Differential Expression of Protein Kinase C Isoenzymes in Normal and Psoriatic Adult Human Skin: Reduced Expression of Protein Kinase C-βII in Psoriasis

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Psoriatic lesions contain elevated levels of 1,2-diacylglycerol, the physiologic activator of protein kinase C (PKC), suggesting that PKC activation may be aberrant in psoriasis. We therefore have investigated the expression and properties of PKC isozymes in normal and psoriatic skin and in human skin cells. Chromatographic and immunoblot analyses revealed the presence of the calcium-dependent PKC isozymes PKC-α and -β, but not -γ, in normal human epidermis. PKC-β was more prominent, constituting two thirds of the total calcium-dependent PKC activity. In psoriatic lesions, expression of both PKC-α and -β was decreased, with preferential reduction (80%) of PKC-β. Northern analysis and semi-quantitative polymerase chain reaction (PCR) indicated no change in the mRNA levels of PKC-α and -β between normal and psoriatic epidermis. In normal epidermis, PKC-α was expressed mainly in the lower epidermis, whereas PKC-β was localized to the upper cell layers, with very intense staining of CD1a+ Langerhans cells. In psoriasis, PKC-α staining was present in the lower epidermis, whereas PKC-β staining was essentially absent, with the exception of some positive inflammatory cells. In addition to PKC-α and β, immunoblot and Northern/PCR analysis revealed expression of four calcium-independent PKC isozymes, δ, ε, ζ, and η, in both normal and psoriatic skin. There were no significant differences in mRNA levels among any of these PKC isozymes, between normal and psoriatic skin. Soluble PKC-ζ protein was modestly increased (twofold) in psoriatic, compared to normal, skin, whereas the levels of PKC-δ, ε, and η were unchanged. Analysis of PKC isozyme expression in the three major cell types of human epidermis revealed that Langerhans cells and keratinocytes were the major sources of PKC-β and PKC-ζ, respectively. These data demonstrate the diversity of PKC isozyme expression in human skin, and suggest that alterations of PKC-β and -ζ may participate in the aberrant regulation of growth and differentiation observed in psoriasis. Key words: protein kinase C isozyme activation/psoriasis/1,2,-diacylglycerol. *J Invest Dermatol 101:553–559, 1993*

Protein kinase C (PKC) [1] is a family of phospholipid-dependent serine/threonine protein kinases that function to transduce extracellular signals across the cell surface (reviewed in [1,2]). These enzymes are activated by 1,2-diacylglycerol (DAG), which is formed as a consequence of agonist-stimulated phospholipid hydrolysis [3,4]. Tumor-promoting phorbol esters such as 12-O-tetradecanoyl-13-acetate (TPA) also activate the PKC family in a manner similar to DAG, and PKCs are the predominant cellular phorbol ester receptors [5–7]. To date, nine mammalian genes encoding ten PKC isozymes have been identified [8,9]. Whereas virtually every cell expresses some form of PKC, the PKC isozyme composition differs among cell types. PKC isozymes can be broadly classified into two groups based on their calcium dependence. PKC isozymes α, βI, βII, and γ are calcium dependent, whereas PKC isozymes δ, ε, ζ, η, θ, and λ are calcium independent. Accumulating evidence indicates that certain PKC isozymes display distinct substrate specificities and requirements for activation, suggesting that they may possess functional specificity [10–13].

We have previously demonstrated that psoriatic lesions contain elevated phosphoinositide-specific phospholipase C activity and DAG content, compared to non-lesional skin [14]. In addition, we have found that calcium-dependent PKC activity, determined by phosphorylation of histone *in vitro*, is significantly reduced in psoriasis compared to normal skin [15]. Based on these observations, we have proposed that misregulation of the PKC signal transduction pathway may be an important participant in the pathophysiology of psoriasis [14,15]. In this study, we extend our observations to demonstrate that reduced PKC activity in psoriatic lesions is largely due to reduced expression of PKC-βII, which in normal skin is highly expressed in epidermal Langerhans cells, and to a lesser extent reduced PKC-α, which is expressed in all cell types of the skin. In addition, we have characterized mRNA and protein expression of calcium-independent PKC isozymes δ, ε, ζ, and η in normal and psoriatic skin.

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Abbreviation: DAG, 1,2-diacylglycerol.

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MATERIALS AND METHODS

Reagents Monoclonal antibodies to PKC-α and PKC-β (which detects both PKC-βI and -βII) were obtained from Seikagaku. Rabbit polyclonal antibodies to PKC-βI, -βII, -γ, -δ, -ϵ, -ζ, and -η were prepared to synthetic peptides and characterized for specificity to each PKC isozyme expressed in the Baculovirus-insect cell expression system [16]. Alkaline phosphatase-conjugated goat anti-rabbit IgG and alkaline phosphatase-conjugated goat anti-mouse IgG were from Bio-Rad Laboratories. Chemiluminescent alkaline phosphatase substrate (ECL) was from Amersham. M-MLV H$^-$ reverse transcriptase and rabbit globin mRNA were from Gibco BRL. TAQ DNA polymerase was obtained from Perkin Elmer Cetus. The ABC vectostain immunohistochemistry reagent kit was from Vector Laboratories. 3-Amino-9-ethyl carbazole was from Sigma Chemical Company. Anti-CD1a antibody was from Becton-Dickenson. Magnetic beads coated with goat anti-mouse IgG were obtained from Dynal.

Tissue Procurement Keratome and punch biopsies from normal and psoriatic epidermis were obtained as previously described [17]. Keratome blade depths were set at 0.2 mm and 0.4 mm for normal and psoriatic skin, respectively, resulting in excision near the epidermal-dermal junction. Biopsies were composed of epidermis with some residual (approximately 5%) dermis, as judged by histologic examination. Biopsies were either used immediately or snap frozen in liquid nitrogen and stored at −70°C until used. All procedures involving human subjects were approved by the University of Michigan Institutional Review Board.

