Staurosporine Induces a Sequential Program of Mouse Keratinocyte Terminal Differentiation through Activation of PKC Isozymes

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Staurosporine (stsp) induces assembly of cornified envelopes in mouse keratinocyte cultures. To clarify whether this effect is the consequence of a coordinated differentiation program similar to that observed in epidermis, we assessed the expression of multiple differentiation-specific markers in stsp-treated keratinocytes. In medium containing 0.05 mM Ca^{2+}, in which the basal cell phenotype is normally maintained, stsp induced dose-dependent increases in keratin 1, epidermal and keratinocyte transglutaminases, SPR-1, loricrin, and profilaggrin mRNA. Based on nuclear run-on analysis, stsp-mediated marker expression was found to be due at least in part to increased transcription. Since protein kinase C (PKC) activation is required for keratinocyte differentiation, we tested whether stsp influenced this signaling pathway. Stsp induced the translocation of multiple PKC isoforms from the cytosol to membrane and/or cytoskeletal fractions, inducing isozyme downregulation within 24 h. Moreover, AP-1 DNA binding activity was elevated in stsp-treated keratinocytes, consistent with the notion that this agent influences keratinocyte-specific gene expression *via* the PKC pathway. Stsp-mediated marker expression was inhibited by the PKC inhibitor GF 109203X. In cells pre-treated with bryostatin 1 to selectively down-modulate specific PKC isoforms, stsp-induced loricrin, filaggrin, and SPR-1 expression was suppressed when PKC α, ϵ, and/or δ were downregulated, suggesting that these isozymes may be necessary for marker expression in response to this agent. Thus, in addition to its effects on cornified envelope assembly, stsp induces a coordinate program of differentiation-specific keratinocyte gene expression that is mediated at least in part by the PKC signaling pathway. *Key words: keratinocytes/protein kinase C/staurosporine. J Invest Dermatol 106:482–489, 1996*

A gradient of rising intracellular calcium (Ca^{2+}) concentration from basal cells towards the outermost skin layers (Malmquist *et al*, 1984; Menon *et al*, 1985) may be an important stimulus for the sequential expression of genes for epidermal differentiation markers (reviewed by Yuspa, 1994). This situation can be emulated *in vitro* using mouse keratinocytes in submerged culture by increasing the extracellular Ca^{2+} concentration from 0.05 mM, in which cells exhibit the proliferative basal cell phenotype, to 0.12 mM, which induces the sequential expression of early (spinous cell) and late (granular cell) differentiation marker proteins (Yuspa *et al*, 1989; Denning *et al*, 1995). The Ca^{2+} signal for differentiation is accompanied by increased phospholipase C activity (Lee and Yuspa, 1991; Punnonen *et al*, 1993), which generates the production of diacylglycerol, an activator of protein kinase C (PKC) (Nishizuka, 1992). Evidence strongly supports the necessity of PKC activation for keratinocyte differentiation. Ca^{2+} changes the subcellular distribution of PKC *in vitro* (Isseroff *et al*, 1989), and the Ca^{2+}-induced expression of genes for the late differentiation markers keratinocyte transglutaminase (TG_K), loricrin, and profilaggrin is dependent upon PKC activation (Dlugosz and Yuspa, 1993, 1994). Conversely, Ca^{2+}-induced expression of the early markers keratins 1 (K1) and 10 (K10) is suppressed by PKC activation (Dlugosz and Yuspa, 1993). The importance of PKC in keratinocyte differentiation is substantiated by the fact that PKC activators such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and synthetic diacylglycerols also induce late differentiation marker expression and cornified envelope assembly *in vitro* in the absence of changes in extracellular Ca^{2+} levels (Dlugosz and Yuspa, 1991, 1993), and TPA inhibits K1 and K10 expression *in vitro* and *in vivo* (Dlugosz and Yuspa, 1993; Toftgard *et al*, 1985).

In several cellular systems, biochemical events have been assigned to modulation of specific PKC isozymes (Goodnight *et al*, 1994). The α, δ, ϵ, η, and ζ isoforms of PKC are expressed in murine keratinocytes (Dlugosz *et al*, 1992; Denning *et al*, 1995). Recent evidence suggests that in keratinocytes *in vitro*, terminal

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Abbreviations: stsp, staurosporine; TG, transglutaminase; TG_K, keratinocyte transglutaminase; TG_E, epidermal transglutaminase.

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differentiation in response to Ca^{2+} is also mediated by specific isozymes, a rise in extracellular Ca^{2+} being followed by translocation of multiple PKC isoforms, and loricrin and filaggrin expression correlating closely with a requirement for PKC α (Denning *et al*, 1995). PKC η is expressed only in the granular layer of epidermis, suggesting a specific role for this isoform in keratinocyte differentiation (Osada *et al*, 1993).

Staurosporine (stsp) is a microbial alkaloid that was initially described as an inhibitor of PKC (Tamaoki *et al*, 1986), but has since been established as a potent inhibitor of many other protein kinases (Ruegg and Burgess, 1989; Herbert *et al*, 1990). In keratinocytes, stsp is able to mimic many of the responses induced by TPA and Ca^{2+}. Ornithine decarboxylase activity is induced by stsp and TPA, and this induction can be inhibited by the PKC inhibitor H-7 or chronic TPA application to downregulate PKC prior to agent application (Yamamoto *et al*, 1992). Like TPA and Ca^{2+}, stsp induces the maturation of normal human and mouse keratinocytes, with the formation of cornified envelopes associated with an increase in transglutaminase (TG) activity (Sako *et al*, 1988; Dlugosz and Yuspa, 1991). In mouse keratinocytes, cornification is partially blocked by initially incubating cells for prolonged periods with 60 nM bryostatin 1 to downregulate PKC, and in human cells co-incubation with the specific PKC inhibitor Ro31-8220 partially abrogates stsp-induced effects (Jones and Sharpe, 1994). Thus, the ability of stsp to mimic certain responses of keratinocytes to TPA and Ca^{2+}, as well as the inhibition of these effects by downregulating or inhibiting PKC, strongly suggests that stsp is capable of activating the PKC signal transduction pathway in these cells. Unlike TPA or Ca^{2+}, stsp is also able to induce cornification in neoplastic mouse keratinocytes (Dlugosz and Yuspa, 1991), suggesting that stsp may have unique as well as common targets with phorbol esters.

