Effects of the Selective Protein Kinase C Inhibitor, Ro 31-7549, on the Proliferation of Cultured Mouse Epidermal Keratinocytes

Wendy B. Bollag, Janet Ducote, and Charles S. Harmon
Preclinical Dermatology Research, Hoffmann-La Roche, Nutley, New Jersey U.S.A.

We have investigated the effects of Ro 31-7549, a selective protein kinase C (PKC) inhibitor, on DNA synthesis and proliferation in two primary mouse epidermal keratinocyte culture systems. In differentiating keratinocytes incubated in medium containing 10% serum and high calcium (approximately 0.5 mM), Ro 31-7549 blocked the inhibitory effect of the phorbol ester 12-0-tetradecanoyl-13-acetate (TPA) (a PKC activator) on keratinocyte DNA synthesis at 24 h [50% maximal response concentration (EC_{50}) = 1 μM], consistent with inhibition of PKC-mediated differentiation. Continuous treatment of the differentiative culture system with the PKC inhibitor resulted in a marked (fourfold) stimulation of [^{3}H]thymidine incorporation at day 7 of exposure, with an EC_{50} of 0.25 μM. The potencies of these effects of Ro 31-7549 are comparable to that reported for inhibition of TPA-induced platelet 47-kD protein phosphorylation [50% inhibitory concentration (IC_{50}) = 4.4 μM]. The time course of [^{3}H]thymidine incorporation indicated that Ro 31-7549 did not directly stimulate DNA synthesis but instead prevented the loss of proliferative capacity associated with continued culture in this medium. Maximal stimulation (2.6 times) of DNA synthesis was observed on day 4, whereas DNA synthesis at day 1 was unaffected. In a highly proliferative culture system using serum-free medium containing 25 μM calcium, TPA dose-dependently inhibited proliferation with an IC_{50} of approximately 0.3 nM. This antiproliferative effect of TPA was largely reversed by 0.1 μM Ro 31-7549. In the proliferative culture system, 0.75 μM Ro 31-7549 also essentially reversed the inhibition of proliferation caused by switching to high (1.0 mM) calcium. These results suggest that the loss of proliferative capacity in differentiating epidermal keratinocyte cultures may be mediated, at least in part, by PKC. *J Invest Dermatol 100:240–246, 1993*

Protein kinase C (PKC), the phospholipid-dependent, calcium-sensitive, diacylglycerol (DAG)–activated protein kinase described by Nishizuka (reviewed in [1]), is known to regulate proliferation and differentiation in a variety of cell types. There are numerous data to support such a role for PKC in the regulation of keratinocyte growth and differentiation. For example, PKC is a well-known cellular receptor for phorbol esters, which bind to and activate this enzyme [1]; in mouse skin *in vivo* phorbol esters are known tumor promoters [2,3]. In primary cultures of murine keratinocytes phorbol esters, such as 12-0-tetradecanoyl-phorbol-13-acetate (TPA), elicit divergent responses of both proliferation and differentiation. Thus, TPA exposure results in the induction of both ornithine decarboxylase (ODC) and transglutaminase (TGA), markers of keratinocyte proliferation and differentiation, respectively [4–6]. TPA also acutely (24 h) inhibits DNA synthesis [7], due to the recruitment of a subpopulation of cells to a differentiated state [5,8]; the differentiated cells detach from the plate within 48 h and those remaining are resistant to the prodifferentiative effects of high calcium or a second TPA treatment [5]. On the other hand, prolonged (2–3 d) TPA treatment results in a marked stimulation of keratinocyte DNA synthesis [7]. This biphasic response to TPA is also observed *in vivo* [2,3]. Thus, using the pharmacologic agent TPA as a PKC activator, several investigators have obtained evidence to indicate the possible involvement of PKC in the regulation of keratinocyte proliferation and differentiation.

Further complicating the interpretation of such TPA effects is the fact that the compound elicits two nearly concurrent events: PKC activation and PKC down-regulation [1]. Several investigators have reported that TPA treatment induces a rapid but incomplete PKC down-regulation in mouse keratinocytes [9,10], as in many other systems. The question remains, however, as to which TPA-elicited effects are mediated by PKC activation and which are the result of down-regulation. Nevertheless, comparisons of the kinetics of PKC down-regulation do not resolve this issue for several reasons, in-

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Wendy Bollag's current address: Department of Biology, Seton Hall University, South Orange, NJ 07079.
Reprint requests to: Dr. C.S. Harmon, Preclinical Dermatology Research, Building 86/716, Hoffmann-La Roche, Nutley, NJ 07110.

Abbreviations:
AMP: adenosine monophosphate
BSA: bovine serum albumin
DAG: diacylglycerol
DMSO: dimethylsulfoxide
EC_{50}: 50% maximal response concentration
FBS: fetal bovine serum
IC_{50}: 50% inhibitory concentration
ITS: insulin, transferrin, and selenious acid
ODC: ornithine decarboxylase
PBS: phosphate-buffered saline
PEN: phosphate, ethylenediaminetetraacetic acid, and sodium chloride
PKC: protein kinase C
SFKM: serum-free keratinocyte medium
TGA: transglutaminase
TPA: 12-0-tetradecanoyl-phorbol-13-acetate

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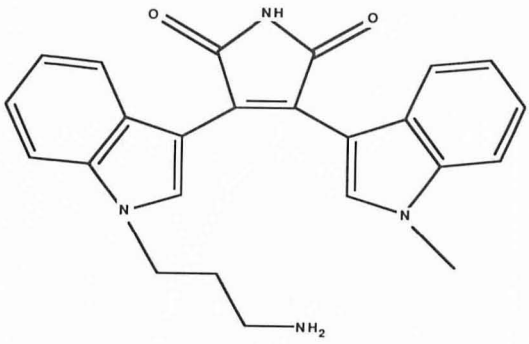

Figure 1. Structure of the bisindolylmaleimide PKC inhibitor Ro 31-7549.

cluding 1) the aforementioned ability of TPA to cause both activation and down-regulation of PKC; 2) the fact that there is a time delay of unknown duration between early signal-transduction events and such complex processes as DNA synthesis, proliferation, and differentiation; and 3) the apparent inability of TPA to induce complete down-regulation [9,10], because the percentage of PKC activity required for a cellular response is unknown. Due to these difficulties, it has become apparent that compounds that selectively inhibit PKC in intact cells are needed to determine the role of this enzyme in cellular processes [11].