Partial Purification and Assay of Soluble Calcium-Dependent PKC Previous studies demonstrated that 70–80% of calcium-dependent PKC activity in human epidermis was in the soluble fraction [18]. Therefore the soluble fraction of epidermal homogenates was utilized to characterize calcium-dependent PKC isozymes. Frozen keratome biopsies from normal and psoriatic skin were ground under liquid nitrogen with mortar and pestle and homogenized in a glass homogenizer in ice in 20 mM Tris-HCl (pH 7.5), 5 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM β-mercaptoethanol, 0.1% leupeptin. The homogenate was centrifuged at 100,000 × *g* for 1 h at 4°C, and the supernatant applied to a diethylaminoethyl-5PW column (7.5 × 0.75 cm), equilibrated with 20 mM Tris-HCl, (pH 7.5), 2 mM EGTA, 2 mM EDTA, 10 mM β-mercaptoethanol, 0.01% leupeptin, connected to a Pharmacia fast protein liquid chromatography system. All chromatography was performed at 4°C in a cold room. The column was washed with 20 ml equilibration buffer and eluted with a linear gradient of NaCl (0–0.5 M). Fractions (0.5 ml) containing PKC activity were pooled, diluted threefold with 5 mM KPO_4 (pH 7.5), 10% glycerol, 0.01% leupeptin, and applied to a high-performance hydroxylapitite column (10 × 0.78 cm) equilibrated with diluent. The column was eluted with a linear gradient of KPO_4 (5–300 mM), and fractions (0.5 ml) were assayed for PKC activity.

Calcium-dependent PKC activity in column fractions was determined by measurement of PKC-catalyzed incorporation of [^{32}P]PO_4 into histone in the presence of calcium, phospholipid, and TPA as previously described [18,19].

Northern Blot Analysis Total RNA was isolated from keratome biopsies of normal and psoriatic epidermis by cesium chloride gradient centrifugation as described [20]. Briefly, frozen keratome biopsies were ground under liquid nitrogen with mortar and pestle, and homogenized in a polytron homogenizer in guanidinium hydrochloride lysis buffer (1 ml/100 mg wet weight). The resulting homogenate was centrifuged to remove insoluble debris and total RNA was obtained by cesium chloride gradient centrifugation. PolyA+ RNA, obtained by passage of total RNA over oligo-dT cellulose, was size fractionated by electrophoresis on 1.2% formaldehyde agarose gels and transferred to nylon membrane. Membranes were probed with [^{32}P]-labeled cDNA probes specific for PKC-α, -β, -δ, -ϵ, -ζ, and -η, generated by reverse transcriptase/polymerase chain reaction (see below). cDNA probes were labeled by random priming.

Western Blot Analysis Soluble and particulate fractions from normal and psoriatic epidermis were prepared as described above, and equal amounts of protein (50 μg) were resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (8% gels) [21]. Proteins were transferred to nitrocellulose membranes and blotted with specific antibodies to PKC-α, -β (βI and βII), -βI, -βII, -γ, -δ, -ϵ, -ζ, and -η, using standard procedures. PKC/antibody complexes were visualized by autoradiography using a chemiluminescent substrate. Recombinant PKC isozymes, obtained by expression in the Baculovirus-insect cell expression system, were used as standards.

Reverse Transcriptase/Polymerase Chain Reaction Total RNA from keratome biopsies of normal and psoriatic epidermis and epidermal cells (keratinocytes, melanocytes, and Langerhans cells) was prepared as described above. RNA (1 mg) was reverse transcribed, using random heximer primers (25 pmol) and 200 U M-MLV H− reverse transcriptase, at 37°C for 30 min in 50 mM Tris (pH 8.3), 0.3 mM $MgCl_2$, 20 mM dithiothreitol, 500 mM dXTP, 20 units RNAse inhibitor. Following this, PKC isozyme-specific primers (50 pmol), and TAQ DNA polymerase (2 U) were added, and polymerase chain reaction was carried out in 40 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 125 mM deoxyribonucleotides (final concentrations) for 30 cycles of 95°C for 30 seconds, 50–56°C (depending on the target being amplified) for 30 sec, and 72°C for 30 sec, with a 3-sec extension per cycle. The primers and annealing temperatures utilized were as follows: PKC-α, 5′GTCGGCAACAAAGTCATCAGTCCCTCTGAAGACAG-GAAACAA, 5′TTTTAACGACTGAAACCCTACACGTTCCTT, 50°C; PKC-β(I/II), 5′TTCCTTACACATGCCAAAATCGGCAATCTT, 5′C-AACCAGCAGAAAAGAAGGCCC-TGGAACCAAGG, 50°C; PKC-βI, 5′AAGGGCATCATTTACCGTGAC, 5′TGGAGGTTGAAGCTGTT-TCTC, 50°C; PKC-βII, 5′AAGGGCATCATTTACCGTGAC, 5′-AATTTTGGGCTCAGTTCTCG, 50°C; PKC-γ, 5′GGTTCTACTCA-CCTCATACAATTCCAGGGGGTAGTT, 5′GTGTGTCGCGGCCA-CGTGCAAGTAGAGCGTCGTTCTTGT, 50°C; PKC-δ, 5′GTGGA-TTGCAAACAGTCTATGCGCAG-TGAG, 5′TCTAGTCTGAGTCG-GAGGAGTCTCGGA, 50°C; PKCϵ, 5′GACAATGAGAGCGTGTG-TTCAGGGAACGC, 5′TAACGACCACGGCTCAGGGGCGTCG-GACGA, 50°C; PKC-ζ, 5′CTAATGTTTGAGATGAGATGGCTGGG, 5′GTATCTGAC-CCTGAACGACCTCTT, 56°C; PKC-η, 5′CCAGC-TGAACCATCGCCAAATAGA 5′ACACAGAGGTCTTAACGTTGG, 52°C. Primers were synthesized by the University of Michigan DNA core facility.

