Putative Conventional Protein Kinase C Inhibitor Gödecke 6976 [12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole] Stimulates Transglutaminase Activity in Primary Mouse Epidermal Keratinocytes

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ABSTRACT
Much data in the literature suggest a role for protein kinase C (PKC) in regulating keratinocyte proliferation and differentiation. Nevertheless, the exact role of this family of isoenzymes is unclear, since PKC agonists (e.g., phorbol esters) are known to stimulate expression of both proliferative and differentiative markers in keratinocytes. Similarly, PKC inhibitors have been demonstrated both to inhibit [2-[1-(3-aminopropyl)indol-3-yl]-3(1-methyl-1H-indol-3-yl)maleimide, acetate (Ro 31-7549) and 3-[1-[3-(aminothio)propyl]-1H-indol-3-1-(1-methyl-1H-indol-3yl) maleimide (Ro 31-8220)] and to induce (staurosporine) keratinocyte differentiation. In this study, we examined the role of the PKC inhibitor, Gödecke 6976 (Gö 6976) [12-(2-cyanoethy)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole], on keratinocyte proliferation, as measured by DNA synthesis, and differentiation, as monitored by transglutaminase activity. This compound is reported to be selective for the conventional PKC isoforms, of which keratinocytes express only PKCα, and for protein kinase D (PKD; also known as PKCμ). We report that Gö 6976 stimulated transglutaminase activity. Consistent with this effect, Gö 6976 also potently inhibited [3H]thymidine incorporation (a half-maximal inhibitory concentration of ~0.1 μM). In addition, Gö 6976 (1 μM) was able to enhance the stimulation of transglutaminase activity by 1,25-dihydroxyvitamin D3 but had no effect on D3-induced expression of keratin-1. Conversely, Gö 6983 [2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide], a similar compound that also selectively inhibits conventional PKCα, but not PKD, had little or no effect on DNA synthesis or transglutaminase activity (up to 1 μM). The effect of Gö 6976 was not due to cytotoxicity as its effect on thymidine incorporation was largely reversible, and its stimulation of transglutaminase activity could be inhibited by another general PKC inhibitor, bisindolylmaleimide I. Therefore, our results suggest a proproliferative, antendifferentiative role for PKD in epidermal maturation.

The epidermis is composed primarily of epidermal keratinocytes, which continuously proliferate and differentiate to maintain this important tissue. Keratinocyte differentiation is characterized by a spatially and temporally regulated program of gene and protein expression, which ultimately results in terminal differentiation and cell death. This program of differentiation is essential for the function of the epidermis as a barrier to water loss, microbial invasion, and mechanical stress. Despite the importance of keratinocyte differentiation to epidermal structure, the signaling pathways that regulate this process are not well understood. Numerous data in the literature indicate a role for PKC in keratinocyte differentiation; however, the exact role of this enzyme is at present unclear (reviewed in Bollag and Bollag, 2001). Thus, PKC-activating phorbol esters elicit events associated paradoxically both with differentiation and proliferation, and a purported PKC inhibitor, staurosporine (Stsp), has been shown to act seemingly as a PKC agonist in keratinocytes (Dlugosz and Yuspa, 1991; Stanwell et al., 1996). Similarly, both keratinocyte mitogens and keratinocyte differentiative agents increase phosphoinositide hydrolysis and, presumably, increase PKC activity. We, therefore, examined the effect of Gö 6976, a selective conventional PKC inhibitor, on keratinocyte differentiation.

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ABBREVIATIONS: PKC, protein kinase C; Stsp, staurosporine; PKD, protein kinase D; D3, 1,25-dihydroxyvitamin D3; Bis, bisindolylmaleimide I; SFKM, serum-free keratinocyte media; PBS, phosphate-buffered saline lacking divalent cations; DMSO, dimethyl sulfoxide; TRK A, tyrosine kinase A; NGF, nerve growth factor; Ro 31-8220, 3-[1-[3-(aminothio)propyl]-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide; Gö 69676, 12-(2-cyanoethy)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; Bis, bisindolylmaleimide.
activate PKC (reviewed in Bikle and Pillai, 1993; Bollag and Bollag, 2001). On the other hand, both adenovirus-mediated transfection studies (Ohba et al., 1998) and antisense ablation experiments (Lee et al., 1997) indicate a prodifferentiative role for PKC.