Calcium is another keratinocyte regulator for which the role of PKC is unclear. Nevertheless, there is evidence to suggest that in this case also PKC activation and differentiation may be coupled: switching keratinocytes from low to physiologic extracellular calcium concentrations elicits both differentiation [12] and the formation of diacylglycerol (DAG) [13,14], the natural activator of PKC. Similarly, the addition of exogenous phospholipase C (PLC) to primary mouse epidermal keratinocytes results in the formation of DAG; like TPA, exogenous PLC also induces TGA and ODC [4]. In addition, synthetic DAGs have been reported to promote modest differentiative responses [15]. Furthermore, Isseroff *et al* [15] have observed effects of high calcium on the subcellular distribution of PKC. Finally, inhibition of PKC activity, either by TPA-induced enzyme down-regulation or with the use of putative enzyme inhibitors, has been shown to prevent the differentiative effect of high extracellular calcium concentrations [16]. On the other hand, these non-selective PKC inhibitors have also been reported both to mimic and prevent the effects of TPA, on for instance ODC and TGA induction [17–19]. Thus, although several lines of evidence indicate that PKC is involved in the regulation of keratinocyte proliferation and differentiation, the exact role of this enzyme is unclear. Again, the utility of a selective PKC inhibitor is obvious.

Recently, a series of bisindolylmaleimides has been shown to possess potent and selective inhibitory activity towards PKC in cell-free assays, with markedly reduced potencies towards cyclic adenosine monophosphate (AMP)–dependent and calcium/calmodulin-dependent protein kinases [20]. In addition, these PKC inhibitors inhibit in cellular assays both TPA-induced 47-kD protein phosphorylation in platelets and phorbol-ester–induced down-regulation of CD3 receptors in human T cells [20]. Furthermore, the bisindolylmaleimide PKC inhibitors have been used by several investigators to demonstrate the involvement of PKC in such cellular responses as cerebellar long-term depression [21], the neutrophil respiratory burst [22], fibroblast proliferation [11], and mast cell histamine release and phospholipase D activation [23]. In the present study we have used one such bisindolylmaleimide PKC inhibitor, Ro 31-7549 (Fig 1), to investigate the role of PKC in the regulation of keratinocyte proliferation.

MATERIALS AND METHODS

Cell Isolation and Culture Mouse epidermal keratinocytes were prepared according to the method of Marcelo *et al* [24] with minor modifications. Briefly, whole skin from 2-d–old neonatal CD-1 mice was incubated at 4°C in HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid]–buffered RPMI 1640 medium containing 0.25% trypsin overnight, followed by an approximate 45-min incubation at 37°C. After mechanical separation of the epidermis from the dermis with forceps, keratinocytes were detached and purified through a stepwise (12–20%) Ficoll gradient. Cells from the lower two layers were pooled, washed, and counted. For studies in a differentiative high-calcium, serum-containing medium, cells were plated in 96- or 6-well plates (Corning, Park Ridge, IL) at a density of 500,000 cells/cm^2 and cultured overnight in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone at 32°C. Cultures were then treated with medium containing the test compounds and were refed every 1–3 d.

For studies in proliferating cultures, cells were plated in six-well plates at a density of 25,000 cells/cm^2 in 10% fetal bovine serum–containing RPMI 1640. Cells were allowed to adhere for 4 h in serum-containing medium after which time the cultures were washed with phosphate-buffered saline lacking calcium and magnesium (PBS) and refed with a serum-free keratinocyte medium (SFKM) according to Yada *et al* [25] with minor modifications. SFKM consisted of calcium-free modified Eagles' medium (Specialty Media, Lafayette, NJ) containing 25 μM calcium, 5 ng/ml epidermal growth factor, 1 mM glutamine, 90 μg/ml bovine pituitary extract, ITS (5 μg/ml insulin + 5 μg/ml transferrin + 5 ng/ml selenious acid), 0.05% bovine serum albumin (BSA), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone. Cells were incubated overnight at 37°C in the low-calcium, serum-free medium and were subsequently treated with medium containing the test compounds; cells were refed every 1–3 d. In some experiments ITS + (6.25 μg/ml transferrin + 6.25 ng/ml selenious acid + 5.35 μg/ml linoleic acid + 0.125% BSA) was substituted for ITS and 0.05% BSA.

Measurement of DNA Synthesis For measurement of [^{3}H]thymidine incorporation, cells were incubated in differentiative 10% FBS-containing medium containing 5–10 μCi/ml [^{3}H]thymidine for the indicated time periods. After labeling, cultures were washed with phosphate-buffered saline and incubated with 1% trypsin at 37°C. Detached cells were collected onto filters using an automated cell harvester, washed, and counted in a liquid scintillation spectrometer. For determination of DNA spcific radioactivity, trypsinized cells were washed thoroughly with saline (0.9% NaCl + 50 mM phosphate, pH 7.4) to remove the enzyme and were lysed in PEN buffer (50 mM phosphate + 2 mM ethylenediaminetetraacetic acid + 2 M NaCl, pH 7.4) by sonication. Aliquots were taken for determination of radioactivity and measurement of DNA content using the fluorescent dye, Hoechst 33258, and calf thymus DNA (Clonetech Laboratories, Palo Alto, CA) as standard [26].

