Arch Dermatol Res (1997) 289 : 540–546 © Springer-Verlag 1997

ORIGINAL ARTICLE

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SCH 47112, a novel staurosporine derivative, inhibits 12-O-tetradecanoylphorbol-13-acetate-induced inflammation and epidermal hyperplasia in hairless mouse skin

Received: 29 July 1996

Abstract Protein kinase C (PKC) regulates keratinocyte growth and differentiation as well as inflammation in skin, processes which are abnormal in skin diseases such as psoriasis. 12-O-tetradecanoylphorbol-13-acetate (TPA) binds to and activates PKC. We investigated the effects of SCH 47112, a novel staurosporine derivative, which interacts with the catalytic domain of PKC, on TPA-induced inflammation and hyperplasia in hairless mouse skin and TPA-induced differentiation in cultured human keratinocytes. Dorsal mouse skin was treated with vehicle, TPA (2.0/2.5 nmol) or SCH 47112 followed by TPA. Epidermal thickness, and epidermal, upper dermal and deep dermal inflammation (assessed on an ordinal semiquantitative scale) were determined in biopsies taken 24 h and 48 h post-treatment. SCH 47112 (100 nmol) inhibited TPA-induced epidermal, upper dermal and deep dermal inflammation by 71%, 45% and 22%, respectively, at 24 h ($n = 3, P < 0.05$). TPA-induced epidermal hyperplasia was inhibited by SCH 47112 (400 nmol) by 38% at 48 h ($n = 3, P < 0.05$). In addition, in cultured human keratinocytes, SCH 47112 inhibited TPA induction of transglutaminase I protein, which catalyzes the formation of crosslinked envelopes. These results indicate that SCH 47112 exhibits biological activity, inhibiting TPA-induced changes in hairless mouse skin in vivo and cultured human keratinocytes in vitro, and suggest that PKC inhibitors may have a therapeutic role in inflammatory skin diseases.

Key words SCH 47112 · TPA · Inflammation · Epidermal hyperplasia · Mouse skin

Supported in part by The British Association of Dermatologists Dowling Travelling Fellowship, The Psoriasis Association (NJR) and The National Institutes of Health Grant RO1-AR42419-X (GJF)
Presented in part at the Annual Meeting of the Society for Investigative Dermatology, Washington, USA, 1996.

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Introduction

Evidence indicates that protein kinase C (PKC) plays an important role in regulating keratinocyte growth and differentiation, as well as cutaneous inflammation [1–4]. Abnormalities of keratinocyte growth and differentiation, together with an influx of inflammatory cells, are found in a number of skin diseases, including psoriasis [5]. Furthermore, alterations in the PKC signal transduction pathway have been identified in psoriasis [6–8], raising the possibility of PKC inhibitors as potential therapeutic agents in skin disease.

PKC inhibitors may be divided into those that interact with the regulatory domain and those that interact with the catalytic domain. The PKC inhibitor sphingosine, a natural metabolize of membrane phospholipids, interacts with the regulatory domain of PKC, which contains the 12-O-tetradecanoylphorbol-13-acetate (TPA) and 1,2-diacylglycerol (1,2-DAG) binding sites [9, 10]. Sphingosine inhibits TPA-induced inflammation, epidermal hyperplasia and ornithine decarboxylase activity in hairless mouse skin [11]. Sphingosine is, however, not entirely specific for PKC, may act through other cellular targets [12] and also inhibits TPA binding to chimaerins [13]. On the other hand, cyclosporin A, which inhibits many of the TPA-induced changes in mouse skin, does not inhibit PKC [3].