In part, the discrepancy may lie in the fact that PKC is a family of isoenzymes. This family can be divided into three types: the conventional type, the activity of which is regulated by calcium and diacylglycerol (PKC-α, -β, and -γ); the novel, calcium-insensitive isoenzymes (PKC-δ, -ε, -η, and -θ), which are activated by diacylglycerol; and the atypical isoforms (PKC-ζ and -σ(α)), which are insensitive to both calcium and diacylglycerol (Nishizuka, 1995). Thus, it is possible that the discrepant data may be the result of a differential involvement of PKC isoenzymes in various proliferative or differentiative events. On the other hand, other diacylglycerol/phorbol ester-responsive protein kinases exist that may have roles distinct from PKC in regulating keratinocyte proliferation/differentiation. One such protein kinase is protein kinase D (PKD; also known as PKCμ). PKD is a newly discovered enzyme that has both similarities to and profound differences from the PKC family of protein kinases (reviewed in Waldron et al., 1999). PKD is similar to classic and novel PKC isoenzymes in that it is activated by phorbol esters (Valverde et al., 1994) and shares some sequence homology with the novel PKC isoforms. However, PKD lacks a pseudosubstrate domain and possesses two motifs that are not found in other PKC isoenzymes: a possible hydrophobic transmembrane region and a pleckstrin homology domain (reviewed in Waldron et al., 1999). In addition, PKD does not appear to phosphorylate typical PKC substrates (e.g., histone or pseudosubstrate ϵ) and, in fact, the preferred PKD substrate in vitro assays is the calcium/calmodulin-dependent protein kinase substrate, syntide 2 (Van Lint et al., 1995). Like calcium/calmodulin-dependent protein kinase II (Soderling, 1996), PKD also demonstrates an autophosphorylation-mediated constitutive activity following its activation (Waldron et al., 1999). PKD activity increases with the addition of factors such as bombesin, vasopressin, and platelet-derived growth factor in Swiss 3T3 cells (Zugaza et al., 1997), and it has been shown that there is an increase in mitogenesis with PKD overexpression in fibroblasts (Rennecke et al., 1999; Zhukova et al., 2001). In addition, PKD levels are increased in cutaneous carcinomas and the proliferating compartment of mouse skin (Rennecke et al., 1999). Thus, we hypothesized that PKD might play an antiproliferative or prodifferentiative role in keratinocytes.

Go6976 (Calbiochem, San Diego, CA) inhibits conventional PKC isoforms, of which keratinocytes are reported to express only PKCα (Dlugosz et al., 1992), as well as PKD (Rennecke et al., 1996). We report that Go6976 stimulated 1,25-dihydroxyvitamin D₃ (D₃)-induced transglutaminase activity, a marker of late keratinocyte differentiation, without affecting the expression of keratin-1, a marker of early keratinocyte differentiation. Go6976 itself also induced transglutaminase activity in a dose-dependent manner and inhibited DNA synthesis, as measured by [³H]thymidine incorporation into DNA. The fact that Go6976 inhibition of DNA synthesis could be rescued by subsequent incubation with vehicle suggested that the action of the inhibitor was nontoxic. In addition, bisindolylmaleimide (BisI) inhibited the ability of Go6976 to stimulate transglutaminase activity, confirming the lack of toxicity. Other related compounds [Go6983 (Calbiochem), Ro 31-8220 (Calbiochem-Novabiochem, San Diego, CA), and BisI] had little or no effect alone on differentiation and inhibited D₃-induced differentiation, consistent with their ability to inhibit PKCα and their low potency toward PKD. Thus, the stimulation of differentiation and inhibition of proliferation by Go6976 suggest a proproliferative role for the Go6976-inhibited enzyme. The relative potencies of the tested compounds to inhibit PKD suggest that this enzyme is the proproliferative, antiproliferative protein kinase inhibited by Go6976 in keratinocytes.