Polymerase chain reaction products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The authenticity of each product was confirmed by size and digestion with two restriction enzymes.

Reverse transcriptase/polymerase chain reaction was also performed under conditions that allowed the relative levels of PKC isozyme transcripts in normal and psoriatic epidermis to be determined [22]. Rabbit globin mRNA (1.2 pg) and RNA from normal or psoriatic epidermis (1 μg) were combined and reverse transcribed as described above. Following this, 3 four-fold dilutions of the reverse transcriptase reaction were made. To each dilution, specific PKC isozyme and rabbit β-globin primers were added, and the target sequences for both co-amplified as described above for 20 cycles. Polymerase chain reaction products were separated by agarose gel electrophoresis, transferred to nylon membrane, and simultaneously hybridized with [^{32}P]-labeled PKC isozyme and β-globin probes (isolated polymerase chain reaction products). Radioactivity in the hybridized bands was quantified using a PhosphorImager (Molecular Dynamics). Results are analyzed as the ratio of counts in the PKC isozyme band to counts in the β-globin band. Over the concentration range of epidermal and rabbit globin mRNA utilized, the amount of polymerase chain reaction products formed after 20 cycles was linearly proportional to the amount of RNA added.

Immunohistochemistry of PKC-α and PKC-β Punch biopsies for immunohistochemistry were oriented and embedded in gum tragacanth, snap frozen in isopentane chilled in liquid nitrogen, and stored at −70°C. Frozen cryostat sections (5 μm) of normal and psoriatic skin were stained with monoclonal antibodies to PKC-α and PKC-β, using an avidin-biotin peroxidase technique as described [17]. Peroxidase/antibody complexes were visualized with 3-amino-9-ethyl carbazole as substrate, and sections were counterstained with 1% hematoxylin. Normal mouse IgG was used as an isotype control and showed no specific staining.

Isolation of Langerhans Cells Keratome biopsies from normal epidermis were trypsinized (0.25%) to produce a cell suspension, which was filtered through nylon mesh to remove particulate debris. The cells were washed three times in calcium-, magnesium-free phosphate buffered saline containing 1% fetal bovine serum, and incubated on ice in RPMI 1640 containing 10% fetal bovine serum, with mouse monoclonal antibody to CD1a, for 30 min. Unbound anti-CD1a antibody was removed by washing the cells three times as above, and the cells were incubated on ice, with gentle agitation, with magnetic beads coated with goat anti-mouse IgG for 45 min [23]. Langerhans cells, rosetted with magnetic beads, were separated from non-CD1a–bearing epidermal cells by placing the epidermal cell suspension next to a fixed magnet for 5 min. The Langerhans cells–depleted epidermal cells were removed by pipette from the magnet-bound Langerhans cells. Langerhans cells were separated from the epidermal cell suspension twice more with additional beads, as above, and the rosetted Langerhans cells pooled. This procedure resulted in an epidermal cell suspension that was depleted of Langerhans cells by greater than 98%, and a preparation of Langerhans cells that was 90% pure, as assessed by CD1a staining.

Culture of Human Keratinocytes and Melanocytes Adult human keratinocytes and melanocytes were cultured from keratome biopsies of

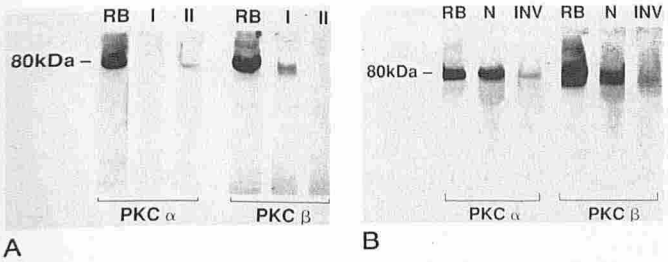

Figure 1. Immunoblot analysis of PKC-α and PKC-β in normal and psoriatic human epidermis following chromatographic separation. *A)* PKC activities in the soluble fraction from normal human epidermis (10 mg) were partially purified by chromatography on a DEAE-5PW column. Fractions containing PKC activity were pooled and applied to a hydroxylapatite column to separate calcium-dependent PKC isoenzymes. This yielded two peaks of PKC activity, which were separately pooled, and analyzed by immunoblot with monoclonal antibodies to PKC-α *(left)* and PKC-β *(right)*. RB, partially purified PKC from rat brain used as a positive control; *lane I,* first peak of PKC activity eluted from hydroxylapatite; *lane II,* second peak of PKC activity eluted from hydroxylapatite. Results are representative of four experiments. *B)* Equal amounts of soluble protein from normal and psoriatic epidermis were chromatographed on DEAE-5PW and hydroxylapatite columns to separate PKC isoenzymes as in *A*. The first and second peaks of PKC activity from normal (N) and psoriatic (INV) epidermis were separately pooled and analyzed by immunoblot with monoclonal antibodies to PKC-α *(left, peak II)* and PKC-β *(right, peak I)*. Results are representative of four experiments.

normal skin as previously described [24,25]. Briefly, keratome biopsies were trypsinized (0.25%) to produce an epidermal cell suspension and primary cultures containing mostly keratinocytes, but including 1–10% melanocytes, were grown in complete MCDB 153 [24]. After approximately 1 week, cultures were briefly treated with trypsin (0.025%) to preferentially remove melanocytes, and the melanocytes were seeded in fresh dishes in complete MCDB 153 containing 10 nM TPA. TPA stimulated melanocyte proliferation, while causing any residual keratinocytes to terminally differentiate and detach from the dish. Keratinocytes remaining after removal of melanocytes were further trypsinized and expanded by passage in complete MCDB 153.