Staurosporine (Fig. 1), an indolocarbazole which occurs naturally, is one of the most potent PKC inhibitors yet described, but appears to be relatively nonselective [14]. Staurosporine interacts with the catalytic domain of

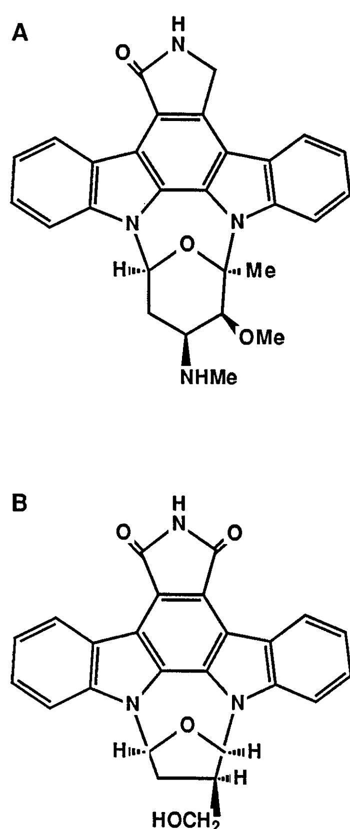

Fig. 1A, B Structural formula of (**A**) staurosporine and (**B**) SCH 47112

PKC, which contains the ATP and substrate binding sites [15]. However, the catalytic domain of PKC shows homology with other serine and tyrosine kinases, and staurosporine also inhibits cAMP-dependent kinases and protein tyrosine kinases in similar concentration ranges. Staurosporine inhibits TPA-induced tumour promotion in mouse skin but fails to inhibit TPA-induced inflammation, or TPA-induced hyperplasia [16]. Furthermore, in cultured mouse keratinocytes, staurosporine induces TPA-like morphological changes and terminal differentiation [17]. Moreover, induction of cornified envelope formation by staurosporine is blocked by pretreatment with the PKC activator, bryostatin-1 [17]. Thus, paradoxically, staurosporine may function as a PKC agonist in cultured mouse keratinocytes as well as other cells [17].

In an attempt to design more selective PKC inhibitors a variety of modifications have been made to the basic staurosporine structure. Initial bisindolylmaleimide derivatives show increased selectivity as PKC inhibitors but are significantly less potent than staurosporine [18, 19]. However, more recently developed bisindolylmaleimide derivatives, such as RO 31-8220 and GF 109203X, show increased potency while retaining specificity [18–20]. The macrocyclic bisindolymaleimide LY333531 has been found to specifically inhibit PKC-β [21]. Go 6976 is a synthetic indolocarbazole derivative that shows selective inhibition of calcium-dependent PKC isozymes compared to calcium-independent isozymes [22, 23]. SCH 47112 (Fig. 1) is a further indolocarbazole derivative, a synthetic analogue of K-252a, that inhibits PKC in vitro at nanomolar concentrations (IC_{50} = 1.7 nM, compared with IC_{50} of 7 nM for staurosporine) [24, 25].

TPA binds to and activates PKC and has been widely utilized as a PKC agonist. However, two other classes of proteins, chimaerins (a GTPase activating protein) and unc-13 (a protein expressed by *Caenorhabditis elegans*), have recently been identified which bind TPA with high affinity [13, 26]. Although the tissue distribution of chimaerins has not been well defined and it is unknown whether they are expressed in skin, a biological response to TPA cannot be formally assumed to reflect the involvement of PKC in that response. In addition, PKC inhibitors targeted to the regulatory domain also inhibit the binding of TPA to chimaerins [13]. On the other hand, PKC inhibitors which act through the catalytic domain, may provide additional supporting evidence for involvement of PKC in a particular response.

The aims of this study were to investigate the effects of SCH 47112, a new potent inhibitor of PKC, on TPA-induced responses in mouse skin in vivo and cultured human keratinocytes.

Methods

Materials

TPA was from Sigma Chemical Company (St. Louis, Mo.). SCH 47112 was provided by Schering-Plough Research Institute (Kenilworth, NJ). Cyclosporin A was a kind gift from Sandoz Pharmaceutical (Basle, Switzerland).

Mice

Male HRS/J hairless mice were purchased from Jackson Laboratories (Bar Harbor, Me.) and were used for experiments between 6 and 10 weeks of age. Mice were cared for in the University of Michigan Animal Facility and experimental protocols were approved by the University of Michigan Committee on Use and Care of Animals.

