Translocation and Downregulation of Protein Kinase C Isoenzymes-α and -ε by Phorbol Ester and Bryostatin-1 in Human Keratinocytes and Fibroblasts

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Protein kinase C isoenzymes can be subdivided into two classes, based on their requirement for calcium. Protein kinase C-α, -βI, -βII, and -γ are calcium dependent, whereas protein kinase C-γ, -ε, -ζ, -η, and -θ are calcium independent. We have examined the expression, translocation, downregulation, and activation of calcium-dependent and -independent protein kinase C isoenzymes in human skin keratinocytes and fibroblasts. Human keratinocytes and fibroblasts expressed protein kinase C-α, -δ, -ε, and -ζ mRNA and protein, whereas protein kinase C-η (L) was detected only in keratinocytes. Protein kinase C-βI, -βII, -γ, and -θ were not detected in either cell type. The protein kinase C activators 12-0-tetradecanoylphorbol 13-acetate and bryostatin-1 (50 nM, for 5 min) induced translocation of protein kinase C-α and -ε cytosol to membrane in both keratinocytes and fibroblasts. 12-0-tetradecanoylphorbol 13-acetate and bryostatin-1, for 18 h, induced complete downregulation (i.e., loss) of protein kinase C-α and -ε in keratinocytes, but only partial downregulation was observed in fibroblasts. The subcellular distribution of protein kinase C-δ, -ζ or protein kinase C-η, in keratinocytes or fibroblasts, did not change in response to 12-0-tetradecanoylphorbol 13-acetate or bryostatin-1. These data indicate differential expression, subcellular distribution, and regulation of protein kinase C isoenzymes in human skin cells. *J Invest Dermatol 103:364–369, 1994*

Protein kinase C (PKC) is a multigene family, encoded by at least nine genes, which plays a key role in signal transduction [1–3]. On the basis of molecular structure and biochemical characteristics, PKC isoenzymes may be classified into conventional isoenzymes (PKC-α, -βI, -βII, and -γ), which require Ca^{++} for activity, novel isoenzymes (PKC-δ, -ε, -ζ, -η (L), and -θ), which are Ca^{++} independent, and atypical isoenzymes, (PKC-ζ and PKC-ι[λ]), which are Ca^{++} independent and are not activated by 1,2-diacylglycerol (1,2-DAG) [1,2]. Conventional and novel PKC isoenzymes are activated by 1,2-DAG, generated from membrane phospholipids in response to activation of cell surface receptors by a variety of extracellular mediators [1,4]. The mechanism of atypical PKC activation is unknown. Conventional and novel PKC isoenzymes are the major cellular receptors for tumor-promoting phorbol esters, such as 12-0-tetradecanoylphorbol-13-acetate (TPA), which bind to and activate PKCs in a manner analogous to 1,2-DAG. PKC isoenzymes exhibit kinetic and substrate specificities *in vitro* and vary in their degree of activation by unsaturated fatty acids [1,5,6]. In addition, PKC isoenzymes display selective organ and cellular expression [2,7].

Depending on the cell type and its state of differentiation, PKC activation may either stimulate proliferation or reduce proliferation and stimulate differentiation. For example, TPA is mitogenic for murine fibroblast cell lines [8], whereas TPA stimulates differentiation, with concomitant reduced cell growth, in human keratinocytes [9], HL-60 cells [10,11], and GH_4 cells [12]. Studies in which specific PKC isoenzymes have been overexpressed in cells have provided evidence for the role of distinct isoenzymes in the control of growth and differentiation. For example, overexpression of PKC-βI in murine or rat fibroblast cell lines [13], PKC-βII antisense oligonucleotide in human erythroleukamic cell lines [14], and a dominant negative mutant of PKC-ζ in Swiss 3T3 cells [15], have demonstrated critical roles for PKC-βI, PKC-βII, and PKC-ζ in mitogenic signaling. On the other hand, in CHO cells, overexpression of PKC-δ resulted in arrest of cell division [16].