Determination of Cell Number Cells were washed with PBS and were incubated at 37°C with 0.5% trypsin to detach cells. Trypsin inhibitor was then added and the cells were triturated through an 18-gauge needle to generate a single-cell suspension. An aliquot of this suspension was added to 10 ml saline and cell number was determined using an electronic particle counter (Coulter Electronics, Hialeah, FL).

Materials TPA (CCR Acquisition Corporation, Edina, MN) was a generous gift of Dr. A. Wood. [^{3}H]Thymidine was purchased from ICN (Irvine, CA). Hoechst 33258 was obtained from Polysciences (Warrington, PA); Ficoll from Pharmacia (Piscataway, NJ); and bovine pituitary extract (P-Neurext) from Upstate Biotechnology (Lake Placid, NY). ITS and ITS+ were purchased from Collaborative Research (Bedford, MA); trypsin and epidermal growth factor from Gibco (Grand Island, NY); RPMI 1640, FBS, glutamine, HEPES, and antibiotics from Hazleton (Lenexa, KS); and trypsin inhibitor, dimethylsulfoxide and bovine serum albumin

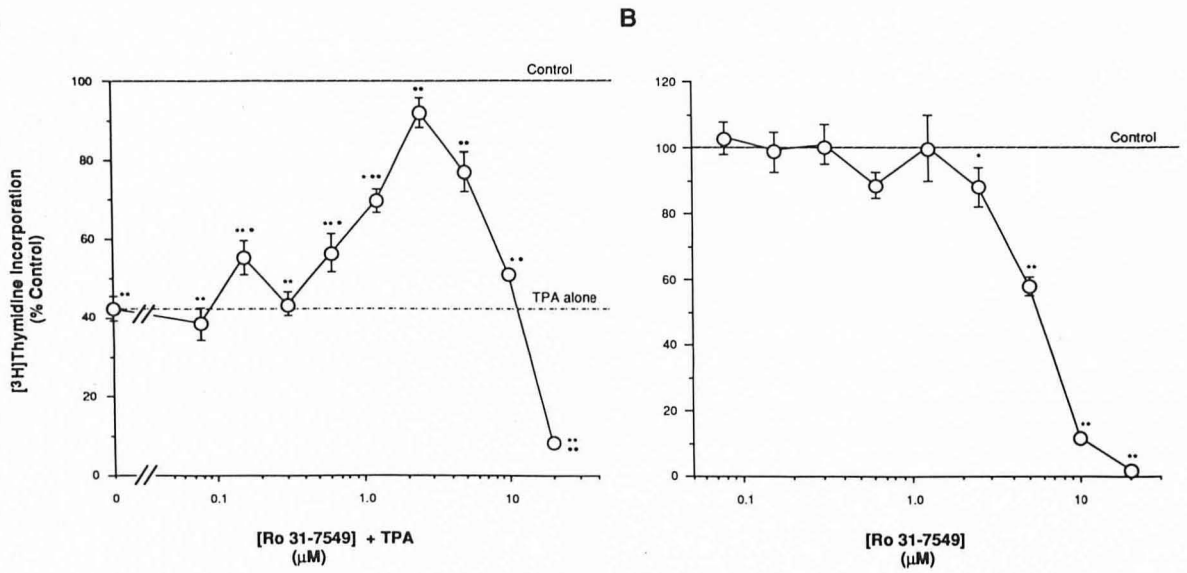

Figure 2. Ro 31-7549 reversed the inhibitory effect of an acute (24-h) exposure to TPA without itself affecting [^{3}H]thymidine incorporation into primary mouse epidermal keratinocytes. *A*) Cells in the differentiative high-calcium, serum-containing medium were treated for 30 min with various concentrations of Ro 31-7549 prior to exposure to 1 μM TPA for 24 h in the continued presence of the PKC inhibitor. [^{3}H]Thymidine incorporation was then determined after a 6-h labeling period. Data points are the means ± SEM (n = 4) of a representative experiment with control values (100 ± 10%) of 1236 ± 149 cpm/well (0.32 cm^2); *p ≤ 0.05, **p ≤ 0.005 versus control; •p ≤ 0.05, ••p ≤ 0.005 versus TPA alone. *B*) Cells in the differentiative high-calcium, serum-containing medium were treated for 24 h with various concentrations of Ro 31-7549 and [^{3}H]thymidine incorporation was determined after a 6-h labeling period. Data points are the means (n = 4) ± SEM of a representative experiment with control values (100 ± 4%, n = 8) of 2717 ± 110 cpm/well (0.32 cm^2); *p ≤ 0.05, **p ≤ 0.0005 versus control.

(fatty acid free) from Sigma (St. Louis, MO). All other materials were of reagent grade.