Treatment of mice

Compounds were dissolved in acetone and applied to the dorsal surface of mice using a pipette. Where indicated, cyclosporin A or SCH 47112 were applied in 100 μl acetone 5 min prior to the application of TPA (2.0/2.5 nmol in 50 μl acetone, applied to a localized area on the back, rostral to the tail). Mice were sacrificed 24 or 48 h later and 4-mm punch biopsies were taken from the treated dorsal skin.

Culture of human keratinocytes

Keratinocytes were cultured from keratome biopsies of adult human skin, as previously described [3, 27], using serum-free low-calcium MCDB-153 medium.

Keratinocyte growth assay

Second passage keratinocytes, from four individuals, were plated into 96-well plates at a density of 5×10^3/well and allowed to attach.

After 18 h, at approximately 25% confluency, cells were treated with the indicated concentrations of compounds or vehicle (DMSO 0.1% final concentration). Cells were fed fresh medium containing compounds (dissolved in DMSO) three times per week until cells reached 90% confluency. This took between 5 and 7 days, depending on the donor. Cell number was then assessed using the neutral red dye assay as described previously [28]. Keratinocyte growth is expressed as a percentage of the growth with vehicle only.

Assay of transglutaminase I protein

Adult human keratinocytes from four individuals (second passage) were grown to 80% confluency in 96-well plates. Cells were then treated with the indicated concentrations of compounds or vehicle (0.1% DMSO), followed 30 min later by the addition of TPA (50 nM) or vehicle (0.1% DMSO). Transglutaminase I (TGase I) protein levels were determined 24 h later by ELISA as described previously [29], using a mouse monoclonal antibody to human TGase I. The results are expressed as a percentage of TPA-treated cells.

Assessment of mouse skin histology

Punch biopsies were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Histological assessments were made by an investigator who was not aware of the treatment conditions. Epidermal thickness,