In vitro bryostatin-1 binds to and activates PKC in a manner similar to TPA [8,17]. When added to cells, however, bryostatin-1 does not always elicit responses similar to TPA. In cultured murine fibroblast cell lines, TPA and bryostatin-1 are both strongly mitogenic [8], whereas in human keratinocytes TPA induces growth arrest and terminal differentiation, as assessed by increased cholesterol sulfotransferase and type I transglutaminase activities and bryostatin-1 does not affect keratinocyte growth or induce terminal differentiation [9,18]. Similarly, in HL-60 [10,11] and GH_4 cells [12], TPA induces growth arrest and differentiation, whereas bryostatin-1 does not. In human dermal fibroblasts we have found that neither TPA or bryostatin-1, alone or in the presence of mitogenic growth factors (insulin and platelet-derived growth factor), affected DNA synthesis (unpublished observation). Furthermore, bryostatin-1 inhibits many TPA-induced responses [8,10], including growth arrest and terminal differentiation of keratinocytes [9,19], and tumor promotion in mouse skin [20]. The mechanisms for the divergent effects of TPA and bryostatin-1 are unknown; however, both qualitative and quantitative differences between TPA and

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Abbreviations: 1,2-DAG, 1,2-diacylglycerol.

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Table I. Protein Kinase C Isoenzyme Primer Sequences

Primer	Sequence (5′ → 3′)	Product Size (bp)
PKC-α 5′	GTCGGCAACAAAGTCATCAGTCCCTCTGAAGACAGGAAACAA	524
PKC-α 3′	TTTTAACGACTGAAACCCTACACGTTCCTT	
PKC-βI 5′	AAGGGCATCATTTACCGTGAC	521
PKC-βI 3′	TGGAGGTTGAAGCTGTTTCTC	
PKC-βII 5′	AAGGGCATCATTTACCGTGAC	641
PKC-βII 3′	AATTTTGGGCTCAGTTCTCG	
PKC-γ 5′	GGTTCTACTCACCTCATACAATTCCAGGGGGTAGTT	561
PKC-γ 3′	GTGTGTCGCGGCCACGTGCAAGTAGAGCGTCGTTCTTGT	
PKC-δ 5′	GTGGATTGCAAACAGTCTATGCGCAGTGAG	525
PKC-δ 3′	TCTAGTCTGAGTCGGAGGAGTCTCGGA	
PKC-ε 5′	GACAATGAAGAGCGTGTGTTCAGGGAACGC	578
PKC-ε 3′	TAACGACCACGGCTCAGGGGCGTCTGACGA	
PKC-ζ 5′	CTAATGTTTGAGATGATGGCTGGG	267
PKC-ζ 3′	GTATCTGACCCTGAACGACCTCTT	
PKC-η 5′	CCAGCTGAACCATCGCCAAATAGA	189
PKC-η 3′	ACACAGAGGTCTTAACGTTGG	
PKC-θ 5′	AACCATGTCGCCATTTCT	241
PKC-θ 3′	CACGTTTTTGCCTTTCAC	

bryostatin-1–induced PKC activation and patterns of substrate phosphorylations have been observed and provide possible explanations [21–23].

PKC regulates epidermal growth, differentiation, and tumor promotion. Thus, skin provides a useful model system to study PKC-mediated signal transduction. Calcium-dependent and -independent PKC activities are detectable in human skin [24,25] and misregulation of PKC signaling has been implicated in the pathogenesis of the hyperproliferative skin disease psoriasis [26,27]. To better understand the role of PKC in skin, we have examined the expression of calcium-dependent and -independent PKC isoenzymes in normal human keratinocytes and fibroblasts at the mRNA and protein levels. In addition, we evaluated whether the divergent actions of TPA and bryostatin-1 on human keratinocytes and fibroblast growth and differentiation could be explained by differential effects on PKC isoenzyme translocation and activation.