RESULTS

Ro 31-7549 has previously been demonstrated to potently inhibit PKC both in cell-free assays and in intact platelets and T cells *in situ* [20]. The ability of Ro 31-7549 to inhibit PKC activity in primary murine epidermal keratinocytes was investigated using the PKC activator TPA to initiate PKC-mediated cellular responses. Keratinocytes cultured in the differentiative high-calcium, serum-containing medium and pre-treated with various concentrations of Ro 31-7549, were exposed to 1 μM TPA for 24 h in the continued presence of the PKC inhibitor, and [^{3}H]thymidine incorporation was measured. As illustrated in Fig 2*A*, TPA inhibited DNA synthesis by approximately 60%. Ro 31-7549 dose-dependently blocked this inhibition with a 50% maximal response concentration (EC_{50}) of about 1 μM. At a concentration of 2.5 μM, Ro 31-7549 almost completely reversed the inhibitory effect of TPA on [^{3}H]thymidine incorporation (Fig 2*A*), indicating that Ro 31-7549 inhibits keratinocyte PKC. In Fig 2*B* is shown the effect of Ro 31-7549 alone on [^{3}H]thymidine incorporation. At concentrations of Ro 31-7549 less than or equal to 2.5 μM, the PKC inhibitor alone had little or no effect on DNA synthesis after a 24-h exposure. At higher concentrations (> 2.5 μM), Ro 31-7549 inhibited [^{3}H]thymidine incorporation, likely as a result of cytotoxicity because protein synthesis decreased concomitantly (data not shown). Taken together, the results shown in Fig 2*A,B* suggest that the reversal of the TPA inhibitory effect is the result of an inhibition of a PKC-mediated loss of proliferative capacity, rather than a direct enhancement of cell proliferation.

We then examined the effect of long-term exposure to various concentrations of Ro 31-7549 on cell growth. Primary keratinocytes cultured in the differentiative high-calcium, serum-containing medium were treated for 7 d with various concentrations of Ro 31-7549, and [^{3}H]thymidine incorporation was determined. At lower concentrations (≤5 μM) Ro 31-7549 alone dose-dependently stimulated [^{3}H]thymidine incorporation with an EC_{50} of approximately 0.25 μM (Fig 3). A maximal increase in radiolabel of fourfold over control values was observed at a dose of 2.5 μM Ro 31-7549; in separate experiments a mean EC_{50} of 0.6 μM and a mean maximal stimulation of 2.6 times were obtained. Doses greater than 5 μM again inhibited DNA synthesis, presumably due to cytotoxicity as measured by a concomitant fall in protein synthetic rates (data not shown).

The time course of the stimulatory effect of Ro 31-7549 on DNA synthesis was subsequently determined. Cells in the differentiative high-calcium, serum-containing medium were treated for various time periods with 2 μM Ro 31-7549 and DNA synthesis was measured. As shown in Fig 4, there was an inverse correlation between proliferative activity in control cultures (i.e., rates of [^{3}H]thymidine incorporation [Fig 4*A*] and DNA specific activity [Fig 4*B*]) and the extent of the stimulation of DNA synthesis elicited by Ro 31-7549.

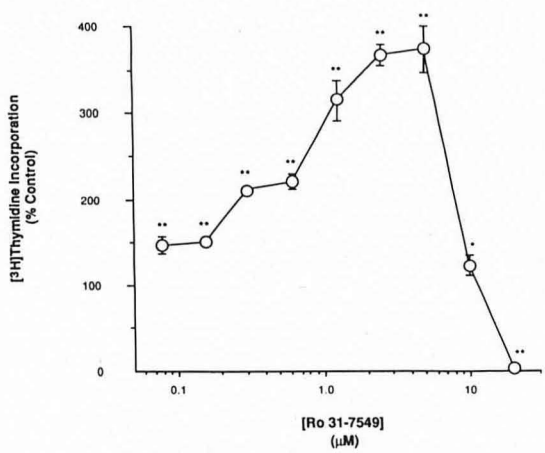

Figure 3. Ro 31-7549 stimulated [^{3}H]thymidine incorporation into primary mouse epidermal keratinocytes at day 7. Cells in the differentiative high-calcium, serum-containing medium were treated for 7 d with various concentrations of Ro 31-7549 and [^{3}H]thymidine incorporation was determined after a 19-h labeling period. Data points are the means (n = 4) ± SEM of a representative experiment with control values (100 ± 3%) of 29,238 ± 817 cpm/well (0.32 cm^2); *p ≤ 0.025, **p ≤ 0.0005 versus control.

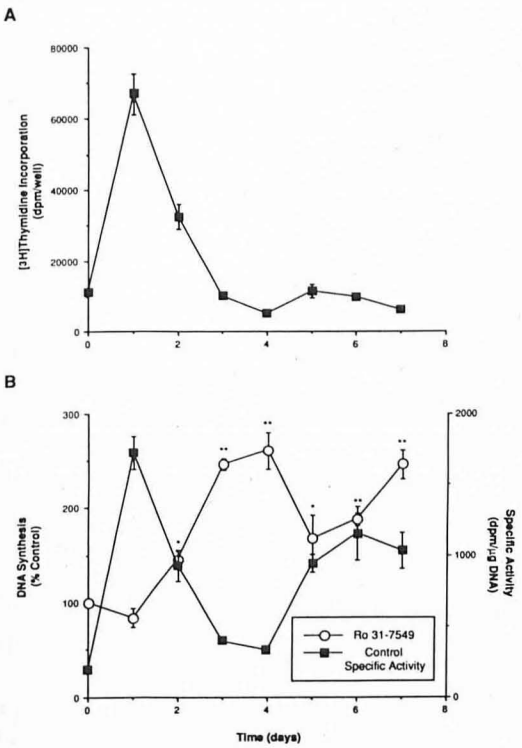

Figure 4. Ro 31-7549 maintained DNA synthesis in differentiating primary mouse epidermal keratinocytes. Cells in the differentiative high-calcium, serum-containing medium were treated for various time periods with 2 μM Ro 31-7549 and (*A*) [^{3}H]thymidine incorporation (dpm/well) and (*B*) DNA specific radioactivity (dpm/μg DNA) were determined after a 4-h labeling period. *Solid squares,* [^{3}H]thymidine incorporation (*A*) or DNA-specific activity (*B*) in the absence of Ro 31-7549; *open circles,* DNA-specific activity in the presence of 2 μM Ro 31-7549, expressed as percent of the control values. Data points are the means ($\pm$ SEM) of at least three samples from a single experiment; *p $\leq$ 0.05, **p $\leq$ 0.005 versus control.