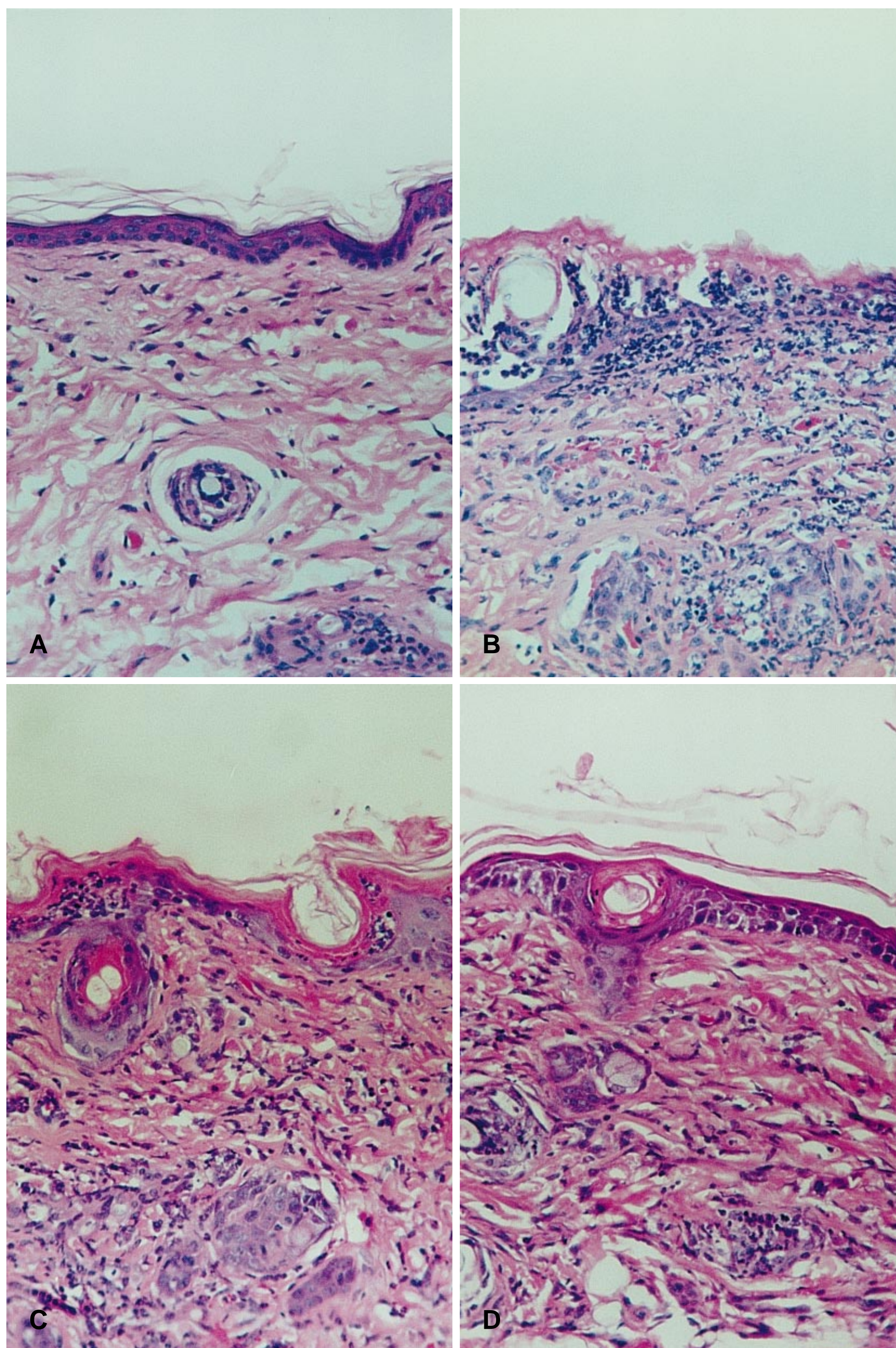

Fig. 2A–D Photomicrographs of skin biopsies from hairless mouse skin 24 h after single applications of (**A**) acetone, (**B**) 2.5 nmol TPA, (**C**) 2.5 nmol TPA plus 30 nmol SCH 47112 and (**D**) 2.5 nmol TPA plus 100 nmol SCH 47112 (original magnification × 250). Note dose-dependent inhibition of TPA-induced inflammatory cell infiltrate by SCH 47112

taken as the distance from the bottom of the stratum corneum to the basement membrane in interfollicular epidermis, was determined from the mean of five measurements using a calibrated micrometer scale, under light microscopy at × 100 magnification. The degree of inflammation was assessed within the epidermis, upper dermis and deep dermis using a semiquantitative ordinal five-point scale in half increments, in which 0 indicated the absence of the characteristic and 4 indicated the maximal degree of the characteristic [27].

Statistical analysis

For measurements of epidermal thickness, and epidermal and dermal inflammation, comparisons among treatment groups were performed with one-way ANOVA and Fisher's least significant difference test. The data were analysed with the Michigan Interactive Data Analysis System (MIDAS, a statistical software package developed by the Center for Statistical Consultation and Research at the University of Michigan, Ann Arbor) or with Minitab for Windows. Summary statistics are reported as means ± standard error of the mean (SEM). *P*-values equal to or less than 0.05 were considered significant.

Results

Effect of SCH 47112 on phorbol ester-induced histological changes at 24 h

As previously reported [11], a single application of TPA (2.5 nmol) to hairless mouse skin resulted in an intense inflammatory infiltrate, composed predominantly of polymorphonuclear leukocytes, within the epidermis, around blood vessels in the upper dermis, and in collections in the deeper dermis, 24 h later (Fig. 2). There was marked epidermal spongiosis and dermal edema; deeper structures, including underlying muscle appeared normal (Fig. 2).

Analysis of the semiquantitative scores showed that TPA induced a significant increase in the degree of epidermal, upper dermal (perivascular) and deep dermal inflammatory infiltrate at 24 h, compared with acetone treatment (Fig. 3). Cyclosporin A (250 nmol) was included as a positive control and significantly reduced the degree of TPA-induced epidermal and dermal infiltrate at 24 h, as previously reported (Fig. 3) [3]. Cyclosporin A, however, does not inhibit PKC [3] and probably acts distally to PKC in inhibiting TPA-induced changes in mouse skin. In animals treated with TPA plus SCH 47112 (30 nmol and 100 nmol), there was significantly less epidermal and dermal inflammation at 24 h compared with animals treated with TPA alone (Figs. 2 and 3). Furthermore, SCH 47112 inhibited TPA-induced inflammation in a dose-dependent manner (Fig. 3).