MATERIALS AND METHODS

Materials TPA was from Sigma. Bryostatin-1 was a generous gift of Dr. G. R. Petitt. Monoclonal antibodies to PKC-α and -β (which recognizes PKC-βI and -βII) were purchased from Seikagaku. Polyclonal isoenzyme-specific antibodies to PKC-γ, -δ, -ε, -ζ, and -η were prepared in rabbits using synthetic peptides [28]. Recombinant human PKC isoenzyme protein standards were prepared using the baculovirus/Sf9 insect cell expression system [28].

Cell Culture Keratinocytes and dermal fibroblasts were cultured from keratome biopsies of adult human skin, as previously described [27,29].

Reverse Transcriptase/Polymerase Chain Reaction Total RNA was prepared from cultured cells by the isothiocyanate-chloroform method using a commercially available kit (Stratagene) according to the manufacture's instructions. RNA was reversed transcribed, using random hexamer primers (2.5 μM) and 2.5 U/μl Moloney murine leukemia virus reverse transcriptase (Perkin Elmer Cetus) at 42°C for 15 min. 5′ and 3′ PKC isoenzyme-specific primers (50 pmol each) were then added and polymerase chain reaction (PCR) amplification carried out for 32 cycles (95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds with 3 seconds extension per cycle). PCR products were electrophoresed in gels containing 1.5% agarose (Gibco BRL) and 1.5% NuSieve (FMC), stained with ethidium bromide, visualized with ultraviolet transillumination, and photographed. Sequences of PCR primers utilized are given in Table I. To validate their authenticity, each PCR product was digested separately with two restriction enzymes, and digests were analyzed by agarose gel electrophoresis.

Western Blot Analysis Cells were placed in growth-factor–free MCDB 153 for keratinocytes and serum-free Dulbecco's minimum essential medium (DMEM) for fibroblasts, for 48 h. Cells were treated with agonists as specified in the text, washed 3 × with ice-cold Ca^{++} and Mg^{++}-free phosphate-buffered saline, and scraped into a buffer (25 mM Tris-HCl, pH 7.5, 2 mM ethylenediaminetetraacetic acid [EDTA], 2 mM ethyleneglycol-bis[β-aminoethyl ether]-N, N, N′, N′ tetraacetic acid [EGTA], 0.5 mM dithiothreitol, 0.02% Triton X-100, 10 mM EDTA, 1 mg/ml leupeptin, 25 μg/ml aprotinin, 25 μg/ml antipain, 25 μg/ml pepstatin, 25 μg/ml chymostatin, and 1 mM phenylmethylsulphonyl fluoride). Samples were homogenized with 20 strokes in a Wheaton glass homogenizer and centrifuged at 100,000 × *g* at 4°C for 1 h. Membrane fractions were dissolved in sodium dodecylsulfate (SDS) sample buffer. Supernatants were concentrated using Centricon 10 concentrators (Amicon Corp.) and mixed with an equal volume (approximately 75 μl) of 2 × SDS sample buffer. Soluble and membrane fractions and PKC isoenzyme standards were separated on 8% polyacrylamide gels [30], and proteins were transferred to nitrocellulose membrane. Blots were blocked overnight in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20, 2.5% powdered milk, and then incubated with anti-PKC antibodies diluted 1 : 200 (PKC-α and -β) or 1 : 1000 (PKC-γ, -δ, -ε, -ζ, and -η). Following washing, immunoreactive proteins were visualized using the enhanced chemiluminescence Western blotting detection system (Amersham). PKC band intensities were quantified by laser densitometry.

Stimulation of PKC Isoenzymes by TPA and Bryostatin-1 Enzymatic activity of partially purified recombinant human PKC isoenzymes was determined as described [31] in the presence of phosphatidylserine (2 μg/ml), and either 1,2-diacylglycerol (2 μg/ml) or the indicated concentrations of TPA or bryostatin-1, using myelin basic protein as substrate (250 μg/ml).