Thus, during the period of relatively high proliferative activity following plating of the cells (days 1–2 of culture), Ro 31-7549 had little or no effect on DNA synthesis as measured by [^{3}H]thymidine incorporation. As proliferation declined over days 3–4 of culture, Ro 31-7549 progressively enhanced [^{3}H]thymidine incorporation/DNA synthesis. A similar, inverse relationship was observed during a second (smaller) peak of DNA synthesis at days 5–6 and the subsequent decline over day 7. A maximal, 2.6-times stimulation of DNA synthesis by Ro 31-7549 was obtained on day 4. A second experiment gave qualitatively similar results, although control [^{3}H]thymidine incorporation declined more slowly and no second peak of DNA synthesis was observed (data not shown). In this experiment also stimulation of [^{3}H]thymidine incorporation by Ro 31-7549 was minimal during the period of relatively high proliferative activity; however, as control [^{3}H]thymidine incorporation declined, the Ro 31-7549–induced enhancement of DNA synthesis progressively increased to reach a maximal stimulation of 2.4-times over control on day 7 (data not shown). Thus, the results of Figs 3 and 4 suggest that with continued culture in a differentiative high-calcium, serum-containing medium, there is a time-dependent PKC-mediated loss of proliferative capacity that is likely the result of PKC activity and can be prevented by Ro 31-7549.

Recently, a serum-free system for the culture of primary mouse epidermal keratinocytes has been developed (see *Materials and Methods*). This medium (SFKM) supports good cell growth such that keratinocytes can be plated at low densities and their proliferation monitored directly by cell counting as they approach confluence (after approximately a week, data not shown). We have used this culture system to examine the effects of TPA, a known antiproliferative agent, on keratinocyte growth as measured by changes in cell number. Keratinocytes cultured in SFKM were treated with various concentrations of TPA and cell number was determined initially and after 4 d of treatment. In the absence of TPA, cell number increased by 2.6-times from day zero (time of addition of TPA). TPA dose-dependently inhibited proliferation with a 50% inhibitory concentration (IC_{50}) of approximately 0.3 nM (Fig 5). Maximal inhibition of 74% was observed at a concentration of 10 nM. In a second experiment similar results were obtained although TPA exhibited a somewhat lower potency (IC_{50} of about 1.5 nM). Thus, TPA potently inhibits proliferation of primary keratinocytes cultured in this serum-free medium.

TPA also elicited a characteristic rapid change in keratinocyte morphology, from the appearance of control cells illustrated in Fig 6*A* to, after a 6-h treatment, the "dendritic" morphology shown in Fig 6*B*. This morphologic change occurred quite rapidly: initial effects were observed after as few as 3 h of exposure to TPA, but the response was enhanced with longer periods (up to 4 d) of exposure (data not shown). As shown in Fig 6*C*, 0.5 μM Ro 31-7549 abrogated this TPA-induced morphologic change. The ability of Ro 31-7549 to block this characteristic shape change was dose dependent in the range 0.1–1.0 μM (data not shown), and incubation with Ro 31-7549 alone had little or no effect on keratinocyte appearance (Fig 6*D*). These results provide additional evidence that Ro 31-7549 is able to inhibit keratinocyte PKC.

We next examined the ability of Ro 31-7549 to prevent the antiproliferative effect of TPA in keratinocytes grown in SFKM. Highly proliferative cells cultured in SFKM were switched to medium containing 10 nM TPA and various concentrations of Ro 31-7549, and cell number was determined after 4 d of treatment. As illustrated in Fig 7, 10 nM TPA reduced cell number by 45%, from a control value of 22,732 $\pm$ 768 cells/cm^2 (representing a 3.1-times increase in cell number from day 0) to 12,467 $\pm$ 185 cells/cm^2. This reduction in cell number was partially prevented by 0.1 μM Ro 31-7549: in the presence of TPA, 0.1 μM Ro 31-7549 returned cell number to 86% of the control value. In several additional experiments, an average TPA-elicited decrease of 41 $\pm$ 3% was returned to 87 $\pm$ 6% of the control cell number by concentrations of the PKC inhibitor ranging from 0.1 to 0.5 μM. Thus, Ro 31-7549 blocked, at least partially, the TPA-elicited inhibition of growth in this proliferative culture system.

Data from several laboratories suggest that PKC may also be involved in the antiproliferative effect of high calcium in primary epidermal keratinocytes [13–16, 27–30]. We therefore examined

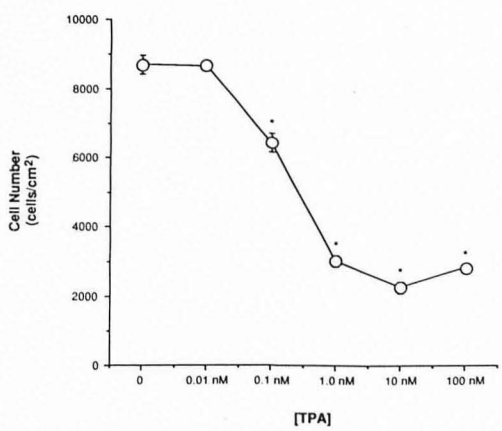

Figure 5. TPA dose-dependently inhibited proliferation of primary mouse epidermal keratinocytes. Highly proliferative cells cultured in the low-calcium, SFKM were switched to medium containing various concentrations of TPA and 0.1% dimethylsulfoxide. Cell number was determined after 4 d of treatment and represented a 2.6-times increase from day 0 ($p \leq 0.0005$ versus control cell number on day 4). Data points are the means (control, n = 5; TPA, n = 3) $\pm$ SEM of a representative experiment; *$p \leq 0.005$ versus control.