Compared with acetone-treated skin, there was no significant increase in epidermal thickness following the application of TPA for 24 h (data not shown). However, in certain areas, particularly where there was intense epidermal spongiosis, there was evidence of keratinocyte cell death and epidermal necrosis, so that an accurate assessment of epidermal thickness was difficult (Fig. 2).

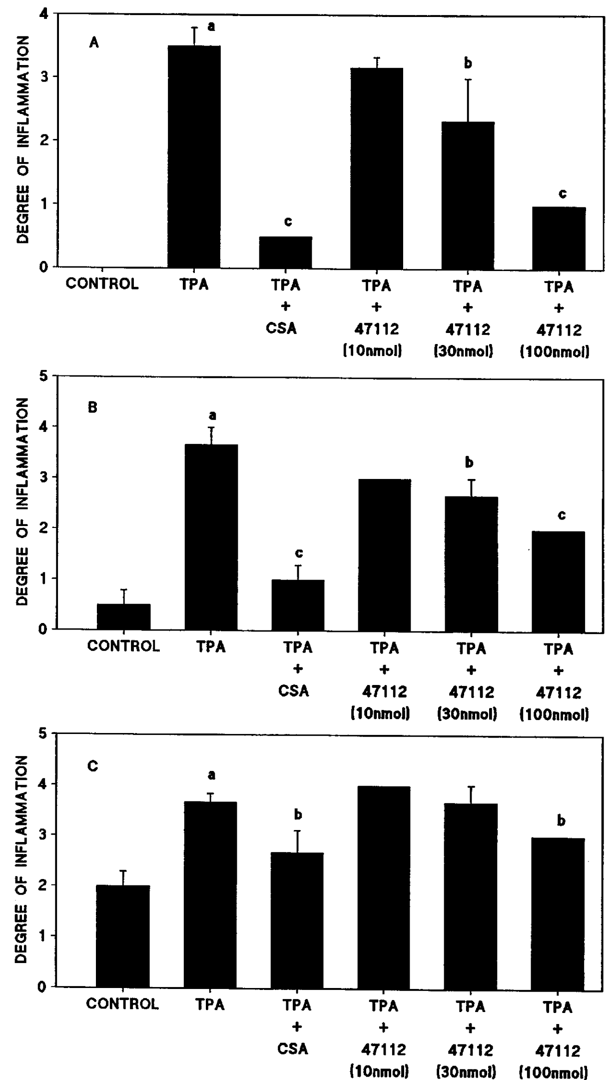

Fig. 3A–C Effect of SCH 47112 on TPA-induced (**A**) epidermal, (**B**) upper dermal and (**C**) deep dermal inflammation at 24 h in hairless mouse skin (CSA cyclosporin A). Data points represent mean values ± SEM for three mice/group. [a] $P < 0.05$ compared with control, [b] $P < 0.05$ compared with TPA, [c] $P < 0.005$ compared with TPA

Effect of SCH 47112 on phorbol ester-induced histological changes at 48 h

In view of TPA-induced epidermal necrosis observed at 24 h, the effects of lower doses of TPA (1.25 nmol and 0.625 nmol) on histological changes at 48 h were investigated. The degree of inflammation induced by 1.25 nmol and 0.625 nmol of TPA was considerably less than that induced by 2.5 nmol, and the effects of inhibitors were difficult to assess at these doses. In further experiments, investigating the effects of inhibitors on TPA-induced histological changes at 48 h, a dose of 2.0 nmol TPA was used.