RESULTS

Measurement of Calcium-Dependent PKC Isozymes in Normal and Psoriatic Epidermis We have previously reported that normal human epidermis expresses PKC activity that is calcium dependent and phosphorylates histone *in vitro* [18], suggesting that human skin expresses one or more of the calcium-dependent PKC isozymes α, β, and γ. To further examine this, PKC activity from normal human epidermis was partially purified by high-performance DEAE chromatography (this yielded a single peak of calcium-dependent PKC activity, data not shown), and the calcium-dependent PKC isozymes resolved by chromatography on hydroxylapatite. Two distinct peaks of calcium-dependent PKC activity, which eluted at 100 mM and 170 mM KPO_4, were obtained from hydroxylapatite chromatography (data not shown). The first peak contained approximately twice as much PKC activity as the second peak. Based on the known elution positions of PKC-α, -β, and -γ [26,27], peaks one and two would be expected to contain PKC-β and -α, respectively. No peak of activity corresponding to the expected elution position of PKC-γ (40 mM KPO_4) was observed. The absence of a PKC-γ peak was not due to failure to resolve PKC-γ from PKC-β, because we were able to resolve PKC-γ and PKC-β in rat brain under similar conditions (data not shown).

To confirm the presence of PKC-α and PKC-β in human epidermis, immunoblot analysis of the two peaks of PKC activity resolved by hydroxylapatite chromatography was performed, using specific monoclonal antibodies. The first peak of activity reacted with antibody to PKC-β, but not antibody to PKC-α, and the second peak reacted with PKC-α antibody, but not PKC-β antibody (Fig 1*A*). Neither peak of PKC activity reacted with antibody to PKC-γ (data not shown). These data demonstrate that normal human epidermis expresses PKC-α and PKC-β in an approximate ratio of 1:2.

We previously reported that psoriatic skin lesions contain significantly less calcium-dependent PKC activity than normal epidermis [53]. We therefore next determined the levels of PKC-α, -β, and -γ in psoriatic lesions by resolution on hydroxylapatite chromatography and immunoblot, as described above. Similar to normal epidermis, two peaks of PKC activity were obtained from psoriatic epidermis, with elution positions corresponding to PKC-β and PKC-α (data not shown). The identity of these two peaks of PKC activity as PKC-α and PKC-β was confirmed by immunoblot (data not shown). Chromatography of equal amounts of protein from normal and psoriatic epidermis on hydroxylapatite revealed that the levels of both PKC-α and -β were reduced in psoriatic epidermis, compared to normal epidermis. PKC-α was reduced $20 \pm 5\%$ ($n = 6$), whereas PKC-β activity was preferentially decreased $80 \pm 9\%$ ($n = 6$), such that the ratio of PKC-α to PKC-β was reversed from that seen in normal epidermis to 2:1. The preferential loss of PKC-β activity in psoriatic epidermis was observed in six of six psoriatic individuals. Taken together, the reduction in activity of PKC-α and -β resulted in a 60% reduction in total calcium-dependent PKC activity. This value is similar to our previously reported value for reduction in PKC activity in psoriasis [15].

Fractions from hydroxylapatite chromatography of normal and psoriatic epidermis containing PKC activity were pooled, concentrated, and analyzed by immunoblot to determine the protein levels of PKC-α and PKC-β. The relative levels of PKC-α and -β immunoreactive proteins in normal and psoriatic epidermis were found to be proportional to the relative levels of PKC-α and -β activities (Fig 1*B*). In psoriatic epidermis, both immunoreactive PKC-α and -β were reduced, compared to normal epidermis, with preferential loss of PKC-β (the lane containing PKC-β from psoriatic epidermis contained four times more protein than the corresponding lane from normal epidermis). The above data demonstrate that the previously observed decrease in calcium-dependent PKC activity in psoriatic epidermis is due to decreases in both PKC-α and -β activity and protein expression, with preferential loss of PKC-β.

Properties of Epidermal PKC-α and -β We next examined the enzymatic properties of PKC-α and -β from normal and psoriatic epidermis with respect to activation by calcium, TPA, and fatty acids. Both PKC-α and -β from normal and psoriatic epidermis required phosphatidylserine and calcium for activity, with maximal activation, in the presence of 100 μM phosphatidylserine, observed at 100 μM calcium (data not shown). PKC-α and PKC-β from normal and psoriatic epidermis were also similarly stimulated by TPA. In the presence of phosphatidylserine (100 μM) and calcium (1 μM) maximal activation (tenfold) was observed at 10 nM TPA (data not shown). Interestingly, both PKC-α and PKC-β from normal and psoriatic epidermis were significantly stimulated by unsaturated fatty acids, in the absence of phosphatidylserine. Stimulation increased with increasing chain length and degree of unsaturation, with arachidonic acid being the most potent of the fatty acids tested. Arachidonic acid stimulated PKC-α to nearly 100% of the maximal value obtained in the presence of phosphatidylserine, calcium, and TPA (Fig 2*A*), whereas PKC-β was stimulated to nearly 60% of maximal (Fig 2*B*). The addition of calcium had a negligible effect on activation of PKC-α or PKC-β by arachidonic acid (data not shown).