RESULTS

PKC Isoenzyme Expression in Human Keratinocytes and Fibroblasts Using reverse transcriptase/PCR (see Table I for sequences of PCR primers), we determined that keratinocytes express PKC-α, -δ, -ε, -ζ, and -η, (L) mRNA **(Fig 1*A*)** and fibroblasts express PKC-α, -δ, -ε, and -ζ, but not -η transcripts **(Fig 1*B*)**. PKC-βI, -βII, -γ, and -θ transcripts were not found in keratinocytes or fibroblasts, although PKC-βII transcripts were present in keratinocyte cultures containing residual melanocytes (data not shown). As a positive control, PKC-βI and -βII were detectable in enriched preparations of Langerhans cells, PKC-γ was amplified from rat brain mRNA, and PKC-θ was amplified from human peripheral blood mononuclear cells (data not shown). Each PCR product yielded the expected size fragments following digestion with two restriction enzymes, thus confirming the authenticity of each product.

We next examined PKC protein expression in soluble and membrane cell fractions prepared from human keratinocytes and fibroblasts, by Western blot analysis, using isoenzyme-specific monoclonal and polyclonal antibodies. PKC isoenzyme protein expression in keratinocytes **(Fig 2)** and fibroblasts **(Fig 3)** corresponded to the mRNAs expressed **(Fig 1*A, B*)**. PKC isoenzyme proteins were identified by their apparent molecular weight on SDS gels and their co-migration with PKC isoenzyme protein standards (*lane 1* in **Figs 2, 3**). In untreated keratinocytes (**Fig 2,** *lane 2*) and fibroblasts

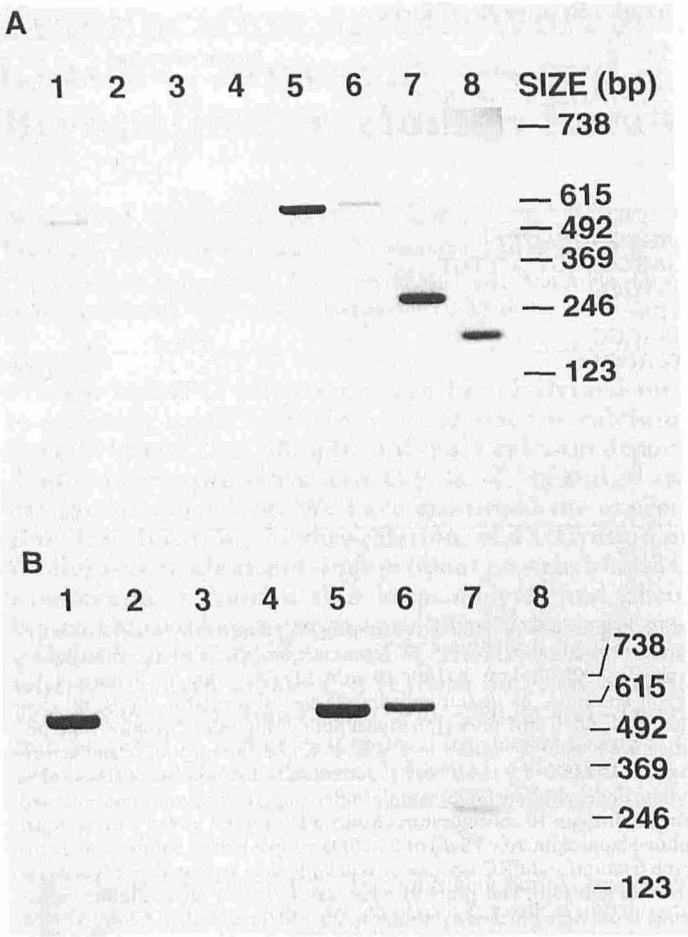

Figure 1. PKC isoenzyme mRNA expression in cultured human keratinocytes *(A)* and dermal fibroblasts *(B)*. mRNA was extracted from cultured cells, reversed transcribed, and PCR performed with isoenzyme-specific primers as described in *Materials and Methods. Lane 1*, PKC-α; *lane 2*, PKC-βI; *lane 3*, PKC-βII; *lane 4*, PKC-γ; *lane 5*, PKC-δ; *lane 6*, PKC-ϵ; *lane 7*, PKC-ζ; *lane 8*, PKC-η (L).