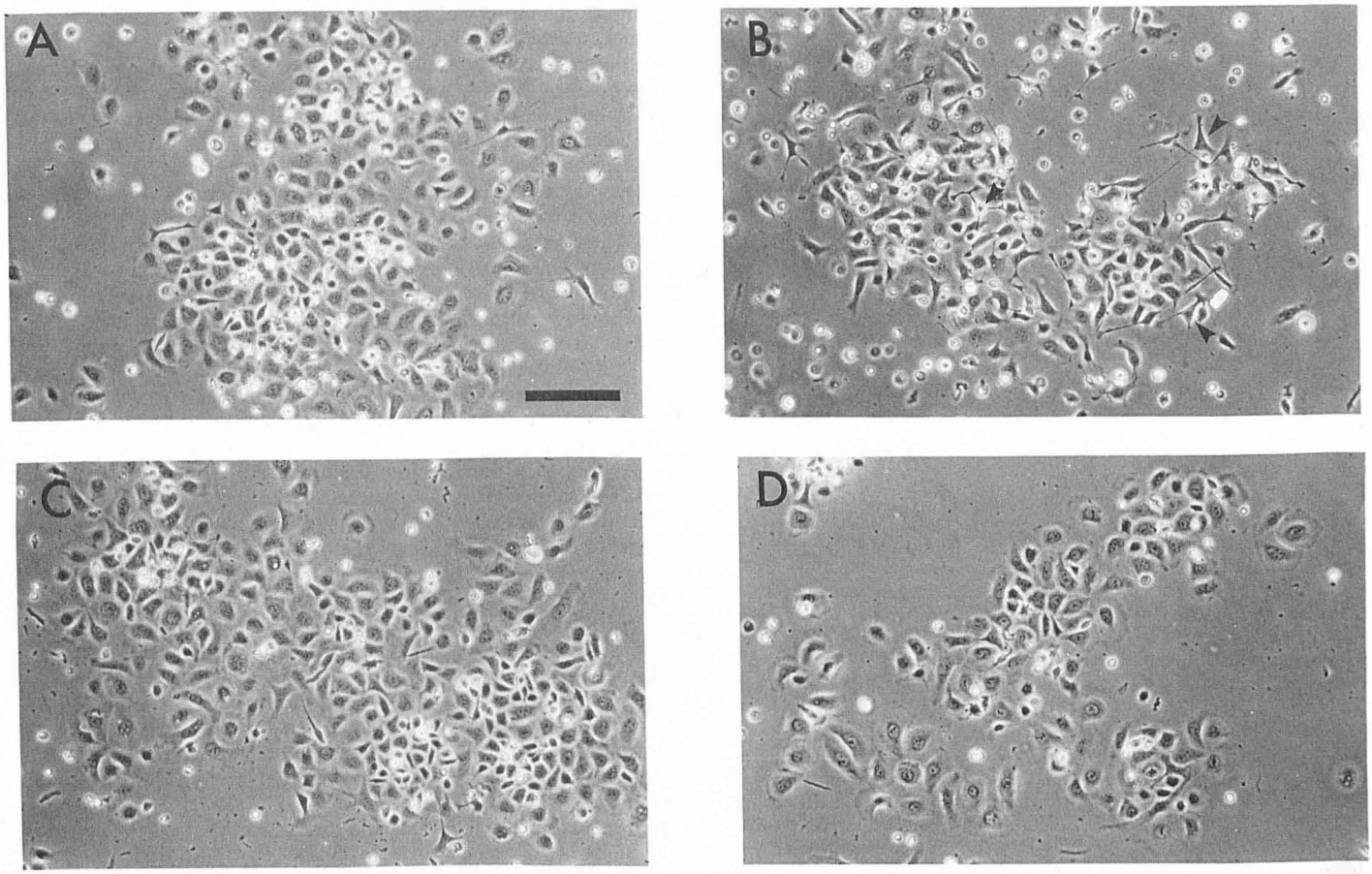

Figure 6. Ro 31-7549 prevents the characteristic "dendritic" morphologic changes elicited by TPA. Highly proliferative cells, incubated in SFKM in the absence *(A,D)* or presence *(B,C)* of 10 nM TPA with *(C,D)* or without *(A,B)* 0.5 μM Ro 31-7549 were photographed after 6-h exposure to the agents. All samples contained 0.1% dimethylsulfoxide. *Arrows,* representative TPA-elicited "dendritic" morphologic changes, which are inhibited by Ro 31-7549. *Bar,* 10 μm.

the ability of Ro 31-7549 to prevent the antiproliferative effect of high (1 mM) calcium. Highly proliferative cells cultured in SFKM were switched to medium containing 1.0 mM calcium and various concentrations of Ro 31-7549, and cell number was determined after 4 d of treatment. In low (25 μM) -calcium (control) and high (1 mM) -calcium cultures, the increase in cell number from day 0 was 3.7 times and 2.7 times, respectively. Thus, switching to high calcium resulted in an approximately 30% inhibition in culture growth over the 4-d period. Figure 8 shows that Ro 31-7549 dose-dependently blocked this growth-inhibitory effect of calcium such that at a concentration of 0.75 μM cell number was returned to control levels. This reversal occurred despite the fact that cell number in the presence of 0.75 μM Ro 31-7549 alone was somewhat reduced (79% of the control value, data not shown). This result suggests that calcium-induced growth inhibition in proliferating keratinocytes may be mediated, at least in part, by PKC.

