experimental Procedures.” Each autoradiogram is representative of at least three others. In panel C, blots were quantified by scanning densitometry (vehicle, Go6983; f, Go6983). Each value represents the mean ± S.E. of at least four individual experiments.
ulated IKKα and IKKβ activity (Fig. 12, A–C). In contrast, trypsin-stimulated IKK activity was differentially inhibited by GF109203X. IKKα activity was reduced by ~50% (49.8 ± 8.2%; Fig. 12, A and C) while IKKβ activity was reduced by ~75% (76.4 ± 9.2%; Fig. 12, B and C). Using G69883 (Fig. 13), which also abolished PMA-stimulated IKK activity, we found that trypsin-stimulated IKKα was reduced still further, by about 80% (Fig. 13, A and C), similar to the reduction in IKKβ activity achieved with this inhibitor treatment (Fig. 13, B and C).

The effect of PKC inhibition was also examined using transfection of FLAG- and Myc-tagged IKK isoforms (Fig. 14). In preliminary experiments transfection of FLAG-IKKα alone generated very low basal and agonist-stimulated activity, whereas transfection with IKKβ alone generated extremely high basal activity with little additional stimulated activity. However, co-transfection of Myc-IKKα with IKKβ at a ratio of 9:1 generated low basal conditions and robust responses to PMA, trypsin, and TNF-α. Under these co-transfection conditions, PMA-stimulated FLAG-IKKβ activity was abolished by the expression of DN-PKCδ, whereas the response to trypsin was also substantially inhibited by this mutant (Fig. 14, A and B). Co-transfection of DN-PKCζ did not, however, result in any further inhibition of IKKβ activity. Myc-tagged IKKα activity could not be reliably measured under these conditions.

The effect of selectively inhibiting PKC isoforms was also assessed at the level of NFκB-DNA binding activity (Fig. 15). Pre-incubation of cells with increasing concentrations of GF109203X or G69883 abolished PMA-stimulated NFκB-DNA binding; however, surprisingly, both interventions only partially inhibited trypsin-stimulated activity (maximal reductions of 67.2 ± 1.7% and 53.2 ± 1.0% for GF109203X and G69883, respectively (Fig. 15, B and D).

As trypsin-stimulated IKK activation was largely abolished by PKC inhibitors, whereas NFκB-DNA binding was only partially inhibited, we assessed the IKK dependence of trypsin and TNF-α-stimulated NFκB reporter activity using DN forms of IKKα and -β (Table I). We found that introduction of either mutant alone or in combination reduced the absolute level of trypsin-stimulated reporter activity. However, this was accompanied by a reduction in basal reporter activity such that the -fold stimulation remained approximately the same (4–7-fold). In contrast, the -fold stimulation with TNF-α was markedly reduced by either DN-IKKα or β alone (-fold stimulation: TNF-α = 7.9-fold, + DN-IKKα = 1.5-fold, + DN-IKKβ = 2.0-fold) and abolished in response to both mutants in combination.

DISCUSSION

In this study we examined the actions of trypsin, mediated through the novel G-protein-coupled receptor PAR-2, upon two key signaling pathways relevant to keratinocyte function: the SAP kinase pathway and the NFκB pathway. To do this we expressed hPAR-2 in NCTC2544 cells, a cell line with characteristics of keratinocytes (32), which does not endogenously express PAR-2 or PAR-1. Trypsin- and SLIGKV-stimulated accumulation of [3H]IP confirmed the coupling of PAR-2 to the IP<sub>3</sub>/Ca<sup>2+</sup> signaling cascade, a phenomenon that has been previously demonstrated in a number of cell types (13, 14). This level of [3H]IP accumulation was comparable with that observed with UTP, suggesting that the level of receptor expression is not prohibitively high relative to endogenous receptor expression. This was confirmed by using RT-PCR, which showed that the level of mRNA for PAR-2 in clone G was comparable to human keratinocytes (Fig. 1). However, it should be noted that these results do not provide an absolute estimate as to the level of cell surface receptor expression in the clone G cells. The level of mRNA expression provides only an indication as to the level of cell surface receptor expression in the clone G cells.

In initial studies we found moderate activation of p42/44 MAP kinase in response to trypsin as assessed by Western.
to a lesser extent, SLIGKV strongly stimulated NFkB signaling in NCTC2544 cells. Both JNK and p38 MAP kinase were activated to a level comparable with the cytokine TNF-α, demonstrating for the first time a link between PAR-2 and SAP kinase activation.