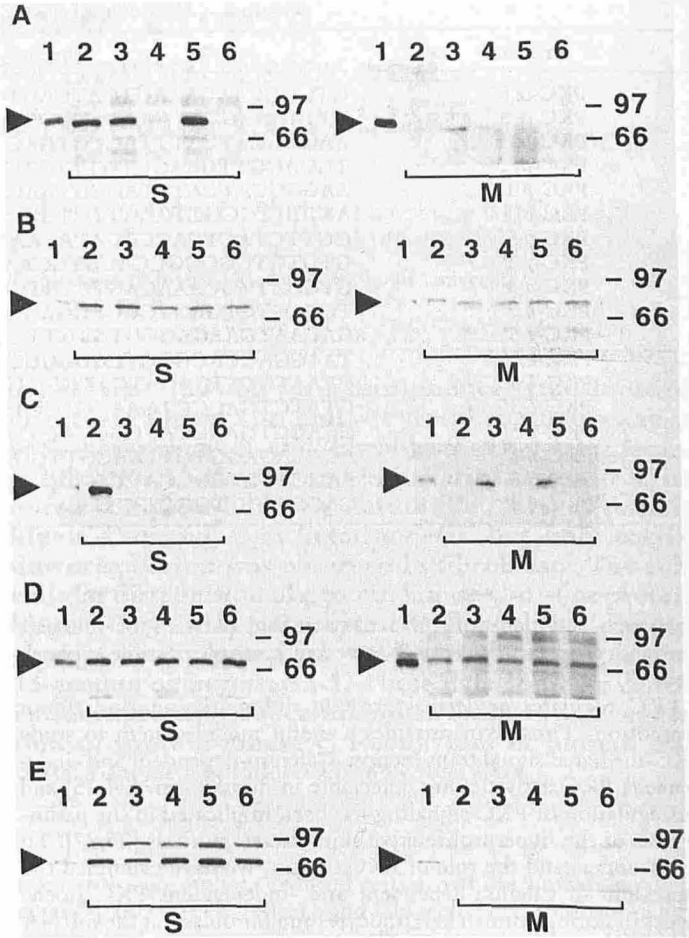

Figure 2. Translocation and downregulation of PKC isoenzymes in human keratinocytes in response to TPA and bryostatin-1. Soluble (S) and membrane (M) cell fractions were prepared from keratinocytes treated with the specified agonists, separated by SDS-PAGE, and immunoblotted as described in *Materials and Methods. A*, PKC-α; *B*, PKC-δ; *C*, PKC-ϵ; *D*, PKC-ζ; *E*, PKC-η. *Lane 1*, PKC isoenzyme standard; *lane 2*, control; *lane 3*, TPA (50 nM) for 5 min; *lane 4*, TPA (50 nM) 18 h; *lane 5*, bryostatin-1 (50 nM) for 5 min; *lane 6*, bryostatin-1 (50 nM) for 18 h.

(**Fig 3,** *lane 2*), PKC-α (**Figs 2*A*, 3*A*,** *lane 2*) and -ζ (**Figs 2*D*, 3*D*,** *lane 2*) were predominantly soluble ($\approx$95% and $\approx$70%, respectively), whereas PKC-δ (**Figs 2*B*, 3*B*,** *lane 2*) was distributed approximately equally between soluble and membrane fractions. In keratinocytes and fibroblasts, PKC-ϵ (**Fig 2*C*,** *lane 2* and **Fig 3*C*,** *lane 2*, respectively) was predominantly soluble (70%–90%), PKC-η in keratinocytes (**Fig 2*E*,** *lane 2*) was also predominantly soluble ($>$ 95%).