A significant increase in epidermal thickness at 48 h was observed in response to TPA (2.0 nmol) compared with the response to acetone at 24 h (Fig. 4, Table 1). Pre-

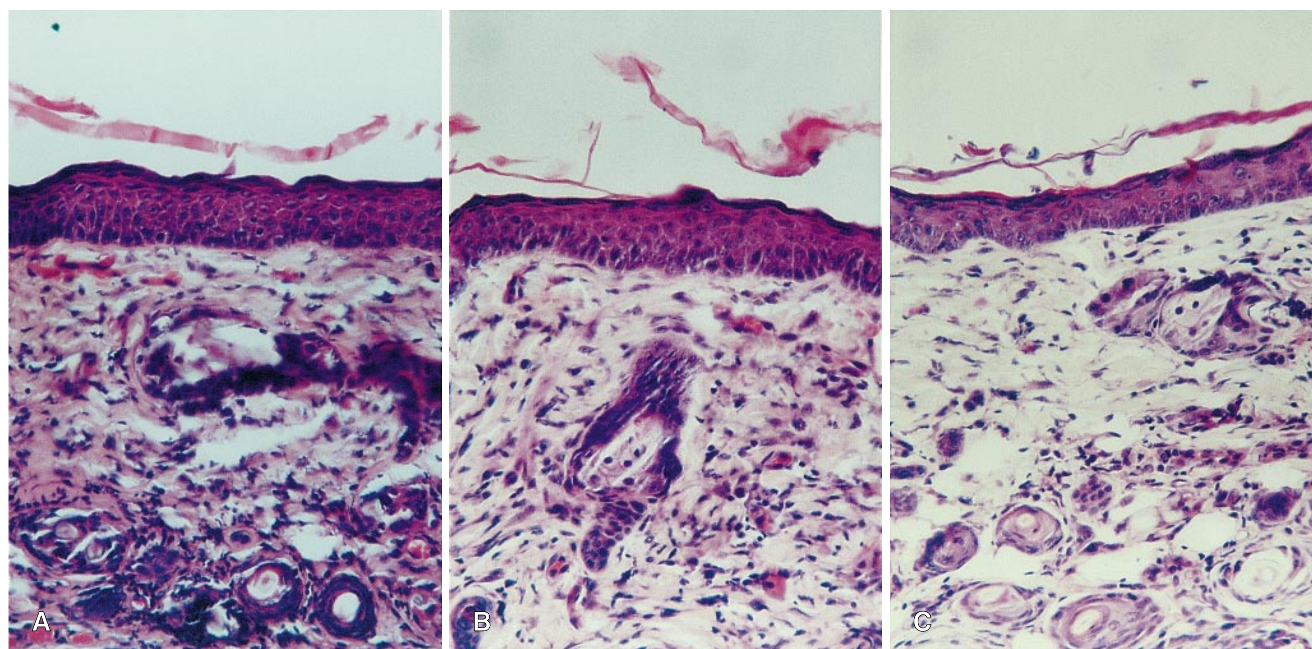

Fig. 4A–C Photomicrographs of skin biopsies from hairless mice 48 h after single applications of (**A**) 2 nmol TPA, (**B**) 2 nmol TPA plus 200 nmol SCH 47112 and (**C**) 2 nmol TPA + 400 nmol SCH 47112 (original magnification × 250). Note dose-dependent inhibition of TPA-induced epidermal thickening by SCH 47112

Table 1 Effect of SCH 47112 on phorbol ester-induced increase in epidermal thickness at 48 h. Compounds were applied to the backs of hairless mice either alone or 5 min prior to TPA, as indicated. Epidermal thickness was assesed in biopsies taken 48 h or, for acetone treatment, 24 h later. Values are means ± SEM for three mice/group

Treatment	Epidermal thickness (μM)
Acetone	24.6 ± 2.5
TPA (2.0 nmol)	49.5 ± 2.7*
TPA (2.0 nmol) + cyclosporin A (250 nmol)	43.5 ± 2.3
TPA (2.0 nmol) + SCH 47112 (200 nmol)	38.9 ± 2.8
TPA (2.0 nmol) + SCH 47112 (400 nmol)	36.7 ± 2.3**
TPA (2.0 nmol) + SCH 47112 (400 nmol)[a]	30.6 ± 0.3***