DISCUSSION

Several investigators have attempted to determine the role of PKC in the regulation of keratinocyte growth and differentiation using reported PKC inhibitors, such as H-7 [1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine], sphingosine, staurosporine, and bryostatin 1, with occasionally unexpected results. Certain inhibitors block some but not all PKC-mediated responses, whereas others may only partially inhibit such responses [18]. Still others behave in keratinocytes as partial agonists/antagonists, preventing some and mimicking other PKC-mediated effects [19]. And finally some "PKC inhibitors" act in these cells as PKC agonists, eliciting in some cases a greater response than the PKC activator TPA [17]. Clearly, none of these PKC "inhibitors" is ideal for an investigation of the role of the enzyme in a cellular response; we, therefore, have used the selective PKC inhibitor Ro 31-7549 to study the possible involvement of PKC in the regulation of keratinocyte proliferation.

The ability of Ro 31-7549 to block the effect of TPA on keratinocyte [^{3}H]thymidine incorporation (Fig 2*A*), morphology (Fig 6), and proliferation (Fig 7) indicates that the compound is able to inhibit keratinocyte PKC. This result is not unexpected, because Ro 31-7837, in addition to inhibiting PKC potently and selectively in cell-free assays, has previously been shown to prevent TPA-induced 47-kD protein phosphorylation in intact platelets and CD3-receptor down-regulation in whole human T cells [20]. Furthermore, a close structural analog of Ro 31-7549, GF 109203X, has been demonstrated to inhibit several PKC-mediated responses [11]. Thus, these bisindolylmaleimides are able to potently inhibit PKC activity both in cellular and cell-free assay systems and to block responses to the phorbol-ester PKC activators. Furthermore, the failure of Ro 31-7549 alone to increase [^{3}H]thymidine incorporation (Fig 2*B*) or cell number (data not shown) suggests that the compound did not stimulate DNA synthesis or proliferation directly but rather reversed the TPA-induced inhibition. Thus, our results suggest that inhibition of keratinocyte PKC maintains cellular proliferation.

The interpretation that PKC plays a role in the loss of proliferative capacity of cultured keratinocytes is further supported by the time course of the Ro 31-7549–elicited increase in DNA synthesis (Fig 4). On those days when control cultures were rapidly synthesizing DNA, the PKC inhibitor induced no or only small increases in [^{3}H]thymidine incorporation and DNA-specific radioactivity, in comparison to the marked stimulatory effect of Ro 31-7549 on cells

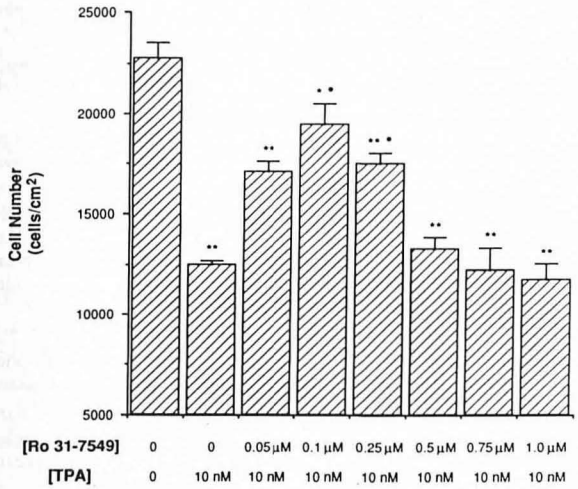

Figure 7. Ro 31-7549 partially reversed the TPA-induced inhibition of proliferation of primary mouse epidermal keratinocytes. Highly proliferative cells in SFKM were pretreated for 30 min with various concentrations of Ro 31-7549 prior to treatment with 10 nM TPA in the continued presence of the PKC inhibitor. Cell number was determined after 4 d of treatment and represented a 3.1-times increase from day 0 ($p \leq 0.0005$ versus control cell number on day 4). Data points are the means (control and TPA, $n = 6$; Ro 31-7549, $n = 3$) ± SEM of a representative experiment; *$p \leq 0.05$, **$p \leq 0.005$ versus control value; •$p \leq 0.05$ versus 10 nM TPA. Additional experiments in which TPA and Ro 31-7549 were added simultaneously gave similar results.

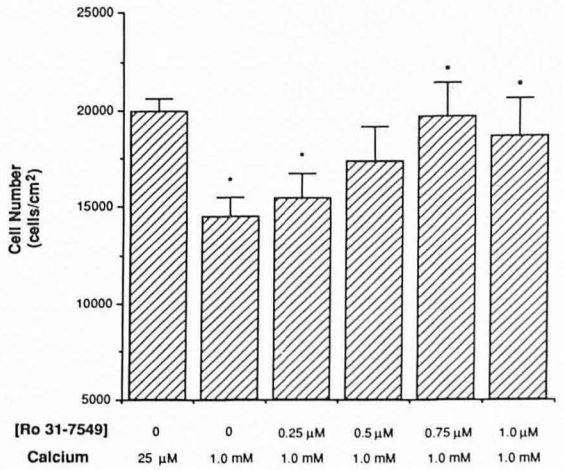

Figure 8. Ro 31-7549 reversed the calcium-induced inhibition of proliferation of primary mouse epidermal keratinocytes. Highly proliferative cells cultured in the low-calcium, serum-free medium were switched to medium containing 1.0 mM calcium and various concentrations of Ro 31-7549. Cell number was determined after 4 d of treatment and represented a 3.7-times increase from day 0 ($p \leq 0.0005$ versus control cell number on day 4). Data points represent the means ± SEM of at least six samples from two separate experiments; *$p \leq 0.005$ versus control value; •$p \leq 0.05$ versus 1.0 mM calcium.

that exhibited minimal DNA synthetic activity. Note that control cells demonstrated a time course of DNA synthesis similar to those reported previously in a nearly identical culture system [24,30].