The aim of this study was to assess the extent of keratinocyte maturation instigated by stsp and clarify the role of PKC isoforms in this process. Currently, it is unknown whether stsp induces sequential differentiation-specific marker expression in murine keratinocytes rather than merely activating TGs to induce cornified envelope assembly *in vitro*. To resolve this issue, stsp-induced expression of proteins and mRNAs specific to epidermal differentiation was characterized. The involvement of PKC isoforms in stsp-induced gene expression was analyzed by determining its effect on PKC isoform redistribution, and specific isoforms were differentially downregulated to discover which were essential for stsp-induced marker expression.

MATERIALS AND METHODS

Reagents Stsp and GF 109203X were purchased from Calbiochem (La Jolla, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO). Bryostatin 1 was obtained from the Pharmaceutical Resources Branch of the National Cancer Institute (Bethesda, MD). All reagents were dissolved in dimethylsulfoxide and stored at −20°C.

Cell Culture Keratinocytes were isolated from BALB/c newborn mouse skin and were grown in Eagle's minimal essential medium lacking Ca^{2+}, with 8% chelex-treated fetal bovine serum and 0.2% penicillin/streptomycin solution. Unless otherwise indicated, the Ca^{2+} concentration in medium was adjusted to 0.05 mM to maintain a basal cell–like population of undifferentiated cells (Hennings *et al*, 1980).

Cornified Envelope Assay The assay measures insoluble cross-linked protein as described (Hough-Monroe and Milstone, 1991), with modifications. Cornified envelopes were prepared by scraping cell monolayers into 2% sodium dodecyl sulfate, 20 mM dithiothreitol in phosphate-buffered saline. Unattached cells were pelleted from medium and were pooled with the scraped cells. Samples were boiled for 10 min. Cornified envelopes were isolated as described (Hough-Monroe and Milstone, 1991) on RC60 membrane on a 96-well dot blot apparatus. Membranes were air-dried, then submerged in 7.5% trichloroacetic acid at 80°C for 30 min. Trichloroacetic acid was decanted and membranes were stained at 50°C for 15 min with 0.5% Coomassie blue G250 in 7% acetic acid. Membranes were destained with 7% acetic acid until the background was white, then were air-dried. Spots were excised and eluted with 1% NH_3OH concentrated solution, 66% methanol overnight. Absorbance of eluate was measured on a Titertek plate reader at 600 nm.

Western Blots Cell fractionation and blotting were performed as described, and antibodies to PKC α, δ, ϵ, η, and ζ were obtained from the sources indicated (Denning *et al*, 1995). For experiments for which preparation of whole cell lysates was required, methods were as described (Yuspa *et al*, 1989), except that analysis was performed on combined floating and attached cells. Antibodies to filaggrin, loricrin, K1, K10, and keratin 14 (K14) were generated within the laboratory (Mehrel *et al*, 1990; Yuspa *et al*, 1989). SPR-1 was detected using an antibody prepared in rabbits to the C terminus of the mouse sequence, which is highly homologous to and cross-reacts with human SPR-1 (Kartasova *et al*, 1996). Immunoreactive proteins were visualized using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Richmond, CA) and Renaissance enhanced chemiluminescence reagents (DuPont, NEN, Boston, MA).

Northern Blots At indicated time points, floating and attached cells were combined, solubilized in 4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% sodium lauroyl sarcosinate, and 0.1 M 2-mercaptoethanol prior to total RNA purification using phenol/chloroform as described (Chomczynski and Sacchi, 1987). RNA was resolved and blots were prehybridized as described (Dlugosz and Yuspa, 1994). TG_K mRNA was detected by hybridization to rat cDNA fragments (Phillips *et al*, 1990) in plasmid vectors kindly donated by Dr. R. Rice of the University of California, Davis. Epidermal transglutaminase (TG_E) mRNA was detected using DNA complementary to bases 2158–2217 (3′ noncoding region) of mouse TG_E (Kim *et al*, 1993) or a 684-bp reverse transcriptase–polymerase chain reaction product generated with primers GGCTTTGGACAAACT-CAAAC (upstream) and GATAGTAGGGAATTTATTGCA (downstream) inserted into a pCR 11 plasmid vector (Invitrogen, San Diego, CA). Either probe gave identical results. SPR-1 was identified with a 420 bp polymerase chain reaction product from the coding portion of mouse SPR-1 (Kartasova *et al*, 1996). The 18S ribosomal RNA (rRNA) probe was obtained from ATCC (Rockville, MD). Plasmid-inserted fragments were used for loricrin (3′ noncoding region) (Mehrel *et al*, 1990), K1, and profilaggrin (Yuspa *et al*, 1989). Probes were labeled with ^{32}P by end labeling for the TG_E 60-mer or by nick translation.

Nuclear Run-On Analysis *In vitro* transcription reactions and hybridization of ^{32}P-labeled transcripts to DNA immobilized on nitrocellulose membranes were performed as described (Dlugosz and Yuspa, 1993). Attached and floating keratinocytes were combined for the analysis. Plasmids and DNA fragments were the same as those used in Northern blotting. For K14, 3′ noncoding fragments (Roop *et al*, 1983) were used in plasmid vectors. Run-on reactions were standardized by hybridizing an equal number of disintegrations per minute from each nuclear reaction to membrane-bound DNA.