*$P < 0.005$ compared with acetone after 24 h, **$P = 0.059$ compared with TPA, ***$P < 0.005$ compared with TPA
[a] SCH 47112 applied simultaneously with TPA

vious studies have revealed a minimal increase in epidermal thickness between 24 and 72 h following acetone treatment; for example, epidermal thickness of hairless mouse skin after daily application of acetone for 4 days was 16.1 + 0.7 μM, $n = 4$ [30]. SCH 47112 inhibited TPA-induced epidermal hyperplasia in a dose-dependent manner, although a significant reduction in epidermal thickness was only observed with 400 nmol SCH 47112 (Fig. 4, Table 1). Overall, our results did not indicate clear differences between simultaneous application of TPA and SCH 47112, and application of SCH 47112 5 min before TPA. Cyclosporin A (250 nmol) did not significantly inhibit TPA-induced epidermal hyperplasia (Table 1).

At 48 h, the degree of epidermal and dermal inflammation in TPA-treated mice (2.0 nmol) was less marked than at 24 h after TPA treatment (2.5 nmol) (Figs. 4 and 5). Nevertheless, SCH 47112 appeared to reduce TPA-induced dermal inflammation at 48 h in a dose-dependent manner (Figs. 4 and 5).

Application of SCH 47112 (200 nmol) alone resulted in no inflammatory or histological changes compared with vehicle only treatment (mean score 0.75, 0.75 and 0.75 for epidermal, upper dermal and deep dermal inflammation, respectively, $n = 2$).

Effect of SCH 47112 in cultured human keratinocytes

As staurosporine acts as a partial PKC agonist and induces differentiation of cultured keratinocytes [17, 31], we investigated the effects of SCH 47112 in cultured human keratinocytes. In a similar manner to staurosporine, SCH 47112 inhibited TPA-induced TGase I protein accumulation (Fig. 6A). However, in contrast to staurosporine, SCH 47112 alone did not induce a fusiform appearance in cultured keratinocytes and did not induce keratinocyte differentiation, as assessed qualitatively from cell appearance (data not shown). SCH 47112 (10–100 nM), like staurosporine, inhibited keratinocyte growth (Fig. 6B).

Discussion

The results presented indicate that SCH 47112 inhibited TPA-induced inflammation and hyperplasia in hairless mouse skin and that these effects were dose-dependent (Table 1, Figs. 2–5). In addition, we showed that SCH 47112 inhibited TPA induction of TGase I protein. SCH 47112 did not itself induce TPA-like changes in mouse skin in vivo or in cultured keratinocytes in vitro,

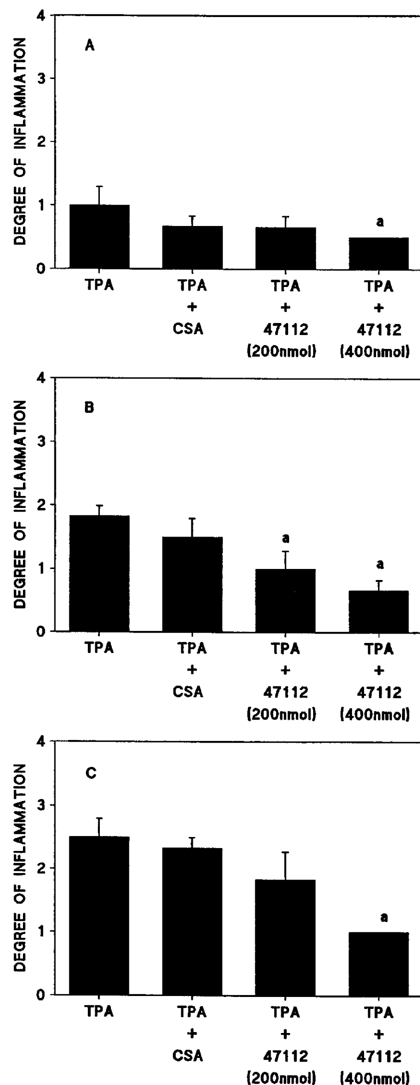

Fig. 5A–C Effect of SCH 47112 on (**A**) TPA-induced epidermal, (**B**) upper dermal and (**C**) deep dermal inflammation at 48 h in hairless mouse skin (CSA cyclosporin A). Data points represent mean values ± SEM for three mice/group. [a] $P < 0.05$ compared with TPA

indicating that it lacks partial agonist activity, as has been reported with staurosporine [17, 31].