Translocation and Downregulation of PKC Isoenzymes in Response to Phorbol Ester and Bryostatin-1 PKC isoenzyme activation is associated with translocation from the cytosol to the membrane, which occurs within minutes of agonist stimulation, and subsequent proteolytic degradation resulting in downregulation, which typically occurs 3–18 h after addition of agonist. TPA and bryostatin-1 exert differential effects on keratinocyte and fibroblast growth and differentiation. We (data not shown) and others [9,18] have demonstrated that TPA, but not bryostatin-1, induces growth arrest and terminal differentiation in human keratinocytes. In contrast, neither TPA nor bryostatin-1, either alone or in the presence of mitogenic growth factors, affected fibroblast growth (data not shown). We therefore examined the effects of TPA and bryostatin-1 on translocation and downregulation of PKC isoenzymes in human keratinocytes and fibroblasts.

Short-term treatment of keratinocytes **(Fig 2)** and fibroblasts **(Fig 3)** with TPA (**Fig 3,** *lane 3*, 50 nM for 5 min) and bryostatin-1 (**Fig 3,** *lane 5*, 50 nM for 5 min) induced partial translocation of PKC-α from the soluble fraction to the membrane. In response to 5 min TPA or bryostatin-1 treatment, less than 10% of PKC-α translocated to the membrane in keratinocytes (**Fig 2*A*,** *lane 3*, and **Table II**), whereas in fibroblasts, TPA or bryostatin-1 induced 35%–65% of PKC-α to associate with the membrane (**Fig 3*A*,** *lane 3*, and **Table III**). In contrast, complete loss of PKC-ϵ from the soluble fraction was observed in both keratinocytes (**Fig 2*C*,** *lanes 3 and 5*, and **Table II**) and fibroblasts (**Fig 3*C*,** *lanes 3 and 5*, and **Table III**), in response to TPA (50 nM) and bryostatin-1 (50 nM) for 5 min. Densitometric analysis of Western blots (data not shown) revealed no significant reproducible changes in the patterns of distribution of PKC-δ, -ζ, or -η in keratinocytes (**Fig 2*B,D,E*,** *lanes 3 and 5*) or fibroblasts (**Fig 3*B,D*,** *lanes 3 and 5*) in response to TPA or bryostatin-1 for 5 min or 15 min (data not shown).

Long-term treatment with TPA or bryostatin-1 (50 nM for 18 h) induced nearly complete downregulation of PKC-α and -ϵ in keratinocytes (**Fig 2*A,C*,** *lanes 4 and 6*, and **Table II**), but only partial downregulation of PKC-α and -ϵ was observed in fibroblasts (**Fig 3*A,C*,** *lanes 4 and 6*, and **Table III**). No consistent downregulation of PKC-δ, -ζ, or -η was observed in response to long-term treatment with TPA or bryostatin-1 in keratinocytes (**Fig 2*B,D,E*,** *lanes 4 and 6*, and data not shown) or fibroblasts (**Fig 3*B,D*,** *lanes 4 and 6*, and data not shown). Borner *et al* [32] observed translocation of PKC-α to the membrane in rat fibroblasts following treatment with 300