AP-1 Binding Assay Nuclear extracts were prepared according to the method of Schreiber *et al* (1989). The binding reactions consisted of 3 μg of nuclear extract incubated for 20 min at room temperature in the presence of 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 4 mM Tris, pH 7.9, 50 mM KCl, 1 mM ethylenediamine tetraacetic acid, 1 mM dithiothreitol, 10% glycerol, 1 μg of polydIdC (Pharmacia, Piscataway, NJ) and 0.1 ng of ^{32}P-labeled AP-1 probe (synthetic consensus sequence purchased from Promega, Madison, WI). The complexes were resolved on a 6% polyacrylamide gel (Novex, San Diego, CA) in 0.5 × TBE buffer at 100V for 80 min. The reactions were visualized through autoradiography. The mutant AP-1 oligomer contained the sequence 5′-GGAGTCA-3′.

RESULTS

Induction of Cornified Envelope Assembly by Stsp Primary mouse keratinocytes were incubated with stsp in medium with 0.05 or 1.4 mM Ca^{2+} for 48 h. Under the assay conditions used in this study, Ca^{2+} alone (1.4 mM) had little effect on cross-linked protein production at this time point **(Fig 1)** (see also Lichti and Yuspa, 1988). In medium with 1.4 mM Ca^{2+}, stsp induced a biphasic dose-dependent increase in cross-linked protein with a maximal effect at 10 nM, in agreement with a previous study (Sako *et al*, 1988). In 0.05 mM Ca^{2+}, similar biphasic dose responses to stsp were observed; however, cornified envelope assembly was greatly reduced **(Fig 1)**. Above 10 nM, lower levels of protein cross-linking were detected, and at 100 and 250 nM, essentially no induction of cross-linked protein was detected.

Stsp Induces a Program of Differentiation Marker Expression in Murine Keratinocytes To assess whether stsp was also capable of influencing differentiation marker expression, Western

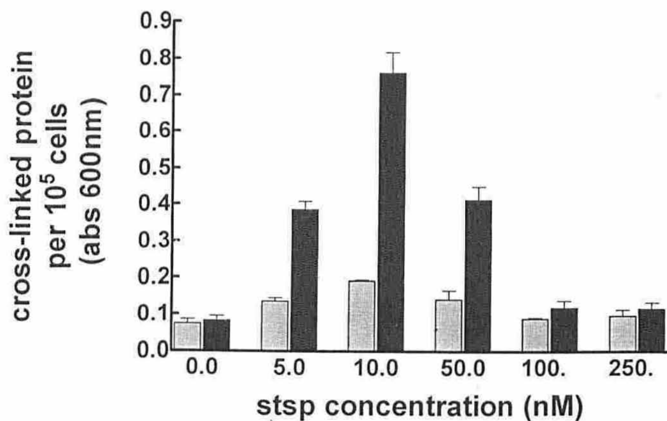

Figure 1. Effect of stsp on cross-linked envelope synthesis in mouse keratinocytes. Cultured keratinocytes were incubated for 48 h with agent in medium with 0.05 mM (▨) or 1.4 mM (■) Ca^{2+}, then envelopes were prepared from pooled detached and attached cells as described in *Materials and Methods*. The inclusion of unattached cells in the assay from 0.05 mM Ca^{2+} cultures increases the baseline value for cross-linked envelopes. Results are of one experiment representative of three; *error bars*, SD. Each experiment was performed in duplicate.

blots were performed on lysates of keratinocytes incubated in the presence of stsp in 1.4 mM Ca^2, conditions under which stsp was the most effective at inducing cornification **(Fig 2*A*)**. In 1.4 mM Ca^{2+} medium without stsp, differentiation marker expression is poor (Yuspa *et al*, 1989) **(Fig 2)**. The suprabasal marker SPR-1 and late (granular cell) markers loricrin and filaggrin were induced by stsp within 24 h. Immunofluorescence of stsp-treated keratinocytes confirmed the results of **Fig 2*A*** for late markers and revealed that, despite the substantial increase in expression levels, loricrin and filaggrin were not expressed in all cells in response to the agent, but that marker expression was localized to distinct regions of the monolayer (not shown), as with incubation of cells in 0.12 mM

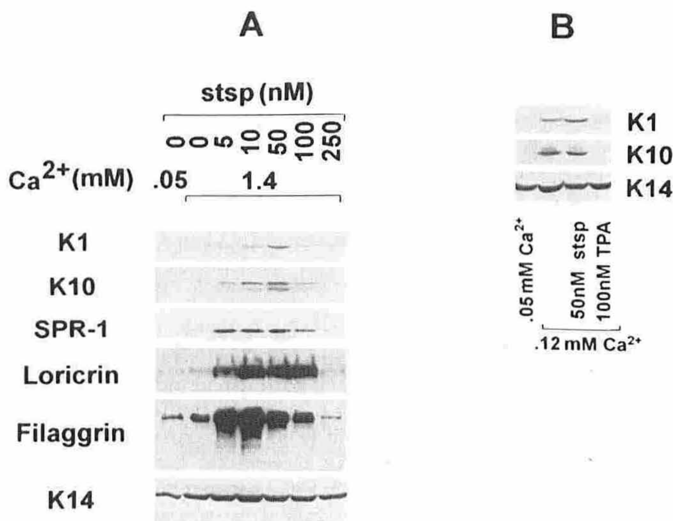

Figure 2. Stsp induces differentiation-specific marker proteins in mouse keratinocytes. *A*) Cells were incubated with stsp for 24 h in 1.4 mM Ca^{2+}. Whole cell lysates were then prepared (Yuspa *et al*, 1989), protein samples were subjected to polyacrylamide gel electrophoresis and Western blotting, then marker proteins were detected with specific antibodies as delineated in *Materials and Methods*. *B*) Cells were incubated in medium with 0.12 mM Ca^{2+} with or without 100 nM TPA or 50 nM stsp for 24 h. Total cell lysates were prepared and analysed as in *Panel A*. Similar results were obtained in two independent experiments.