Materials and Methods

Cell Culture. Primary mouse epidermal keratinocytes were prepared from 1- to 3-day-old neonatal ICR mice and were plated in six-well dishes in a medium consisting of modified Eagle’s medium containing 25 μM calcium, 2% dialyzed fetal bovine serum, 2 mM glutamine, 5 ng/ml epidermal growth factor, ITS⁺ (6.25 μg/ml insulin + 6.25 μg/ml transferrin + 6.25 ng/ml selenious acid + 5.35 μg/ml linoleic acid + 1.25% bovine serum albumin), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (Griner et al., 1999). After an overnight incubation, the cells were refed with serum-free keratinocyte medium (SFKM), in which 2% dialyzed fetal bovine serum was replaced with 90 μg/ml bovine pituitary extract. Cells were refed with fresh medium every 1 to 3 days and treated at near (75–90%) confluence.

DNA Synthesis. Keratinocytes were incubated for the indicated times in SFKM containing the appropriate agents. Subsequently, [³H]thymidine (1 μCi/ml final concentration) was added, and the cells were incubated an additional 60 min. The radioactivity in 5% trichloroacetic acid-precipitable DNA was then quantified by liquid scintillation spectroscopy, following washing and solubilization in 0.3 M NaOH as in Jung et al. (1999).

Transglutaminase Activity Assay. Keratinocytes were incubated for 24 h in SFKM containing the appropriate agents. After the cells were scraped into homogenization buffer (0.1 M Tris-acetate, pH 7.8, 2 μg/ml aprotinin, 2 μM leupeptin, 1 μM pepstatin A, 0.2 mM EDTA, and 20 μM 4-2-aminoethyl)benzenesulfonyl fluoride) and subjected to one freeze-thaw cycle, transglutaminase activity was determined by monitoring the incorporation of [³H]putrescine into casein as described in Jung et al. (1999).

Western Blot Analysis. Keratinocytes were incubated for 24 h in SFKM containing the appropriate agents. After the cells were scraped into lysis buffer (0.1875 M Tris-HCl, pH 8.5, 3% SDS, and 1.5% EGTA), protein concentration was analyzed by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. Loading buffer (final concentration of 15% glycerol, 7.5% β-mercaptoethanol, and 0.5% bromphenol blue) was added to the remaining sample, which was heated to 100°C for 8 min. Twenty to 25 μg of protein were separated on an 8% polyacrylamide gel and transferred to 0.45-μm Immobilon-P nitrocellulose membrane (Millipore Corp., Bedford, MA). Membranes were incubated for 1 h with 10% blocking solution [10% casein and 10% Tween 20 in phosphate-buffered saline lacking divalent cations (PBS⁻) for 1 h with rabbit anti-mouse keratin-1 (1:500) in blocking solution and for 30 min with 125I-labeled goat anti-rabbit secondary antibody (1:500) in blocking solution. Membranes were washed with 1% blocking solution (1% casein and 1% Tween 20 in PBS⁻) several times between incubations. Immunoreactive proteins were visualized using a Molecular Dynamics PhosphorImager system (Sunnyvale, CA).

Statistical Analysis. The significance of differences between mean values was determined using analysis of variance with a Student-Newman-Keuls post hoc test, as performed by the program Instat (GraphPad Software, San Diego, CA).

Results

Go6976 Enhances D₃-Stimulated Transglutaminase Activity. To determine the potential roles of the PKC/PKD pathway in keratinocyte differentiation, we determined the
effect of Go6976 on D₃-induced late differentiation, as measured by transglutaminase activity. Near-confluent mouse keratinocyte cultures were treated for 24 h in SFKM containing vehicle (0.05% ethanol and 0.1% DMSO; control), 1 µM Go6976, 250 nM D₃, or the combination of Go6976 and D₃. Cells were then scraped from the plate, and transglutaminase activity was monitored as [³H]putrescine incorporation into casein, as described under Materials and Methods. As previously shown (Griner et al., 1999), D₃ induced a significant (approximately 2-fold) increase in transglutaminase activity (Fig. 1). However, unexpectedly, we also observed a 2-fold increase in activity with Go6976 alone, and a 5-fold increase in transglutaminase activity in the combination of Go6976 and D₃.