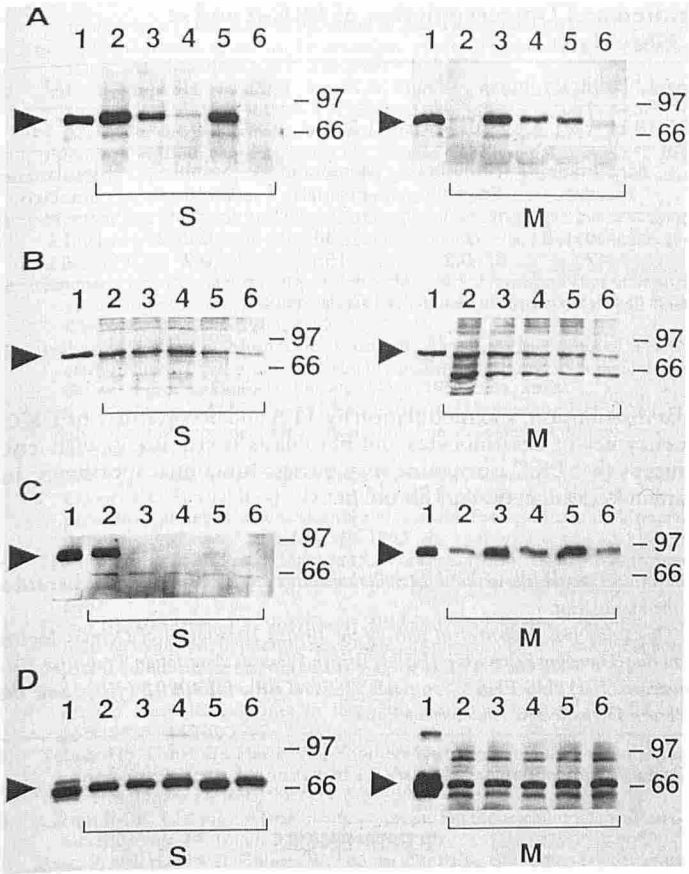

Figure 3. Translocation and downregulation of PKC isoenzymes in human dermal fibroblasts in response to TPA and bryostatin-1. Soluble (S) and membrane (M) cell fractions were prepared from fibroblasts treated with the specified agonists, separated by SDS-PAGE, and immunoblotted as described in *Materials and Methods*. *A*, PKC-α; *B*, PKC-δ; *C*, PKC-ϵ; *D*, PKC-ζ. *Lane 1*, PKC isoenzyme standard; *lane 2*, control; *lane 3*, TPA (50 nM) for 5 min; *lane 4*, TPA (50 nM) 18 h; *lane 5*, bryostatin-1 (50 nM) for 5 min; *lane 6*, bryostatin-1 (50 nM) for 18 h.

nM TPA but not 10 nM TPA. We therefore examined the effects of high-dose TPA and bryostatin-1 treatment on the subcellular distribution of PKC isoenzymes in keratinocytes and fibroblasts. Similar changes in PKC isoenzyme translocation and downregulation were observed with 200 nM TPA or bryostatin-1, compared to 50 nM, in keratinocytes and fibroblasts (data not shown). Thus, the absence of PKC-δ, -ζ, or -η translocation or downregulation is unlikely to be due to low dosage of activators.

Activation of Protein Kinase C-α, -δ, -ϵ, -ζ, and -η *In Vitro* by TPA and Bryostatin-1 Finally, we examined the ability of 1,2-DAG, TPA, and bryostatin-1 to activate *in vitro* each of the PKC isoenzymes expressed in human keratinocytes and fibroblasts. Enzymatic activities of recombinant human PKC-α, -δ, -ϵ, -ζ, and -η were determined under standard assay conditions in the presence of phosphatidylserine alone and with 1,2-DAG, TPA, or bryostatin-1, using myelin basic protein as substrate. PKC-α, -δ, -ϵ, and -η were activated to similar extents by 1,2-DAG, TPA, and bryostatin-1 **(Fig 4)**. Activation of PKC-α, -δ, -ϵ, and -η by TPA and bryostatin-1 was dose dependent between 1 and 50 nM. Interestingly, stimulation of PKC-δ and -ϵ by low-dose (1 nM) TPA or bryostatin-1 was significantly less than for PKC-α and -η. As expected, PKC-ζ was not significantly activated by either TPA or bryostatin-1 compared to phosphatidylserine alone.