Ro 31-7549 at several concentrations significantly stimulated [^{3}H]thymidine incorporation after 7 d of treatment in a differentative medium, with maximal stimulation seen at 2.5 μM and an EC_{50} of approximately 0.25 – 0.6 μM (Fig 3). This potency is greater than that obtained previously for the inhibition of TPA-induced 47-kD protein phosphorylation in platelets (IC_{50} of 4.4 μM) [20]. However, this value is consistent with IC_{50} values reported for inhibition by GF 109203X (the Ro 31-7549 analog) of PKC-mediated cellular responses (IC_{50}s of 0.2 to approximately 1.5 μM) [11]. Other bisindolylmaleimide PKC inhibitors with IC_{50}s in the range of 0.7 to 1.2 μM in the platelet 47-kD protein phosphorylation assay also stimulated [^{3}H]thymidine incorporation at day 7 with potencies of 0.6 to 1.5 μM.* Therefore, it is likely that the stimulatory activity of Ro 31-7549 on [^{3}H]thymidine incorporation is related to its ability to inhibit PKC.

The selectivity of Ro 31-7549 also would suggest that the agent is functioning through inhibition of PKC activity. In *in vitro* assays Ro 31-7549 demonstrates an approximate 60- and 200 times selectivity for PKC over cyclic AMP-dependent protein kinase (PKA) and calcium/calmodulin-dependent protein kinase, respectively [20]. The analog (GF 109203X) has also been shown to be highly selective for PKC both in cellular and cell-free assays [11]. Therefore, the ability of Ro 31-7549 to stimulate [^{3}H]thymidine incorporation is likely a result of its PKC inhibitory activity. This interpretation is also supported by the ability of the compound to block effects of TPA (Figs 2, 6, and 7), which is known to be a potent PKC activator. At higher concentrations, however, Ro 31-7549 would be predicted to inhibit many, if not all, cellular protein kinases; thus, the cytotoxicity exhibited by Ro 31-7549 at high doses (approximately 2.5 to 5 μM and above) is not unexpected. Nevertheless, because the primary effect we observed in this study was a stimulation of DNA synthesis and proliferation, the issue of cytotoxicity is irrelevant.

* C. S. Harmon, J. Ducote, and D. Lutz (unpublished observations).

Taken together, our results thus suggest that continued culture in a differentiative high-calcium, serum-containing medium results in a time-dependent PKC-mediated loss of proliferative capacity which is inhibited by Ro 31-7549.

Our data suggest, therefore, that in keratinocytes PKC activation may play a role in a loss of proliferative potential, because blocking the activity of this enzyme stimulates proliferation. Paradoxically, the PKC-activating phorbol esters are also tumor promoters and prolonged exposure causes epidermal hyperplasia *in vivo* [2,3]. However, in addition to their ability to activate PKC, with continual exposure phorbol esters can also cause the down-regulation of PKC and depletion of its activity [1,9,10,31]. In melanocytes, Brooks *et al* [32] have correlated phorbol ester-induced proliferation with down-regulation, and not transient activation, of PKC. Thus, the ability of phorbol esters to stimulate keratinocyte proliferation with chronic treatment may be related to their ability to cause down-regulation of PKC. This interpretation is further supported by the study of Hansen *et al* [33] who demonstrated that only those regimens of treatment with TPA or *sn*-1,2-didecanoylglycerol that induce PKC down-regulation also result in epidermal hyperplasia. It is not clear whether inhibiting PKC activity also inhibits down-regulation of the enzyme [34,35]. Thus, further investigation is required to determine the effect of PKC inhibitors on down-regulation and tumor promotion.

The results presented here also suggest that PKC may be involved in the calcium-induced growth inhibition of keratinocytes (Fig 8). Such involvement is consistent with previous findings that high extracellular calcium concentrations induce phosphoinositide turnover and DAG production [1,9,10,31], events that usually result in PKC activation. Further support is provided by the observation that calcium induces subcellular redistribution of PKC [15]. Dlugosz *et al* [16] have reported that bryostatin 1 treatment and TPA-induced down-regulation of PKC inhibit calcium-induced differentiation, again suggesting a role for PKC in the regulation of keratinocyte growth and differentiation. Nevertheless, the lack of detectable PKC activation in Balb/MK cells exposed to high calcium [14] argues against an involvement of PKC in calcium-induced differentiation; however, it should be noted that this cell line differentiates poorly relative to primary cultures of Balb/c keratinocytes [36]. Thus, although further study is necessary, it seems likely that PKC

may play some role in calcium-induced regulation of growth and differentiation.

In this study we have demonstrated that the PKC inhibitor Ro 31-7549 prevented the PKC-mediated inhibition of DNA synthesis elicited by acute exposure to the phorbol ester tumor promoter, TPA. Furthermore, in cells cultured for several days in a differentiative high-calcium, serum-containing medium, Ro 31-7549 dose-dependently stimulated DNA synthesis with an EC_{50} of approximately 0.6 μM. The time course of DNA synthesis in cells cultured in the differentiative medium indicated that Ro 31-7549 did not directly stimulate DNA synthesis but instead maintained [^{3}H]thymidine incorporation at elevated levels. Finally, in proliferating cells cultured in a serum-free medium, Ro 31-7549 prevented both calcium- and TPA-induced inhibition of proliferation. Thus, our results suggest that cells cultured with agents which promote differentiation suffer a gradual loss of proliferative capacity which may be mediated, at least in part, by protein kinase C.

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