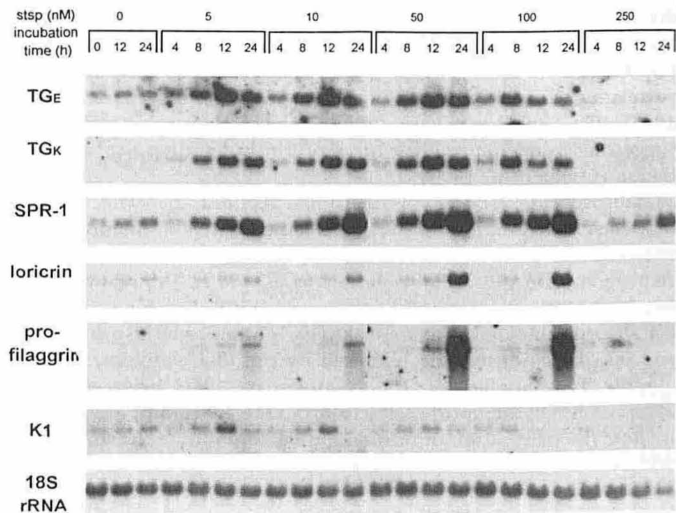

Figure 3. Stsp induces differentiation marker mRNA in mouse keratinocytes. Cells were incubated in medium with 0.05 mM Ca^{2+} and stsp at the indicated concentrations and time intervals before preparation of total cell RNA and Northern blot analysis. Experiments were repeated twice with similar results.

Ca^{2+} (Yuspa *et al*, 1989). Additionally, expression of the spinous markers K1 and K10 was increased by stsp. This is unlike the effect of TPA on keratin expression, as TPA does not induce expression of K1 and 10, and actually suppresses 0.12 mM Ca^{2+}–induced expression of these proteins (Dlugosz and Yuspa, 1993). As antibodies to mouse TGs are not available, these proteins could not be evaluated.

To confirm the differential effects of stsp and TPA on keratin expression, keratinocytes were incubated in the presence of either agent for 24 h in medium with 0.12 mM Ca^{2+}, which is optimal for the induction of spinous cell–specific keratins **(Fig 2*B*)**. Under these conditions, stsp could not further enhance K1 and K10 expression, but TPA suppressed both keratins, suggesting some divergence in mechanism of action for these two agents.

To determine whether raised levels of marker proteins resulted from changes in gene expression and to assess whether expression of markers was a coordinated sequential process, the time course for induction of marker mRNA by stsp was studied by Northern blotting **(Fig 3)**. Keratinocytes were incubated with stsp in medium with 0.05 mM rather than 1.4 mM Ca^{2+}, as Ca^{2+} alone induces a marked increase in TG_K mRNA compared with basal conditions in 0.05 mM Ca^{2+} (Dlugosz and Yuspa, 1994). Stsp induced a time- and dose-dependent increase in transcripts for both TG isoforms similarly, mRNA being induced within 8 h with 5–100 nM stsp. The window of stsp concentrations in which TG activity increased (1–10 nM) (Sako *et al*, 1988; Dlugosz and Yuspa, 1991) and protein cross-linking occurred (5–50 nM) **(Fig 1)** in response to stsp was narrower than that for induction of TG_K mRNA. This suggests that post-translational mechanisms required for TG function are also modified by stsp. Loricrin, profilaggrin, SPR-1, and K1 **(Fig 3)** transcripts were upregulated by stsp in a coordinated sequential fashion, with K1 mRNA being expressed maximally at 12 h and transcripts for SPR-1, loricrin, and filaggrin at 24 h.

To determine at which level stsp upregulates mRNA for markers of keratinocyte differentiation, run-on transcription assays were performed. Transcription of genes for TGs and K1, for which mRNA was induced within 8 h **(Fig 3)**, was assessed after an 8-h incubation with stsp, and a 20-h time point was used for profilaggrin and SPR-1 run-ons as mRNA for these markers was detected maximally by Northern blot analysis at 24 h **(Fig 3)**. Increased transcription of genes for all of these markers of differentiation was demonstrated in response to stsp, but in contrast, there was

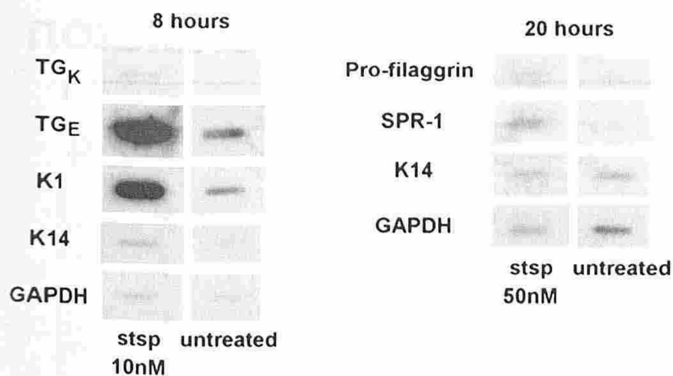

Figure 4. Ca^{2+} accelerates stsp-induced expression of TG_K and TG_E mRNA in mouse keratinocytes. Keratinocytes were incubated for the indicated times with 10 nM stsp in 0.05 or 1.4 mM Ca^{2+}. Cells were processed for total RNA extraction, and 20 μg RNA was electrophoresed, then blotted onto nitrocellulose and probed for TG_E or TG_K as described in *Materials and Methods*. The effect of Ca^{2+} was assessed in two separate experiments with similar results.

relatively little effect on the basal cell marker K14 or glyceraldehyde-3-phosphate dehydrogenase **(Fig 4)**.