Immunohistochemical Localization of PKC-α and -β in Normal and Psoriatic Human Epidermis We next examined by immunohistochemistry the cellular localization of PKC-α and -β in normal and psoriatic skin. PKC-α immunoreactivity in normal skin was observed throughout all the layers of living cells of the epidermis, with the highest intensity in the lower layers of keratinocytes (Fig 3*A*). A similar staining pattern, although of decreased intensity, was observed within the epidermis of psoriatic skin, and, in addition, PKC-α staining was observed in inflammatory cells within the dermis (Fig 3*B*). In contrast to PKC-α, PKC-β immunoreactivity was observed primarily in the upper two thirds of normal epidermis, with intense staining within a small number of scattered cells (Fig

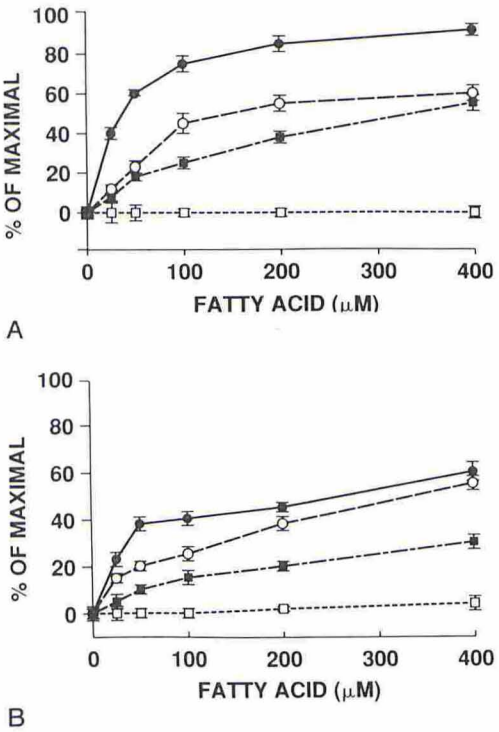

Figure 2. Stimulation of PKC-α and PKC-β from normal human epidermis by free fatty acids. Phosphorylation of histone by partially purified PKC-α *(A)* and PKC-β *(B)* was determined in the presence of the indicated concentrations of free fatty acids. Results are plotted as percent of maximal activity observed in the presence of calcium, phosphatidylserine, and TPA. □, stearic acid; ■, oleaic acid; O, linoleic acid; ●, arachidonic acid. Results are means ± SD of duplicate determinations from one of three representative experiments.

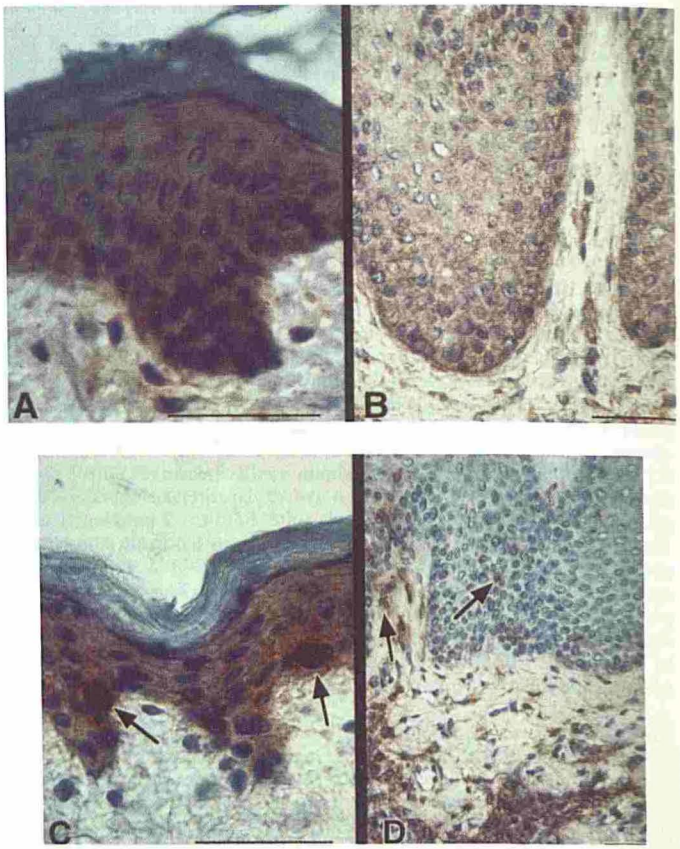

Figure 3. Immunohistochemical localization of PKC-α and PKC-β in normal and psoriatic human epidermis. Frozen sections (5 μm) from normal (*A* and *C*) and psoriatic (*B* and *D*) epidermis were stained with monoclonal antibodies to PKC-α (*A* and *B*) and PKC-β (*C* and *D*) by immunoperoxidase. Sections were counter stained with hematoxalin and eosin. In *C*, *arrows* point to intense PKC-β staining in Langerhans cells in normal epidermis. In panel *D*, *arrows* point to inflammatory cells within the epidermis and dermis in psoriatic lesions. *Scale bar*, 40 μm.

3*C*). In psoriatic skin, PKC-β staining was greatly diminished within the epidermis. Intense focal staining of cells observed in normal epidermis was essentially absent (Fig 3*D*). Prominent staining of inflammatory cells within the dermis and epidermis, which was absent in normal skin, was observed in psoriatic skin.

The location and appearance of the intensely PKC-β staining cells observed in normal epidermis identifies them as Langerhans cells. To determine whether loss of Langerhans cell PKC-β immunoreactivity in psoriasis was due to reduced numbers of Langerhans cells, or to reduced Langerhans cell PKC-β expression, tissue sections of normal and psoriatic skin were stained with antibodies to CD1a, a specific marker for epidermal Langerhans cells. The morphology and location within the epidermis of CD1a-positive Langerhans cells were similar in normal epidermis and psoriatic lesions (data not shown). There was no difference in the number of CD1a-positive Langerhans cells in tissue sections from normal epidermis (14.9 ± 0.5/HPF, $n = 7$) and psoriatic lesions (15.7 ± 1, $n = 7$). Taken together, the above data demonstrate that although CD1a-positive Langerhans cells are present in normal numbers in psoriatic skin, they express little, if any, immunoreactive PKC-β.