PAR-2-mediated activation of JNK and p38 MAP kinase was further confirmed using the peptide SLIGKV, which mimics the N-terminal sequence generated by trypsin mediated cleavage of PAR-2. However, it should also be noted that stimulation of SAP kinase activity is more robust with the enzyme relative to the peptide. This phenomenon has been observed previously in relation to thrombin and TRAP stimulation of PAR-1 associated signaling events (42, 43) and reflects differences in the efficiency of receptor activation by trypsin relative to the peptide. Indeed, many studies indicate that concentration of 1–200 μM SLIGKV may not maximally activate PAR-2 (13, 14).

A previous study from our laboratory has shown that, in another cell type, where PAR-2 is endogenously expressed, PAR-2 is specifically linked to p42/44 MAP kinase activity (19), suggesting that coupling to SAP kinase may be due to the levels of expression of PAR-2 in NCTC2544. However, UTP, acting through the G-protein-coupled P2Y2 receptor, also stimulated p42/44 MAP kinase, indicating a similarity in receptor levels and efficiency in coupling to downstream signaling systems between exogenous PAR-2 and endogenous P2Y2. It is thus possible that PAR-2 is more efficiently connected to SAP kinase signaling in certain cell types, and, in preliminary experiments with primary cultures of endothelial cells and human keratinocytes, we have found a similar pattern of JNK activation induced through endogenous PAR-2.

In examining the NFkB pathway, we found that trypsin and, to a lesser extent, SLIGKV strongly stimulated NFkB-DNA binding. This in itself is not unusual, as several agents, including some G-protein-coupled receptor agonists, have been shown previously to induce modest increases in NFkB activation through indirect mechanisms, possibly involving p60 

or p42/44 MAP kinase/p90 

activation (31, 44). However, in a number of preliminary studies, we found no evidence of p42/44 MAP kinase involvement in the regulation of PAR-2-mediated NFkB activity, as PD98059 did not modify NFkB-DNA binding activity. Rather, in this cell type, we have found that activation of PAR-2 results in stimulation of the upstream mediator IKK. A similar result was observed for TNF-α, raising the possibility that PAR-2 and the TNF-α receptor expressed in this cell type could share some common signaling elements relevant to IKK activation. PAR-2 coupling to NFkB signaling is not restricted to transfected cell systems, since we have shown that trypsin also stimulates a robust increase in NFkB-DNA binding in primary cultures of human keratinocytes (Fig. 5). Indeed, a recent study has shown PAR-2-mediated activation of NFkB in coronary smooth muscle cells (29) and the activation of IKK through other G-protein-coupled receptors such as bradykinin (45), indicating the potential relevance this pathway has in the cellular actions mediated through this class of receptor.

We found that activation of PMA strongly stimulated SAP kinase activation and IKK signaling. PMA has been shown previously to be a moderate activator of SAP kinase signaling in some cell types (46, 47) while being inactive in others (16) and has also been linked to inhibition of SAP kinase activity (39, 48). However, activation of PKC is strongly implicated in keratinocyte cell differentiation (48) through effects upon AP-1 and CREB, events known to be distal to JNK and p38 MAP kinase activation (49). PMA-stimulated NFkB DNA binding has similarly been demonstrated in keratinocytes (50, 51), and it is possible that, at least in this cell type, PAR-2 may activate both cascades by a common PKC-mediated pathway.

However, our experiments indicated clear differences in the regulation of PAR-2-mediated SAP kinase and IKK signaling by PKC isoforms. Inhibition of PKC isoforms by GF109203X, Go6983, and introduction of DN-PKCa only partially reduced trypsin-stimulated JNK activity, suggesting a small involvement for conventional PKCs. Experiments using GF109203X and Go6983 also suggest a lack of involvement for PKCε and some other novel or atypical isoforms, as these compounds are able to inhibit isotypes such as PKCc-δ, and -ζ (34). Using the pleotropic DN-PKCδ and -ζ mutants supported these observations, particularly since these mutants have the ability to inhibit several different isoforms in addition to PKCε and PKCc-δ. Although these results point to a predominantly PKC-independent pathway for PAR-2-mediated regulation of JNK, some PKC isoforms have been omitted, and it is possible that an isoform highly expressed in keratinocytes, such as PKCη (52, 53), may be involved.