Stsp-induced Expression of TG_K and TG_E mRNA Is Accelerated in 1.4 mM Compared with 0.05 mM Ca^{2+} Because the effect of stsp on protein cross-linking is enhanced in 1.4 mM Ca^{2+}, the ability of stsp to induce late differentiation markers in medium with this Ca^{2+} concentration was compared with induction in 0.05 mM Ca^{2+}. Stsp induced respective ~5- and 7-fold increases in expression of loricrin and profilaggrin mRNA in 1.4 mM Ca^{2+} compared with 0.05 mM Ca^{2+} (data not shown), which if translated into more abundant proteins may contribute to the increased quantity of cross-linked protein detected in 1.4 mM Ca^{2+}. However, the maximum levels of TG_E and TG_K transcripts induced by stsp were not dramatically higher in 1.4 mM Ca^{2+} compared with 0.05 mM **(Fig 5)**, suggesting that increased gene expression for TGs does not explain the enhanced cornification by stsp in 1.4 mM Ca^{2+} medium **(Fig 1)**. Interestingly, 1.4 mM Ca^{2+} had a distinct effect on the time course for stsp mRNA induction,

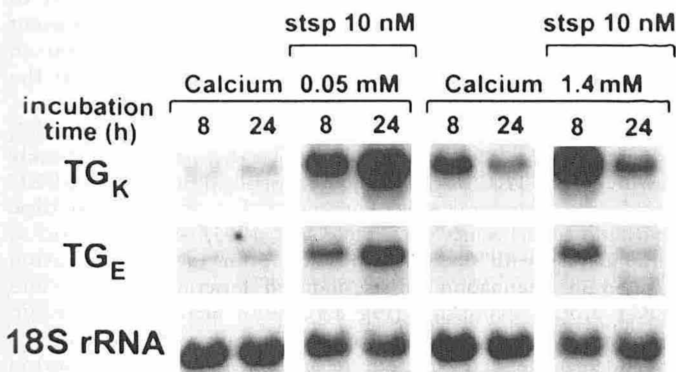

Figure 5. Stsp induces gene transcription for differentiation markers in mouse keratinocytes. Nuclei from cells grown in medium with 0.05 mM Ca^{2+}, and dimethylsulfoxide vehicle or stsp (50 nM) for 8 or 20 h were isolated. Transcript elongation proceeded *in vitro* in the presence of ^{32}P UTP for radiolabeling. Transcripts were hybridized for 3 d at 42°C to probes immobilized on nitrocellulose. The final wash stringency was 0.2 × sodium citrate/sodium chloride buffer, 0.1% sodium dodecyl sulfate at 65°C followed by treatment with RNAse A, 5 μg/ml in 2 × sodium citrate/sodium chloride buffer (37°C, 30 min). Hybridization was visualized by autoradiography. The experiment was repeated with 6 and 15 h exposures to stsp with similar results.

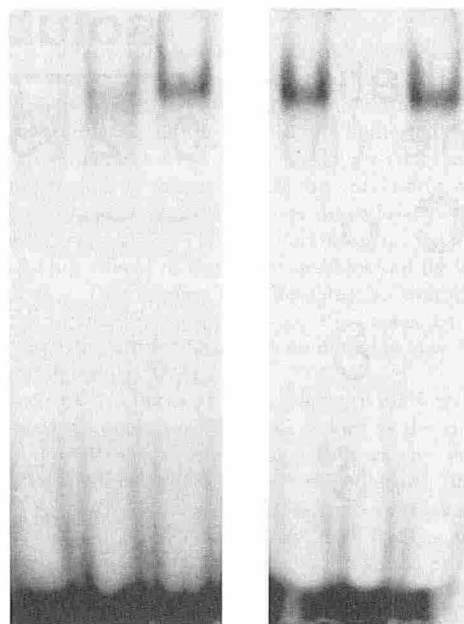

Figure 6. Stsp augments AP-1 binding in mouse keratinocytes. Keratinocytes were incubated in 0.05 mM Ca^{2+} in the presence of 50 nM stsp for the time periods indicated. Cells attached to dishes were harvested and nuclei were isolated. Nuclear extracts were incubated with a ^{32}P-labeled AP-1 binding sequence and levels of AP-1 binding activity were determined by gel mobility shift and autoradiography. To determine the specificity of the binding reaction, unlabeled oligonucleotides (as indicated) were added to the binding reaction, using extracts prepared 24 h after treatment with stsp, 10 min before the addition of the radiolabeled probe. Results are representative of two determinations.

when compared with treatment in 0.05 mM Ca^{2+} medium. **Figure 5** shows that in 0.05 mM Ca^{2+} medium, induction of transcripts for TG_K and TG_E by stsp was detected at higher levels at 24 than at 8 h, whereas in 1.4 mM Ca^{2+} stsp induced higher levels of TG mRNAs at 8 than at 24 h of treatment. The attenuation of TG mRNA expression at 24 h in 1.4 mM Ca^{2+} medium may be due to the commencement of cross-linking, which is within this time frame in stsp-treated mouse keratinocytes (Dlugosz and Yuspa, 1991), but may also be a result of an additive effect of stsp and Ca^{2+} on PKC function. Switching extracellular Ca^{2+} from 0.05 to 1.4 mM induces the translocation of PKC isoforms in mouse keratinocytes (Denning *et al*, 1995), and the presence of stsp may further modulate PKC, resulting in accelerated but transient TG mRNA expression.

Stsp Induces AP-1 Binding To gain insight into the potential mechanism by which stsp coordinately activates keratinocyte-specific gene expression, its ability to enhance binding of the AP-1 transcription factor to a TPA response element was tested using a gel shift assay. **Figure 6** shows that stsp increased AP-1 DNA-