Although the effects of TPA on mouse skin have been assumed to reflect activation of PKC, it is now known that TPA binds to other classes of proteins in addition to PKC [13, 26]. Furthermore, PKC inhibitors have shown variable effects, although this could, in part, be a reflection of nonspecificity. Thus, for example, although staurosporine inhibits TPA-induced tumour promotion in CD-1 mice, no inhibition of TPA-induced hyperplasia or inflammation has been observed [16]. In addition, novel indolocarbazole derivatives, including Go 6976, which exhibit potent and selective PKC-inhibitory activity in vitro, fail to significantly inhibit TPA-induced inflammation and hyperproliferation in mouse skin [23]. IC_{50} values of Go 6976 are lower for calcium-dependent PKC isozymes than for calcium-independent isozymes [22, 23], and this may be relevant to the action of Go 6976 on TPA responses in mouse skin. SCH 47112 potently inhibits PKC

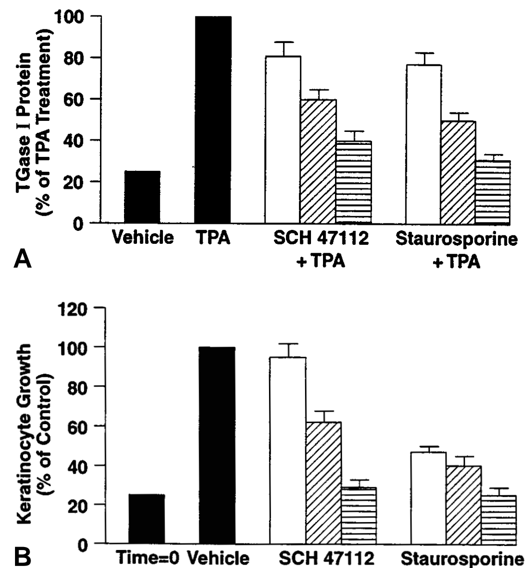

Fig. 6A, B Effect of SCH 47112 on (**A**) TPA induction of TGase I protein levels in cultured human keratinocytes and (**B**) keratinocyte growth. TGase I protein levels were determined using ELISA 24 h after treatment of keratinocytes with vehicle or TPA (50 nM). Keratinocyte growth was assessed using a neutral red dye assay when vehicle-treated keratinocyte cultures reached 90% confluency at 5–7 days, depending on the individual donor (*open bars* 10 nM of indicated inhibitor; *diagonally-hatched bars* 30 nM of indicated inhibitor; *horizontally hatched* bars 100 nM of indicated inhibitor; $n = 4$)

in vitro through interaction with its catalytic domain [24, 25]. Thus, inhibition of TPA-induced inflammation and hyperplasia in mouse skin by SCH 47112 suggests the involvement of PKC in these processes. This is further supported by inhibition of TPA-induced inflammation in mouse ears by GF109203X [32]. SCH 47112, however, also inhibits cAMP-dependent kinase (IC_{50} 18.5 nM) and myosin light-chain kinase, albeit with a higher IC_{50} value than PKC (1.7 nM) [24, 25]. Therefore, interaction with other ATP-dependent kinases cannot be excluded, although whether this is relevant to inhibition of TPA-induced effects is unknown.

Recent studies have indicated that PKC may play a role in the pathophysiology of psoriasis [7, 8, 33, 34]. Interestingly, dithranol, a potent topical therapeutic agent in psoriasis, inhibits PKC [35]. Thus, PKC inhibitors may have a potential therapeutic role in psoriasis. Inhibition of TPA-induced inflammation and hyperplasia in hairless mouse skin may prove to be a useful model for assessing potential new treatments for psoriasis.

Acknowledgements We thank Ted Hamilton, M.S., for performing the statistical analyses.

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