**Induction of Transglutaminase Activity.** To determine the specificity of the induction of differentiation by Go6976, we examined the effect of other protein kinase inhibitors on transglutaminase activity. Keratinocytes were incubated for 24 h in SFKM containing vehicle (0.1% DMSO; control) or 1 µM Go6976, 250 nM D₃, or BisI, and transglutaminase activity was monitored as described above. We observed that only Go6976 enhanced transglutaminase activity (Fig. 2A). 2-fold over control, whereas Go6976 and BisI had no effect on transglutaminase activity. On the other hand, K252a (Nocardiosis; Calbiochem-Novabiochem), a relatively nonselective kinase inhibitor, also stimulated transglutaminase activity (Fig. 2B).

In addition, we performed studies to determine the dose dependence of the Go6976-induced transglutaminase activity. We observed a 2-fold increase in transglutaminase activity between 0.1 and 2 µM Go6976 concentration with a half-maximal dose of about 0.1 µM and a maximal response at 0.5 to 1 µM (Fig. 3).

**Specificity of Go6976.** Because Go6976 is a closely related analog of Go6976, with similar potencies against most of the inhibited conventional PKC isozymes, but not PKD (Gschwendt et al., 1996), we tested whether Go6976 exhibited the same enhancing effect as Go6976 on D₃-stimulated differentiation. Keratinocytes were incubated for 24 h in SFKM containing vehicle (0.05% ethanol and 0.1% DMSO; control), 1 µM Go6976, 250 nM D₃, or the combination of Go6976 and D₃, and again, transglutaminase activity was monitored as described above. D₃ stimulated a 2-fold increase in transglutaminase activity, whereas Go6976 inhibited transglutaminase activity slightly but not significantly (Fig. 4A). However, Go6976 inhibited D₃-induced transglutaminase activity because the combination of the two compounds showed a 50% decrease relative to D₃ alone and returned the activity to a value not significantly different from the control level. Similarly, another general PKC inhibitor, Ro 31-8220, also had no significant effect on transglutaminase activity itself but reduced D₃-stimulated activity (Fig. 4B).

**Inhibition of DNA Synthesis by PKC/PKD Inhibitors.** To determine whether Go6976 or Go6976 had an effect on keratinocyte proliferation, we tested the ability of these compounds to inhibit DNA synthesis. Keratinocytes were incubated with various concentrations of the two agents for 24 h, and DNA synthesis was monitored as [³H]thymidine incorporation into DNA. Go6976 exhibited an IC₅₀ of approximately 0.1 µM and a maximal inhibition at 0.5 µM, whereas Go6983 had no significant effect on DNA synthesis (Fig. 5).

**Induction of Keratin-1 Expression.** To test the effect of Go6976 on D₃-induced early keratinocyte differentiation, we incubated keratinocytes with Go6976, D₃, or Go6976 and D₃, and then measured the protein levels of keratin-1, a marker of early differentiation (Dlugosz and Yuspa, 1993), via Western blot analysis. We noted a significant 40% increase in keratin-1 expression with D₃ treatment, which was not affected by Go6976 (Fig. 6). Go6976 alone also had no effect on keratin-1 expression.

**Reversibility of Go6976.** To determine whether the effect of Go6976 could be related to nonspecific toxicity, we tested the reversibility of the compound in terms of its ability to inhibit DNA synthesis. Keratinocytes were incubated with either vehicle (0.1% DMSO) or 1 µM Go6976 for 24 h. Cells were then washed twice with PBS and then with SFKM, and either vehicle or Go6976 was added for another 24 h of incubation. DNA synthesis was then monitored as described under Materials and Methods. Upon removal of Go6976 and a subsequent 24 h of incubation with control medium, DNA synthesis returned to within 30% of the control value (Fig. 7A). Continuous exposure to Go6976, on the other hand, inhibited DNA synthesis by 67% after a total of 48 h of treatment.