The finding that p38 MAP kinase activation was much more sensitive to Go6983 than GF109203X indicates differences in the roles played by PKC isoforms in the regulation of either JNK or p38 MAP kinase following PAR-2 activation. Unexpectedly, experiments using DN mutants of PKCa and -ζ also suggested no role for these isoforms, although p38 MAP kinase was inhibited by Go6983, which has been shown to inhibit PKCζ (40, 54). Preliminary experiments in our laboratory have shown that PAR-2-mediated p38 MAP kinase activation is Ca2+-independent but is abolished by PMA pre-treatment. This suggests a role for an unidentified Ca2+-independent, diacylglycerol-dependent isoform of PKC, such as PKCy or -δ, which Go6983 has been also shown to inhibit (40, 54). Apart from this study, little evidence supports differential regulation of p38 MAP kinase and JNK through different PKCs. Rather, other recent studies have indicated a role for both PKC-dependent and -independent inputs into SAP kinase activation mediated through G-protein-coupled receptors (47).

In contrast, conventional PKC isoforms were found to play a more substantial role in the regulation of IKK signaling. Trypsin-mediated activation of IKKβ was largely abolished by GF109203X, Go6983, and chronic treatment with PMA (results not shown), consistent with a role for typical PKC isoforms in this pathway. Indeed, PKCa has been implicated in PMA regulation of IKKβ in HeLa cells (26), and our present study shows that this is also an intermediate involved in PAR-2 stimulation. Trypsin-mediated activation of IKKα was only partially affected by GF109203X and chronic PMA pretreatment (results not shown) and was further decreased but not abolished by Go6983. This may also imply an additional minor role for atypical PKCζ isoforms in the regulation of IKKα; however, in general, PKC-mediated regulation of IKKα has not been shown to be a common feature of cytokine receptor stimulation. This may therefore be a feature of G-protein-coupled receptors that strongly activate PKC.

Studies to confirm the role of PKC isoforms in the regulation of PAR-2-mediated IKK activation were only partially successful. Transfection of clone G cells with IKKβ generated a very high basal activity that was not increased upon agonist stimulation. This high basal activity could be lowered by co-transfection of IKKα, such that agonist stimulation could be revealed. This confirms a recent study indicating that IKKα functions, in part, to regulate the endogenous activity of IKKβ (55), therefore suggesting that under normal conditions endogenous IKKα and IKKβ levels are comparable or favor IKKα.
This was confirmed by immunoblotting of IκKα and IκKβ, which showed comparable levels of the two kinases in clone G cells (data not shown). Using this co-transfection procedure, we were, nevertheless, able to confirm the important role for PKCa in the regulation of IκKβ and the lack of effect of PKCζ. However, assay of IκKα either following transfection alone or with IκKβ did not give consistent agonist-stimulated activity. Given that IκKα and IκKβ exist as a complex involving IκKκ, NIK, and possibly other intermediates (56–58), transfection of exogenous IκK is likely to disturb the equilibrium between the kinases such that this may be reflected in the lack of IκKα activity.

Our studies also suggest that PKC-mediated regulation of both IκKα and IκKβ may only play a minor role in the regulation of PAR-2-mediated NFκB-DNA binding activity. Treatment with GF109203X only partially affected NFκB-DNA binding activity, whereas Gö6983, which is more effective against IκKα, caused a similar reduction (Fig. 13). This suggests that, in the case of PAR-2, either PKC-independent mechanisms are involved in the regulation of NFκB or sufficient IκB phosphorylation and degradation can be initiated via the remaining IκK signal. In support of the former hypothesis, we found that introduction of DN-IκKα and IκKβ did not reduce trypsin-stimulated NFκB reporter activity, although their presence abolished the response to TNF-α. However, these interpretations are difficult since, as discussed above, IκKα can unidirectionally regulate IκKβ activity in cells both in a negative and positive manner (55). Therefore, introduction of DN mutants, particularly IκKα, may enhance endogenous IκK activity.

Alternatively, it is possible that IκKα may be involved in other aspects of signaling that are directly involved in NFκB translocation and DNA binding, and these possibilities are currently being investigated in our laboratory. However, the fact that TNF-α-stimulated IκK activity was not affected by PKC inhibitors, while being abolished by DN-IκKs, indicates differences in the coupling of cytokine and G-protein-coupled receptors to IκK signaling events.

The results in this study do not necessarily exclude the possibility that other PKC-independent events may regulate IκK activity following PAR-2 stimulation. This includes involvement of NIK and protein kinase B (24, 25). Furthermore, given the co-activation through PAR-2 of both IκKα and SAP kinase signaling, it is also possible that there are upstream intermediates regulating both IκK and SAP kinase signaling in response to PAR-2 activation. For example, it has been shown that MEKK-1 can substitute for NIK in the regulation of IκK in response to PAR-2 activation. For example, it has been shown that MEKK-1 can substitute for NIK in the regulation of JNK and p38 MAP kinase, MEKK-2 shown that MEKK-1 can substitute for NIK in the regulation of IκK activity following PAR-2 stimulation. This includes in-

Acknowledgments—We acknowledge the people who kindly donated reagents and constructs used in this study.