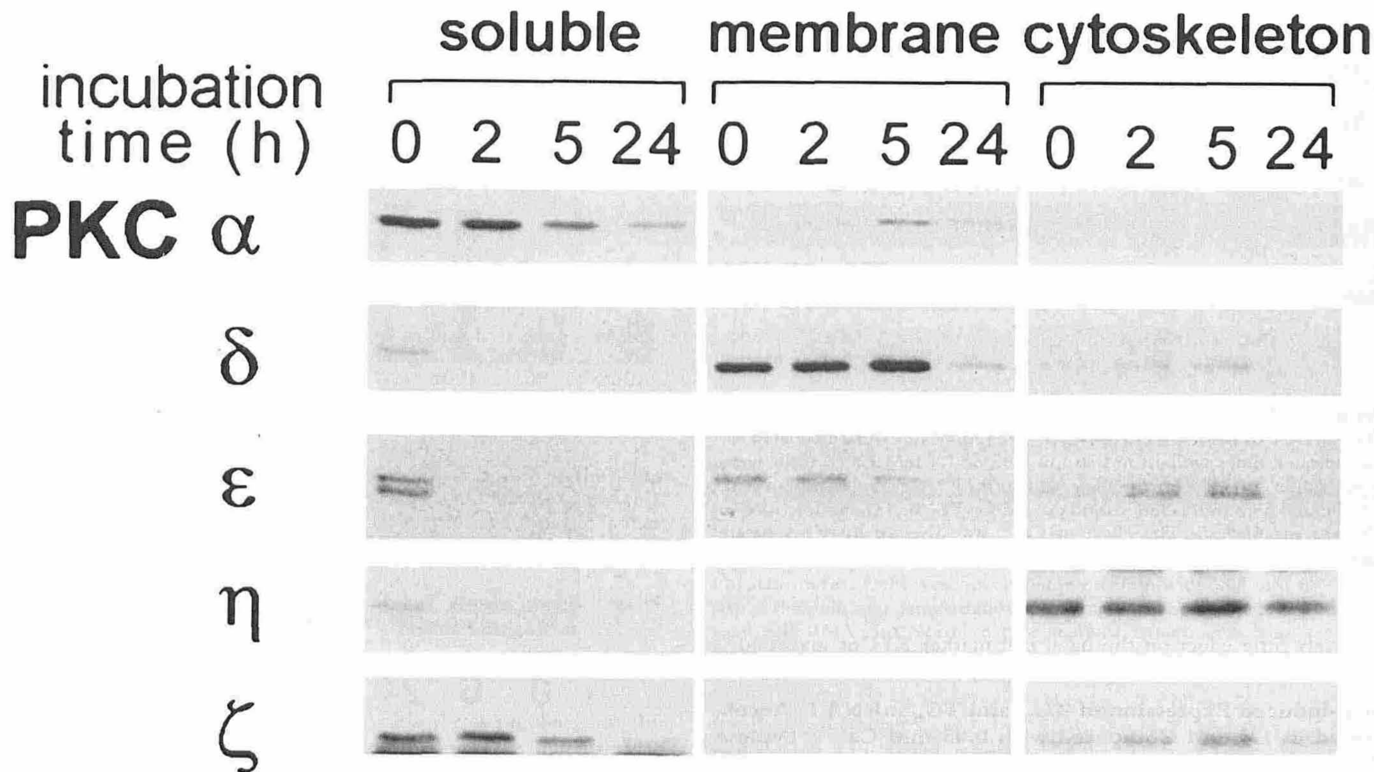

Figure 7. Translocation and downregulation of PKC isoforms in mouse keratinocytes by stsp. Cells were incubated with 50 nM stsp for the indicated time points in medium with 0.05 mM Ca^{2+}. Cells were then separated into soluble, triton-soluble membrane, and cytoskeletal fractions (Denning *et al*, 1995). The cytoskeletal fraction was obtained by sonication of triton-insoluble material in Laemmli sample buffer. Sample proteins were separated by electrophoresis, blotted onto nitrocellulose and PKC isoforms were detected as described in *Materials and Methods*. Results were replicated in another experiment. Results were similar but less pronounced with 10 nM stsp (not shown).

binding activity within 6 h, and that this activity increased further by 24 h of incubation with agent. This effect was specific for AP-1, as it could be blocked by adding excess cold AP-1 consensus sequence to the binding reaction but not mutant sequence. As the genes for K1, loricrin, profilaggrin, SPR-1, and TG_K harbor a TPA response element (Huff *et al*, 1993; DiSepio *et al*, 1995; An *et al*, 1993; Yamanishi *et al*, 1992; Presland *et al*, 1992), the AP-1 transcription factor complex may be an important regulator of stsp-mediated changes in gene expression.

Stsp Induces the Translocation and Downregulation of PKC Isoforms in Mouse Keratinocytes The induction of terminal differentiation in mouse keratinocytes requires activation of PKC (Denning *et al*, 1995; Dlugosz and Yuspa, 1993; Dlugosz and Yuspa, 1994). Since stsp induces PKC agonist effects in this cell type (Dlugosz and Yuspa, 1991), we assessed whether stsp causes changes in the distribution or abundance of PKC isoforms. Fifty nanomolar stsp induced a redistribution of PKC α, δ, ϵ, and ζ to new cellular locations. PKC α translocated to the triton-soluble particulate fraction within 5 h, PKC δ to the cytoskeletal and, to a lesser extent, particulate fractions, whereas the ϵ and ζ isoforms preferentially redistributed to the cytoskeletal fraction within 2 h. PKC η was not translocated in response to stsp. All isoforms were decreased or lost from the soluble pool **(Fig 7)**. At 24 h, there was a substantial downregulation by stsp of all isoforms detected in mouse keratinocytes with the exception of PKC η, which was only marginally affected.

Stsp-Induced Marker Expression Is Inhibited by PKC Inhibition and Downregulation of Specific PKC Isoforms Due to the capacity of stsp to modulate multiple PKC isoforms during the induction of keratinocyte differentiation, we tested the ability of the specific PKC inhibitor GF 109203X to inhibit stsp-induced marker expression. The expression of loricrin, filaggrin, and SPR-1 were greatly reduced in the presence of GF 109203X **(Fig 8*A*)**, suggesting that stsp activates PKC to increase differentiation marker expression.