**Inhibition of Go6976-Stimulated Transglutaminase Activity.** The ability of Go6976, a PKC/PKD inhibitor, to act as an apparent PKC agonist to stimulate keratinocyte differentiation is reminiscent of the effects of the PKC inhibitor Stsp (Dlugosz and Yuspa, 1993). Stsp-induced differentiation can be blocked by PKC inhibitors, such as bryostatin I and BisI (Stanwell et al., 1996). Therefore, we investigated the capacity of BisI to block Go6976-elicited transglutaminase activity. We incubated keratinocytes with SFKM containing vehicle (0.2% DMSO; control), 1 µM Go6976, BisI, or Go6976 and BisI, and transglutaminase activity was monitored as described under Materials and Methods. Again, Go6976 stimulated a 2-fold increase in transglutaminase activity. BisI alone inhibited slightly (25%) but not significantly (Fig. 7B). However, BisI significantly inhibited Go6976-induced transglutaminase activity by over 50%.
Effect of Nerve Growth Factor on G6976-Induced Stimulated Keratinocytes. G6976 has been reported to inhibit Trk A (IC$_{50}$ = 10 nM) (Behrens et al., 1999), a nerve growth factor (NGF) receptor recently suggested to mediate human keratinocyte proliferation (Di Marco et al., 1993; Pincelli et al., 1997; Pincelli and Yaar, 1997; Pincelli, 2000). We addressed whether the effect of G6976 on keratinocyte differentiation could be due to inhibition of antiderivative, proproliferative nerve growth factor receptor signaling by testing the effect of NGF on G6976-altered transglutaminase activity and DNA synthesis. We observed no effect of NGF on G6976-stimulated transglutaminase activity or on G6976-inhibited DNA synthesis in these cells (Table 1). We also examined the effect of an NGF-neutralizing antibody on [3H]thymidine incorporation in the mouse keratinocytes. Using a 1:10,000 and 1:1,000 dilution of the antibody reported to neutralize NGF activity, we saw no effect on [3H]thymidine incorporation (1.04 ± 0.04- and 1.07 ± 0.20-fold over control, respectively, n = 3).

Discussion

Data in the literature (reviewed in Bikle and Pillai, 1993; Dlugosz and Yuspa, 1993; and Bollag and Bollag, 2001) suggest a role for several PKC isoforms in keratinocyte differentiation. In particular, PKC$\alpha$ has been implicated in the formation of adherens junctions between keratinocytes (Lewis et al., 1995), an early step in differentiation, as well as other steps in keratinocyte differentiation (Denning et al., 1995; Yang et al., 2000). We tested the ability of the classical PKC/PKD inhibitor G6976 to affect D$_3$-induced transglutaminase activity, a marker of late differentiation. G6976 is reported to be selective for conventional PKC isoforms (IC$_{50}$ = 2.3 nM for PKC$\alpha$) but does not inhibit novel PKC$\delta$, -$\epsilon$, and -$\zeta$ isoforms even at micromolar levels (Martiny-Baron et al., 1993). Surprisingly, the G6976-induced stimulation of transglutaminase mirrored that seen with D$_3$. In fact, G6976 enhanced D$_3$-stimulated transglutaminase activity to a value approximately 5-fold over control. This suggested to us one of two interpretations. The first is that G6976 is in fact a PKC agonist, as has been reported for staurosporine, a G6976 analog that both inhibits proliferation and stimulates differentiation (Stanwell et al., 1996). Stsp was suggested to act as a PKC agonist since treatment with this compound elicits the translocation of PKC isoforms to the plasma membrane, a process thought to reflect activation of these enzymes (Stanwell et al., 1996). However, Stsp acts by interfering with the binding of
ATP to the active site of PKC (Martiny-Baron et al., 1993), raising the question as to the activity of the Stsp-translocated PKC isoforms. Nevertheless, BisI can inhibit Stsp-induced keratinocyte differentiation (Stanwell et al., 1996). Thus, the data support a role for PKC in Stsp-induced keratinocyte differentiation; however, it is not clear whether this is a direct effect of Stsp to stimulate enzyme activity or whether this occurs downstream of other effects of Stsp. Indeed, the relatively nonspecific