The intensity of Langerhans cell PKC-β staining suggests that Langerhans cells, which comprise only 1–2% of the total cells in human epidermis, may contain a disproportionately large amount of PKC activity. To determine the contribution of Langerhans cell PKC-β to the total calcium-dependent PKC activity in normal epidermis, epidermal cell suspensions were depleted of Langerhans cells by immunoabsorption onto magnetic beads. In three experiments, Langerhans cells depletion resulted in 12–25% decreases in the total calcium-dependent PKC activity (Table I).

mRNA Levels of Calcium-Dependent and -Independent PKC Isozymes in Normal and Psoriatic Epidermis The above data indicate that human epidermis expresses PKC-α and -β, but not PKC-γ, and that PKC-β is preferentially decreased in psoriatic epidermis. We next examined expression of the calcium-independent PKC isozymes in normal and psoriatic epidermis. PKC mRNA expression was determined by reverse transcription/polymerase chain reaction, using primers specific for eight human PKC isozymes. Polymerase chain reaction products of the correct sizes were obtained for PKC-α, as expected, and for PKC-βI, PKC-βII, PKC-δ, PKC-ϵ, PKC-ζ, and PKC-η (Fig 4). Consistent with biochemical studies, no mRNA for PKC-γ was detected (data not shown). PKC-βII mRNA was detected in all samples examined, whereas that of PKC-βI was detectable in approximately one-half of the samples. The frequency of occurrence of PKC-β1 mRNA was similar in normal and psoriatic epidermis (data not shown). The authenticity of each polymerase chain reaction product was confirmed by digestion with two restriction enzymes (data not shown). Northern analysis of polyA+ RNA from pooled samples of normal and psoriatic epidermis (pooling of biopsies from three to four individuals was necessary to obtain sufficient quantities of total RNA to prepare polyA+ RNA) revealed transcripts for each of the PKC isozymes identified by polymerase chain reaction (Fig 5). The sizes of the transcripts were similar to those reported for PKC isozymes from humans and rodents [28–34]. Transcripts from PKC-δ, -ζ, and -η were detectable on Northern blots of total RNA from normal and psoriatic epidermis, whereas those for PKC-α, -β, and -ϵ were

Table I. Effect of Langerhans Cell Depletion on Epidermal PKC Activity[a]

	Total Activity (nmol/min)	Total Protein (mg)	Specific Activity (nmol/min/mg)	Relative % Decrease of Total Activity
Experiment 1				
Control	0.46	0.83	0.56	25.2%
Depleted	0.46	1.1	0.42	
Experiment 2				
Control	0.62	0.59	1.05	12.3%
Depleted	0.45	0.49	0.92	
Experiment 3				
Control	1.18	0.52	2.28	19.2%
Depleted	0.78	0.42	1.85	

[a] Epidermal cell suspensions were prepared from keratome biopsies of normal human skin by trypsinization. Cells ($8 - 10^7$) were incubated with magnetic beads coated with anti-CD1a antibody or isotype IgG (control) to adsorb CD1a+ Langerhans cells. Beads were removed with a magnet, and control and Langerhans cell-depleted cell suspensions were homogenized and assayed for protein kinase C activity as described in *Materials and Methods*.

not (data not shown), suggesting that these latter mRNAs were less abundant. In the pooled samples shown, no apparent differences in relative band intensities between normal and psoriatic epidermis were observed for any of the PKC isozymes.

Because it is difficult to prepare sufficient amounts of polyA+ RNA, needed for Northern analysis, from a single individual's skin biopsy, PKC isozyme mRNA levels in normal and psoriatic individuals were determined by reverse transcriptase/polymerase chain reaction, under quantitative conditions. This method utilizes total RNA and is at least 1,000 times more sensitive than Northern analysis. mRNA levels for each of the six PKC isozyme genes expressed in human skin were determined in biopsies from six normal and six psoriatic subjects. This analysis revealed no significant differences between normal and psoriatic epidermis, for any of the PKC isozymes (data not shown). These data indicate that the observed decrease in PKC-α and -β activity and protein in psoriasis (Fig 1) is not associated with a corresponding decrease in their mRNA levels.

Protein Levels of Calcium-Independent PKC Isozymes in Normal and Psoriatic Epidermis We next determined the relative protein levels of the calcium-independent PKC isozymes in biopsies from normal and psoriatic epidermis. Biopsies were separated into soluble and particular fractions and PKC isozymes measured by immunoblot. PKC-δ was equally distributed between the soluble and membrane fractions, PKC-ϵ was predominantly found in the particulate fraction, whereas PKC-ζ and -η were mostly in the soluble fraction (Fig 6). The levels of PKC-δ, PKC-ϵ, and PKC-η were similar in normal and psoriatic epidermis. In contrast, soluble PKC-ζ was elevated twofold in psoriatic, compared to normal, epidermis (Fig 6).

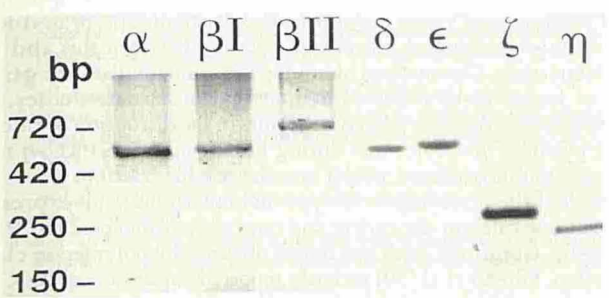

Figure 4. PKC isozyme mRNA expression in normal human epidermis. Total RNA (1 μg) from normal human epidermis was reverse transcribed as described in *Materials and Methods*. Sequences of PKC isozymes $\alpha, \beta, \gamma, \delta, \epsilon, \eta$, and ζ were amplified by polymerase chain reaction, using PKC isozyme-specific primers. Reaction products were separated by agarose gel electrophoresis. The migration positions of DNA size standards is shown on the *left*.

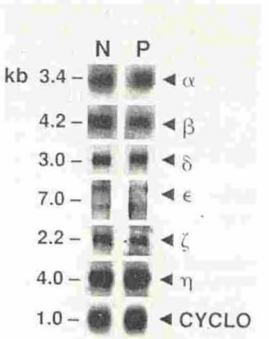

Figure 5. Northern blot analysis of PKC isozymes in normal and psoriatic epidermis. PolyA+ RNA (10 μg/lane) was prepared from three pooled biopsies from normal (N) and psoriatic (P) skin, and hybridized with cDNA probes for PKC-$\alpha, \beta, \delta, \epsilon, \eta$, and ζ, generated by polymerase chain reaction (see Fig 6). The size of the major transcripts for each PKC isozyme is shown on the *left*.