To demonstrate a specific requirement for individual PKC isoforms in stsp-induced differentiation, keratinocytes were incubated with different concentrations of bryostatin 1 for 16 h to downregulate selectively PKC isoforms **(Fig 8*B*)** prior to treatment with stsp and Western blot analysis **(Fig 8*A*)**. Incubation of keratinocytes for 16 h with 0.1–1 nM bryostatin resulted in the downregulation of PKC δ and partial downregulation of PKC α, ϵ, and η in agreement with previous reports (Szallasi *et al*, 1994; Denning *et al*, 1995). One micromolar bryostatin completely downregulated PKC α selectively and partially downregulated PKC ϵ and δ as seen previously, with protection of PKC δ at high bryostatin concentrations (Denning *et al*, 1995). Pre-incubation of keratinocytes with 0.1 nM bryostatin prior to stsp application resulted in attenuation of stsp-induced loricrin, filaggrin, and SPR-1 protein expression **(Fig 8*A*)**. With increasing bryostatin concentrations, SPR-1 and loricrin expression was further reduced or undetectable. This pattern of marker expression inhibition suggests that there may be a requirement for PKC α and/or ϵ for loricrin and SPR-1 expression. Filaggrin appears to be regulated differently in that expression was impeded with increasing concentrations of bryostatin up to 10 nM, but at 1 μM there was a partial reversal of this effect, suggesting a role for the δ isoform. Attenuation of marker expression after pretreatment with bryostatin at concentrations as low as 0.1 nM may be due to a necessity for threshold levels of a single or multiple PKC isoforms, below which marker expression is reduced. It is unlikely that the η and ζ isoforms of PKC are involved in marker expression as these were little affected by pretreatment with bryostatin **(Fig 8*B*)**. Toxicity was not

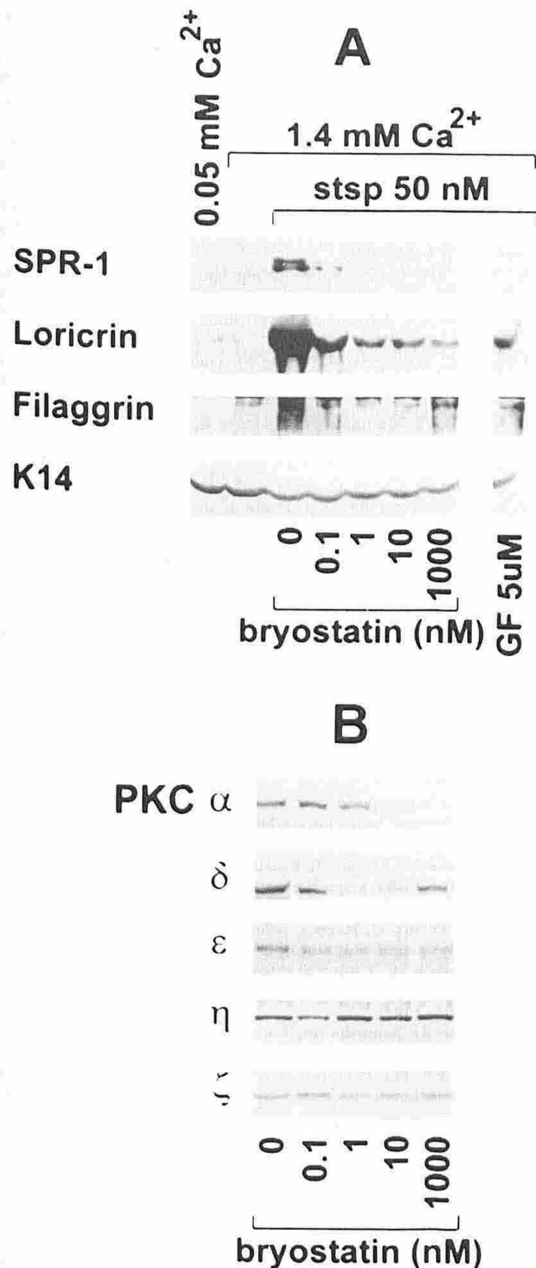

Figure 8. Reversal of stsp-induced marker expression by prior incubation with GF 109203X and bryostatin 1. *A*) Keratinocytes were incubated for 16 h with bryostatin 1 in medium with 0.05 mM Ca^{2+}, then the Ca^{2+} concentration was switched to 1.4 mM and 50 nM stsp was added, or 5 μM GF 109203X was added 1 h prior to stsp and Ca^{2+} switch. Cells were incubated for a further 24 h before total cell sample preparation (Yuspa *et al*, 1989). *B*) Keratinocytes were incubated with 0–1,000 nM bryostatin 1 for 16 h in medium with 0.05 mM Ca^{2+} prior to whole cell lysate sample preparation. Samples were subjected to polyacrylamide gel electrophoresis and were blotted onto nitrocellulose before detection with specific antibodies to differentiation-specific marker proteins (*A*) or to PKC isoforms (*B*) (Denning *et al*, 1995). Experiments were repeated twice with similar results. The slight reduction in PKC ζ expression in response to bryostatin was not observed in the repeat experiment or in a previous study (Denning *et al*, 1995).

significantly increased as measured by the MTT assay (Mosmann, 1983), when bryostatin and stsp were applied together, compared with stsp alone (results not shown), demonstrating that inhibition of marker expression was not due to overt toxicity after application of multiple agents.

DISCUSSION

Stsp is a potent inducer of TG activity and cornification in cultured keratinocytes. In this report, we have demonstrated that this response is part of a coordinated and sequential program of terminal differentiation involving multiple epidermal differentiation markers. Stsp increases AP-1 DNA-binding activity and influences marker expression at least in part by increasing keratinocyte-specific gene transcription. Stsp causes subcellular redistribution of multiple PKC isoforms in cultured keratinocytes, and the differentiation-inducing effects of this agent are blocked by inhibiting the PKC pathway. Taken together, these results establish stsp as a powerful activator of epidermal-specific gene expression and strongly suggest that this response is mediated at least in part by the PKC signaling pathway.