Fig. 4. A, Go6983 inhibits 1,25-dihydroxyvitamin D₃-induced transglutaminase activity. Keratinocytes were incubated for 24 h in SFKM containing vehicle [0.05% ethanol and 0.1% DMSO; control (Con)], 1 μM Go6983, 250 nM D₃, or the combination of Go6983 and D₃. Cells were then scraped from the plate, and transglutaminase activity was monitored as above. Values represent the means of five separate experiments performed in triplicate; *, p < 0.05 versus control. B, Ro 31-8220 inhibits 1,25-dihydroxyvitamin D₃-induced transglutaminase activity. Keratinocytes were incubated for 24 h in SFKM containing vehicle (0.05% ethanol and 0.1% DMSO; Con), 1 μM Ro 31-8220, 250 nM D₃, or the combination of Ro 31-8220 and D₃. Cells were then scraped from the plate, and transglutaminase activity was monitored as above. Values represent the means of five separate experiments performed in triplicate; *, p < 0.05 versus the control value.

Fig. 5. Go6976, but not Go6983, inhibits DNA synthesis. Keratinocytes were incubated with various concentrations of the two agents for 24 h, and DNA synthesis was monitored as [³H]thymidine incorporation into DNA as described under Materials and Methods. Values represent the means from three to six separate experiments performed in triplicate; *, p < 0.01 versus control value.

Fig. 6. Go6976 does not induce keratin-1 expression or inhibit D₃-induced K-1 expression. Keratinocytes were incubated for 24 h in SFKM containing vehicle [0.05% ethanol and 0.1% DMSO; control (Con)], 1 μM Go6976, 10 nM D₃, or the combination of Go6976 and D₃. Cells were then scraped from the plate, and keratin-1 induction was monitored by Western blot as described under Materials and Methods. Values represent the means of four separate experiments performed in duplicate; *, p < 0.001 versus the control value.

Stsp also inhibits the tyrosine phosphorylation of PKCζ that accompanies differentiation (Denning et al., 1993), as well as tyrosine kinases in general (Ruegg and Burgess, 1989).
BisI, and transglutaminase activity was monitored as described under Materials and Methods. Values represent the means of five separate experiments performed in triplicate; * p < 0.01 versus the control value; † p < 0.05 versus G6976/Con washout. B, BisI inhibits G6976-stimulated transglutaminase activity. Keratinocytes were incubated with either SFKM containing vehicle [0.05% ethanol and 0.2% DMSO; control (Con)], 1 μM G6976, BisI, or G6976 and BisI, and transglutaminase activity was monitored as described under Materials and Methods. Values represent the means of five separate experiments performed in triplicate; * p < 0.01 versus control; † p < 0.05 versus G6976 alone.

**TABLE 1**

NGF has no effect on G6976-induced stimulation of transglutaminase activity or inhibition of [3H]thymidine incorporation. Near confluent primary keratinocytes were treated for 24 h with either medium alone or 1 μM G6976, 1 μM Ro 31-8220, or BisI. Values represent the means of five separate experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC₅₀ vs. PKD</th>
<th>Effect on TGase Activity</th>
<th>Interaction with 1,25-Dihydroxyvitamin D₃</th>
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<tbody>
<tr>
<td>K252a</td>
<td>0.007a</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>G6976</td>
<td>0.02a</td>
<td>+</td>
<td>Enhancement</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>0.04a</td>
<td>+</td>
<td>N.D.</td>
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<tr>
<td>Bisindolylmaleimide I</td>
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<td>None</td>
<td>Inhibition</td>
</tr>
<tr>
<td>G6983</td>
<td>20a</td>
<td>None</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Ro 31-8220</td>
<td>&gt;1b</td>
<td>None</td>
<td>Inhibition</td>
</tr>
</tbody>
</table>

N.D., not determined; TGase, transglutaminase.