PKC Isozyme Expression in Individual Cell Types in Human Skin The above data demonstrate that normal and psoriatic human epidermis express PKC isozymes α, βI, βII, δ, ϵ, ζ, and η. As epidermis is composed primarily of three different cell types, keratinocytes, melanocytes, and Langerhans cells, we determined the PKC isozyme composition in each cell type. For this purpose primary cultures of keratinocytes and melanocytes were propagated from biopsies of normal human epidermis. Because it is not possible to culture pure Langerhans cells, these cells were isolated from fresh epidermal cell suspensions by immunoabsorption on magnetic beads. The presence of transcripts for each of eight PKC isozymes in these three cell types was determined by reverse transcriptase/polymerase chain reaction. Keratinocytes were found to express transcripts for each of the six PKC isozymes present in human epidermis, PKC-α, -β, -δ, -ϵ, -ζ, and -η (Fig 7*A*). Melanocytes and Langerhans cells, however, expressed distinct subsets of these enzymes. Melanocytes contained transcripts for PKC-α, PKC-β, PKC-δ, and PKC-ζ (Fig 7*B*), whereas Langerhans cells expressed predominantly PKC-βII, PKC-δ and PKC-ζ (Fig 7*C*). These data indicate that within human epidermis PKC-ϵ and PKC-η are expressed exclusively by keratinocytes, whereas the remaining four PKC isozymes are expressed in two or more cell types.

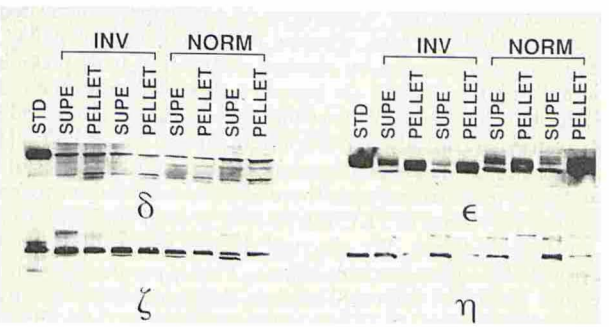

Figure 6. Immunoblot analysis of calcium-independent PKC isozymes in normal and psoriatic epidermis. Normal and psoriatic epidermis were separated into soluble (SUPE) and particulate (PELLET) fractions and analyzed for PKC-δ, ϵ, ζ, and η, using specific polyclonal antibodies. Each lane contained equal amounts of protein (50 mg) from normal (NORM) and psoriatic (INV) epidermis. Results shown are from two normal and two psoriatic individuals. Similar results were observed in a total of six normal and six psoriatic samples. STD, recombinant PKC isozyme. The migration position of molecular weight standards is shown on the *left*.

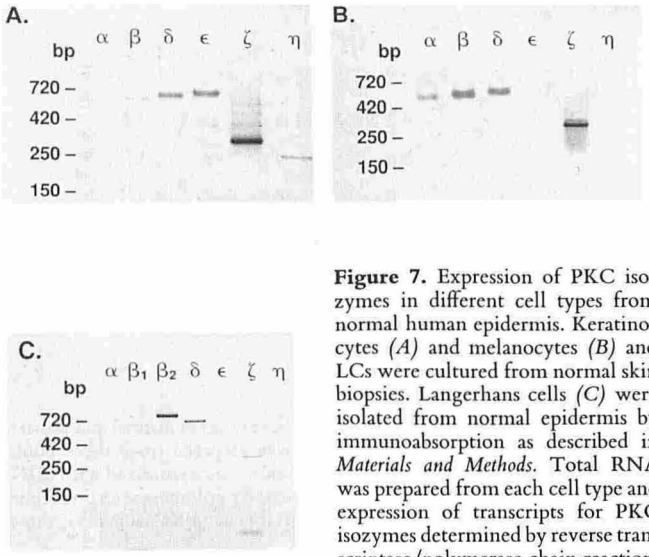

Figure 7. Expression of PKC isozymes in different cell types from normal human epidermis. Keratinocytes *(A)* and melanocytes *(B)* and LCs were cultured from normal skin biopsies. Langerhans cells *(C)* were isolated from normal epidermis by immunoabsorption as described in *Materials and Methods.* Total RNA was prepared from each cell type and expression of transcripts for PKC isozymes determined by reverse transcriptase/polymerase chain reaction. The migration of positions of DNA size standards is shown on the *left.*

DISCUSSION

Skin is well known to be a sensitive target for the actions of tumor-promoting phorbol esters such as TPA. The biologic effects of these agents are primarily mediated by PKC. Topical treatment of human or rodent skin with TPA results in a robust response characterized by keratinocyte activation (i.e., both growth and terminal differentiation are accelerated) and inflammation [35,37]. Our demonstration that six of the genes that encode members of the PKC family are expressed within human epidermis provides insight into the molecular basis for the actions of TPA in skin, and provides evidence for the potential complexity of PKC-mediated signaling pathways in this tissue.

The enzymatic properties of human skin PKC-α and -β were similar to those reported for these PKC isozymes in a variety of other tissues [38,39]. The observed activation of PKC-α and -β by unsaturated fatty acids, particularly arachidonic acid, is of interest because arachidonic acid levels are highly elevated in psoriatic skin [40]. The levels of arachidonic acid in psoriatic epidermis are approximately 50 μM, which *in vitro* was sufficient to activate epidermal PKC-α and -β to 100% and 60% of maximal, respectively. Recent evidence indicates that arachidonic acid preferentially activates soluble, rather than membrane-associated, PKC [41]. In psoriatic epidermis, therefore, soluble PKC-α and -β may be activated by elevated arachidonic acid, whereas membrane-associated PKC-α and -β may be activated by elevated DAG. We have previously reported that DAG levels are increased threefold in psoriatic skin [14]. In addition, recent evidence indicates that arachidonic acid and DAG can synergistically activate protein kinase C-β, -ϵ, and -γ [42].