REFERENCES

1. Nystedt, S., Emilsson, K., Wahlestedt, C., and Sundelin, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9208–9212
2. Nystedt, S., Emilsson, K., Larsson, A. K., Strombeck, B., and Sundelin, J. (1998) FASEB J. 12, 540–544
3. Vu, T. K. H., Hung, D. T., M., Whitmore, T. E., Presnell, S. R., Yee, D. P., Ching, A., Darrow, A. L., Santulli, R. J., Br, F., and Andrade-Gordon, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9151–9155
4. Bölüm, S. K., Wang, W., B., B., Mertz, J. M., Cough, H., C., and Chows, J. M. (1998) Immunity 94, 356–362
5. Santulli, R. J., Derian, C. K., Darrow, A. L., Tomko, K. A., Eckardt, A. J., Seiberg, M., Scarborough, R. M., and Andrade-Gordon, P. (1996) J. Biol. Chem. 271, 24387–24395
6. Ido, R., A., and Weigert, J. B. (1999) Cell Mol. Life Sci. 94, 1250–1254
7. Efremova, T., LaCelle, P., Welter, J. F., and Eckert, R. L. (1998) J. Biol. Chem. 272, 544–548
8. Belham, C. L., Tate, R. J., Scott, P. H., Pemberton, A. D., Miller, H. R. P., Wadsworth, R. M., Gould, W., and Plevin, R. (1996) Biochem. J. 326, 929–946
9. May, M. J., and Ghosh, S. (1998) Immunol. Today 19, 80–88
10. Didonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) Nature 388, 548–554
11. Regnier, C. H., Song, H. Y., Gao, X., Goddel, D. V., Cao, D. Z., and Rothe, M. (1997) Cell 90, 373–383
12. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) Cell 91, 243–252
13. Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997) Nature 385, 540–544
14. Ozsoy, O. N., Mayo, L. D., Gustine, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. R. (1999) Nature 402, 85–92
15. Lallena, M. J., Diaz-Meco, M. T., Bren, G., Paya, C. V., and Moscat, J. (1999) Mol. Cell. Biol. 19, 2180–2188
16. Krames, V. V., Pan, Z. F., and J. H., Herbert, J. M., Ulevitch, R. J., and Y. E. (1995) J. Biol. Chem. 270, 14928–14934
17. Pan, Z. K., Ye, R. D., Christiansen, S. C., Jagels, M. A., Bokoch, G. M., and Zunaw, B. L. (1998) J. Immunol. 160, 3038–3045
18. Breiteneder, E., Kaufmann, R., Braun, M., Wittpath, M., Glusa, E., Nowak, G., and Schrör, K. (1999) Br. J. Pharmacol. 128, 1735–1740
19. Han, Y. Q., Runge, M. S., and Brasier, A. R. (1998) Circ. Res. 84, 693–703
20. Hoshi, S., Goto, M., Koyama, N., Nomoto, K., and Tanaka, H. (2000) Am. J. Physiol. 278, R157–R164
21. Paul, A., Torrie, L. J., McLaren, G. J., Kennedy, C., Gould, G. W., and Plevin, R. (1999) J. Biol. Chem. 274, 10084–10089
22. Rea, K., Karlsson, L. D., Ray, E. B., Bakka, and Hovig, T. (1981)AAM 89, 73–80
23. Touch, D., Piant, F., Cost, H., Bellevueque, P., Grandperret, T., Ajakane, M., Baudet, V., Brodez, Y., Boreel, N., and Plevin, R. (1998) J. Biol. Chem. 273, 544–548
24. Garima-Paramio, P., Cabrero, Y., Borresc, N., and Parker, P. J. (1998) Biochem. J. 333, 631–636
25. Kondo, S., Kono, T., Sauдер, D. N., and McKinney, R. C. (1993) J. Invest. Dermatol. 101, 689–695
26. Wood, K. M., Ruff, M., and Hay, R. T. (1999) Oncogene 16, 2131–2139
27. Plevin, R., Kelloch, N. A., Wakeham, M. J. O., and Wadsworth, R. (1994) Br. J. Pharmacol. 112, 311–315
28. Paul, A., Torrie, L. D., McLare, G. J., Kennedy, C., Gould, G. W., and Plevin, R. (2000) J. Biol. Chem. 275, 13243–13249
29. Schreiber, B., Mathies, P., Muller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6149