* From Gschwendt et al. (1996).

b Concentrations of 0.1 μM (K252a and staurosporine) or 1 μM (all others) were used.

c From Jung et al. (1999).

d From Zugaza et al. (1997).

The second interpretation of our data is that G6976 may inhibit another enzyme whose role is to prevent differentiation. Both G6976 and Stsp inhibit PKD, also known as PKCμ, with a high potency (IC₅₀ = 20 and 40 nM, respectively) (Gschwendt et al., 1996) (Table 2). The interpretation that proproliferative PKD might be involved in the differentiative response to G6976 was suggested by the lack of effect of G6983, BisI, and Ro 31-8220 on transglutaminase activity. G6983 and BisI inhibit PKD only at high concentrations (Gschwendt et al., 1996) (Table 2), but inhibit PKCα potently (IC₅₀ = 7 nM and 10 nM, respectively). G6976 also does not inhibit PKD in vitro at a dose of 1 μM (Zugaza et al., 1997). In addition to an inability to stimulate transglutaminase activity alone, G6983 and Ro 31-8220 also inhibited D₃-induced differentiation (Fig. 7B), supporting our hypothesis that G6976 enhances differentiation through its ability to inhibit PKD rather than a conventional PKC (see Table 2). Thus, the generic PKC inhibitor BisI likely antagonizes G6976-induced differentiation through inhibition of PKC isoenzymes that are required downstream of PKD for the expression of late differentiative markers, such as transglutaminase. The ability of G6976 to elicit transglutaminase activity, a marker of late differenti-
ation, implies that PKD may function as an antidermativative agent. However, G6976 had no effect on the expression of an early keratinocyte differentiation marker, keratin-1, either basally or stimulated by D₂ (Fig. 6). Although this result might argue against a possible antidermativative action of G6976-inhibited PKD, PKCα is known to induce differentiation (Denning et al., 1995; Ohba et al., 1998). Thus, the inability of G6976 to alter keratin-1 levels may be related to its dual capacity to inhibit prodermativitative PKCα and antidermativitative PKD. In addition, we showed that the action of G6976 was not an artifact due to nonspecific toxicity, in that we were able to inhibit its effects using Biased (Fig. 7B).

Because G6976 has been reported to inhibit Trk A, a nerve growth factor receptor recently implicated in human keratinocyte proliferation (Di Marco et al., 1993; Pincelli et al., 1997; Pincelli and Yaar, 1997; Pincelli, 2000), we tested whether the prodermativitative effect of G6976 was mediated through inhibition of signaling through this receptor. We observed no inhibition of [3H]thymidine incorporation in cells incubated with an NGF-blocking antibody or any effect of NGF on G6976-stimulated transglutaminase activity or on G6976-inhibited DNA synthesis in these cells. In contrast, Di Marco et al. (1993) observed a dose-dependent inhibition of [3H]thymidine incorporation into primary human keratinocytes treated with an antibody to NGF. Taken together, these observations suggest that the effect of NGF on keratinocyte proliferation may be species-specific. Furthermore, our results suggest that G6976 does not stimulate transglutaminase activity through inhibitory effects on Trk A in mouse keratinocytes.

Our results implicate a protein kinase with a proproliferative, antidermativitative role. There is evidence in the literature that the identity of this enzyme is PKD. Thus, the similarity in action between two purported PKC inhibitors, Stsp and G6976, may relate to their ability to potently inhibit PKD. We have shown that another potent inhibitor of PKD, K252a, also stimulates transglutaminase activity (Fig. 2B). These effects appear to be specific in that the related compound, G6983, as well as other PKC inhibitors, neither functions to increase differentiation markers nor inhibits PKD. In addition, PKD, activated in response to mitogenic peptides and growth factors (Zugaza et al., 1997; Zhukova et al., 2001), has also been shown to activate the mitogen-activated protein kinase (p42) pathway (Hausser et al., 2001). Therefore, we speculate that PKD functions as an antidermativitative, proproliferative enzyme in keratinocytes, such that the inhibition of this enzyme results in rapid progression of keratinocytes to late differentiation.

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References


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