Because many of the biochemical and histologic features of the skin disease psoriasis resemble those of TPA-treated skin, we compared the expression of PKC isozymes in normal epidermis to that of psoriatic epidermis. We observed that the major difference was decreased PKC-β in psoriatic lesions. This is consistent with our previous finding of decreased calcium-dependent PKC activity in psoriatic epidermis [15]. Unexpectedly, in view of their relatively small number, Langerhans cells were found to be a significant source of PKC-β in normal epidermis. These cells, which represent 1–2% of the total epidermal cells, contained approximately 20% of the total calcium-dependent PKC activity (i.e., PKC-α + PKC-β). Given that PKC-α and PKC-β are present in a ratio of approximately 1:2 in normal epidermis, and that Langerhans cells do not express PKC-α, Langerhans cells account for approximately 30% of the total PKC-β in normal human epidermis. This indicates that on a per-cell basis Langerhans cells contain 10–20 times more PKC-β than keratinocytes. This conclusion is consistent with data demonstrating high levels of PKC-β expression in Langerhans cells in mouse skin [73].

In psoriatic skin, Langerhans cells PKC-β was virtually absent, as determined by immunohistochemistry. Although this may have been due to altered epitope availability rather than decreased PKC-β protein, additional biochemical and immunologic data demonstrated that this was not the case. Chromatographic resolution of calcium-dependent PKC isozymes revealed preferential loss of PKC-β activity in psoriatic epidermis, and this finding was consistent with reduced PKC-β as determined by immunoblot analysis. The physiologic significance of the preferential loss of PKC-β from Langerhans cells in psoriasis remains to be determined. It is especially intriguing, however, in view of accumulating evidence identifying the importance of the immune system in the pathophysiology of psoriasis. Findings from clinical and laboratory studies suggest that abnormal T-cell activation serves as a key mechanism in this disease [44,45]. Because Langerhans cells function, similar to macrophages, to process and present antigens to T cells, they are intimately involved in the regulation of T-cell activation. Evidence suggests that PKC is involved in macrophage activation (46–48] and may therefore function similarly in Langerhans cells.

Reduced PKC-β in Langerhans cells in psoriatic epidermis may reflect PKC activation, with subsequent downregulation. Activation of PKC increases its susceptibility to proteolytic degradation, resulting in reduced PKC levels in a process termed "downregulation." We [48] have previously demonstrated that calcium-dependent PKC activity is downregulated in mouse and human skin following topical treatment with TPA. Alternatively, reduced PKC-β in Langerhans cells in psoriatic epidermis may reflect reduced synthesis, although the finding that PKC-β mRNA levels were not reduced suggests that this is less likely. It is also possible that the reduction of PKC-β in Langerhans cells, rather than in addition to its activation, may have important consequences for Langerhans cell function in psoriasis. Elucidation of this issue must await further studies.

Within keratinocytes, PKC-α was expressed predominantly in the lower epidermal layers, whereas PKC-β was present in the more mature cells in the upper epidermal layers. This suggests that the expression of these two calcium-dependent PKC isozymes may be regulated as a function of keratinocyte maturation, and may therefore act to transduce signals in a differentiation-specific manner. Similarly, the calcium-independent PKC isozymes may also be expressed at different stages of keratinocyte maturation. Our attempts to localize the calcium-independent PKC isozymes within the epidermis by immunohistochemistry using the polyclonal antibodies used for immunoblot were unsuccessful due to high background. This is a common problem encountered when staining skin sections with rabbit antibodies, due to cross reactivity to skin keratins.

In normal and psoriatic skin, PKC-η was predominantly found in the soluble fraction. This is in contrast to a recent report demonstrating nuclear localization of PKC-η in A-431 cells [49]. This difference may be due to differential localization of PKC-η in normal keratinocytes versus transformed epidermoid cells, or to differences in preparation of subcellular fractions between this and the previous study. Our method of homogenization involves the grinding of frozen tissue followed by thawing. In separate studies, we have observed that this procedure results in the disruption of nuclei. It is possible, therefore, that during homogenization PKC-η may have been released from nuclei into the soluble fraction.

Each of the three major cell types in human epidermis expressed at least one calcium-dependent and one calcium-independent PKC isozyme. Consistent with our results obtained by polymerase chain reaction, Gherzi et al [50] recently reported expression of PKC-α, -δ, and -η mRNA in cultured human keratinocytes using Northern analysis. These investigators failed, however, to detect mRNA for PKC-β, -ϵ, and -ζ. This may have been due to lower sensitivity, as oligonucleotide probes based on mouse PKC isozyme cDNA sequences were utilized. Co-expression of calcium-dependent and independent PKC isozymes occurs in many cell types, and suggests that PKC isozymes possess functional specificity [1,8,9]. Increased

levels of DAG and arachidonic acid present in psoriatic lesions provide potential mechanisms through which PKC may be activated *in vivo* in psoriasis. Recent clinical studies demonstrating the efficacy of cyclosporine A and other immunosuppressive agents in the treatment of psoriasis have provided compelling evidence in support of a prominent role of activated T cells in the maintenance, and perhaps the initiation, of psoriatic lesions. Antigen-presenting cells, including Langerhans cells, in psoriatic lesions express cell-surface antigens associated with an activated phenotype. We speculate that PKC-β participates in the transduction of signals involved in the regulation of Langerhans cell activation, and that chronic activation leads to PKC-β downregulation. PKC activation may therefore be a key participant in both the misregulation of epidermal cell homeostasis and aberrant cellular immunity characteristic of psoriatic lesions.

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