30. Gschwendt, M., Dieterich, S., Rennecke, J., Kuttten, W., Mueller, D. H., and Johannes, F. (1996) FEBS Lett. 399, 77–80
31. Maramp, I., Laniou, V., Mats, G. J., and Holleben, M. D. (1992) Can. J. Physiol. Pharmacol. 70, 996–1003
32. Glusa, E., Painitz, M., and Breiteneder, E. (1996) Semin. Thromb. Hemost. 22, 261–265
33. Li, D. J., Feng, W. J., Galup, M., Kim, J. H., Gum, J. Y., and Basbaum, C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5718–5723
34. Xie, P., Browning, D. D., Hay, N., Macknn, N., and Ye, R. D. (2000) J. Biol. Chem. 275, 24907–24914
46. Nagao, M., Yamauchi, J., Kaziro, Y., and Itoh, H. (1998) J. Biol. Chem. 273, 22892–22898
47. Alexandrov, A., Keffel, S., Goepel, M., and Michel, M. C. (1999) Biochem. Biophys. Res. Commun. 261, 372–376
48. Ohba, M., Ishino, K., Kashiwagi, M., Kawabe, S., Chida, K., Huh, N. H., and Kuroki, T. (1998) Mol. Cell. Biol. 18, 5199–5207
49. Banks, E. R., Crieh, J. F., and Eckert, R. L. (1999) Biochem. J. 337, 507–512
50. Tobin, D., Nilsson, M., and Toftgard, R. (1996) Oncogene 12, 785–793
51. Li, J. J., Westergaard, C., Ghosh, P., and Colburn, N. H. (1997) Cancer Res. 57, 3569–3576
52. Denning, M. F., Dlugosz, A. A., Williams, E. K., Szallasi, Z., Blumberg, P. M., and Yuspa, S. H. (1995) Cell Growth Differ. 6, 149–157
53. Takahashi, H., Asano, K., Manabe, A., Kinouchi, M., Ishida-Yamamoto, A. M., and Iizuka, H. (1998) J. Invest. Dermatol. 110, 218–223
54. Way, K. J., Chou, E., and King, G. L. (2000) Trends Pharmacol. Sci. 21, 181–187
55. O’Malley, A., Lin, X., Gelezunavas, R., and Greene, W. C. (2000) Mol. Cell. Biol. 20, 1170–1178
56. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) Cell 93, 1231–1240
57. Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. (1998) Nature 395, 297–300
58. Mercurio, F., Murray, B. W., Shevchenko, A., Bennett, B. L., Young, D. B., Li, J. W., Pascaul, G., Motiwalla, A., Zhu, H. Y., Mann, M., and Manning, A. M. (1999) Mol. Cell. Biol. 19, 1526–1538
59. Nemoto, S., DiDonato, J. A., and Lin, A. N. (1998) Mol. Cell. Biol. 18, 736–7343
60. Blank, J. L., Gerwins, P., Elliott, E. M., Sather, S., and Johnson, G. L. (1996) J. Biol. Chem. 271, 5361–5368
61. Gerwins, P., Blank, J. L., and Johnson, G. L. (1997) J. Biol. Chem. 272, 8288–8295
62. Zhan, Q., and Lee, F. S. (1999) J. Biol. Chem. 274, 8355–8358
63. Li, Q. T., Lu, Q. X., Hwang, J. Y., Buscher, D., Lee, K. F., Izpisua-Belmonte, J. C., and Verma, I. M. (1999) Genes Dev. 13, 1322–1328
64. Hu, Y. L., Baud, V., Delhase, M., Zhang, P. L., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999) Science 284, 316–320
65. Li, Z. W., Chu, W. M., Hu, Y. L., Delhase, M., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999) J. Exp. Med. 189, 1839–1845
Proteinase-activated Receptor-2-mediated Activation of Stress-activated Protein Kinases and Inhibitory κB Kinases in NCTC 2544 Keratinocytes

Toru Kanke, Scott R. Macfarlane, Michael J. Seatter, Emma Davenport, Andrew Paul, Roderick C. McKenzie and Robin Plevin

J. Biol. Chem. 2001, 276:31657-31666. doi: 10.1074/jbc.M100377200 originally published online June 18, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100377200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 65 references, 30 of which can be accessed free at http://www.jbc.org/content/276/34/31657.full.html#ref-list-1