The effects of stsp on murine keratinocyte differentiation *in vitro* closely emulates this series of events *in vivo* as the time course of stsp-induced gene expression *in vitro* follows the same trend as differentiation marker expression in the epidermis, but is within a compressed time frame, demonstrated by the earlier expression of mRNA for the suprabasal markers SPR-1, K1, TG_E, and TG_K than for markers specific to the granular layer such as profilaggrin and loricrin. A number of approaches were taken to analyze potential mechanisms of action of the nonspecific kinase inhibitor stsp on induction of the very specific series of events leading to terminal differentiation. Stsp-treated keratinocytes *in vitro* express the spinous markers K1 and 10 in an analagous fashion to treatment with 0.12 mM Ca^{2+}, but in contrast to the PKC-activating agent TPA which suppresses K1 and 10 expression. PKC activation is the signaling event responsible for the spinous to granular phenotypic transition, with consequent loss of K1 and 10 expression rather than induction (Dlugosz and Yuspa, 1993). Therefore, it is possible that expression of keratins in response to stsp may be a part of the differentiation process regulated by a mechanism independent of PKC. However, the majority of evidence presented here and elsewhere (Dlugosz and Yuspa, 1991; Jones and Sharpe, 1994) strongly suggests that stsp, like TPA and Ca^{2+}, induces activation of PKC, and it is this signaling event that triggers the sequential expression of the other differentiation marker proteins studied. This is supported by the inhibition of stsp-induced marker expression by pre-incubation with the phorbol ester deoxyphorbol phenylacetate (results not shown) and bryostatin 1, which downregulate PKC, and by the specific PKC inhibitor GF 109203X **(Fig 8*A*)**. In addition, stsp increased AP-1 transcription factor binding, which could be the result of changes in transcription factor phosphorylation downstream from stsp-induced PKC activation (Abate *et al*, 1991; Goode *et al*, 1992; Baker *et al*, 1992).

The mechanisms by which stsp induces PKC activation are unclear. Stsp inhibits a wide range of serine/threonine and tyrosine specific kinases (Ruegg and Burgess, 1989). The cumulative effects of stsp on many kinases within one cell type may result in paradoxical increases in protein phosphorylation and activation of signaling pathways. In PC12 cells, stsp induces differentiation which correlates with the phosphorylation of a 145-kDa protein on tyrosine residues (Rasouly and Lazarovici, 1994), and stsp activates serine/threonine kinases from human platelets (Kocher and Clemetson, 1991). Alternatively, reduced phosphorylation of certain proteins in response to stsp may result in activation of the PKC signaling pathway. For example, in mouse keratinocytes expressing oncogenic *ras*, PKC δ is phosphorylated on tyrosine residues and this phosphorylated PKC isoform has a reduced capacity for activation. Unlike TPA, stsp can induce cornification in these cells while concomittantly inhibiting tyrosine phosphorylation of PKC δ (Denning *et al*, 1993), and thus may permit activation of PKC by this post-translational modification.

The redistribution of PKC from cytosol to the membrane or other intracellular sites is the corollary of enzyme activation (Kraft and Anderson, 1983). As described in other cell types (Wolf and Baggiolini, 1988; Kiley *et al*, 1992; Courage *et al*, 1995), stsp induced the translocation of PKC isoforms to new cellular locations

in cultured keratinocytes at concentrations at which differentiation-specific gene expression was induced. PKC α and to a lesser extent PKC δ redistributed to the membrane fraction, bringing enzymes in close proximity with membrane components which can increase PKC activity (Nishizuka, 1992). In human neutrophils (Nigam *et al*, 1992), diacylglycerol levels were raised in response to stsp and in platelets stsp enhanced the binding of phorbol-12,13-dibutyrate to its receptor by increasing binding affinity and capacity in concern with increased membrane localization (Rais *et al*, 1994). These phenomena may also occur in keratinocytes after stsp treatment with enhanced binding of the endogenous activator diacylglycerol, which binds to the same site on PKC as phorbol-12,13-dibutyrate (Nishizuka, 1992). Translocation to other cellular locations may also be important, such as to the cytoskeleton **(Fig 7)** or the nucleus, demonstrated for PKC ϵ in A549 cells by stsp at low nM concentrations (Courage *et al*, 1995). Translocation to new cellular sites is achieved by the interaction of PKC isoforms with binding proteins such as RACKS (Ron *et al*, 1994), and it is feasible that this protein-protein interaction alone may be sufficient to initiate certain signaling events of PKC. Stsp is a selective PKC inhibitor in that it does not inhibit PKC ζ and is 5.6 and 2.8 times less effective for inhibition of PKC δ and ϵ, respectively, than for PKC α (Seynaeve *et al*, 1994), which will affect the potential of each isozyme for activation at any particular stsp concentration.

The results in **Fig 8** suggest a necessity of PKC α, ϵ, and/or δ for expression of SPR-1, filaggrin, and loricrin in response to stsp. However, it is difficult to assign definitively a particular PKC isoform to expression of markers, as stsp clearly has multiple effects on all isoforms, demonstrated by its effects on isozyme translocation and downregulation, which may work in concert with one another. Indeed, dose-response studies for induction of transcripts for differentiation markers **(Fig 3)** also suggest that stsp could have multiple targets. Future studies using an anti-sense approach to eliminate selectively individual PKC isoforms may give further insight into the role of specific PKC isoforms in stsp-induced marker expression.

In conclusion, our results demonstrate that stsp induces coordinate changes in expression of multiple differentiation-specific genes in mouse keratinocytes *in vitro*, emulating the differentiation program of keratinocytes in the epidermis. Evidence presented here suggests that stsp induces marker expression, at least in part, through PKC activation independently of changes in the level of extracellular Ca^{2+}, but that calcium is necessary for augmentation of the final stages of the keratinocyte differentiation program during cornified envelope assembly. As stsp is effective in causing tumor regression in a mouse experimental model (Strickland *et al*, 1993), this drug could be a prototype for the treatment of cutaneous diseases where aberrant differentiation is involved in pathogenesis.

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