DISCUSSION

In this study we report expression of calcium-dependent and -independent PKC isoenzymes in human keratinocytes and fibroblasts. The expression of PKC-α, -δ, -ϵ, -ζ, and -η mRNA and protein, but not PKC-βI, -βII, or -γ, in human keratinocytes is consistent with the reported mRNA expression in mouse keratinocytes [33]. In contrast, Gherzi *et al* [34] reported the presence of PKC-α, -δ, and -η but not βI, -βII, -γ, -ϵ, or -ζ mRNA, by Northern analysis in human keratinocytes cultured as a skin equivalent. Whether the culture conditions account for the difference is unknown. PKC-η was originally cloned from a mouse epidermis cDNA library and was found to be most highly expressed in skin, lung, and heart [35,36]. PKC-η does not, however, appear to be expressed in dermal fibroblasts. Human dermal fibroblasts express PKC isoenzymes similar to rat embryo fibroblasts and Swiss 3T3 cells [32,37]. The presence of Ca^{++}-independent PKC isoenzymes in keratinocytes and fibroblasts provides signaling pathways through which agonists that induce 1,2-DAG formation without increased intracellular Ca^{++} (i.e., through phosphatidylcholine hydrolysis) [38] may act.

A significant proportion of PKC-ϵ in fibroblasts was membrane associated, whereas in keratinocytes PKC-ϵ was nearly completely cytosolic. These findings suggest that the subcellular distribution of PKC isoenzymes is cell type dependent, rather than determined by the specific PKC isoenzyme *per se*. PKC-η has been localized to the nuclear membrane fraction in A431 cells and immortalized keratinocytes [39]. However, in our study PKC-η was almost entirely in the cytosolic fraction. Furthermore, we have been unable to detect PKC-η in nuclear fractions prepared from cultured human keratinocytes (data not shown). Koizumi *et al* have recently reported localization of PKC-η immunostaining in the cytoplasm in keratinocytes in skin [40]. These data suggest that the subcellular distribution of PKC-η may differ between normal human keratinocytes and transformed keratinocyte cell lines.

Of the isoenzymes expressed in keratinocytes and fibroblasts, only PKC-α and -ϵ translocated to the membrane and underwent downregulation in response to TPA and bryostatin-1. In addition, there were differences in responses to TPA and bryostatin-1 between the two cell types studied. Translocation of PKC-α to the membrane in response to TPA and bryostatin-1 was more marked in fibroblasts compared to keratinocytes. Also, downregulation of membrane-associated PKC-α and -ϵ was less complete in fibroblasts compared to keratinocytes. Selective translocation and downregulation of PKC isoenzymes in response to TPA have been reported in other cell types and may reflect differential susceptibility to proteolysis or differences in subcellular localization [41]. Our findings indicate that although long-term treatment with TPA or bryostatin-1 results in decreased Ca^{++}-dependent PKC activity by 90% in

Table II. TPA- and Bryostatin-1–Induced Translocation and Downregulation of PKC-α and -ϵ in Cultured Human Keratinocytes[a]

	Relative PKC Levels[b] (arbitrary units)									
	Control		TPA 5 Min		TPA 18 H		Bryostatin-1 5 Min		Bryostatin-1 18 H	
PKC Isoenzyme	Soluble Fraction	Membrane Fraction	Soluble Fraction	Membrane Fraction	Soluble Fraction	Membrane Fraction	Soluble Fraction	Membrane Fraction	Soluble Fraction	Membrane Fraction
PKC-α	100	0.2	96	5.0	12	0.3	140	3.8	2.9	0.2
PKC-ϵ	100	7.9	0.2	53	0.2	1.5	0.2	43	0.2	20

[a] Values were obtained by densitometry of Western blots as shown in Fig 2, and are representative of three experiments that yielded similar results.
[b] Values are relative to soluble PKC-α or -ϵ, which were set to a value of 100.

Table III. TPA- and Bryostatin-1–Induced Translocation and Downregulation of PKC-α and -ε in Cultured Human Fibroblasts[a]

	Relative PKC Levels[b] (arbitrary units)									
	Control		TPA 5 Min		TPA 18 H		Bryostatin-1 5 Min		Bryostatin-1 18 H	
PKC Isoenzyme	Soluble Fraction	Membrane Fraction	Soluble Fraction	Membrane Fraction	Soluble Fraction	Membrane Fraction	Soluble Fraction	Membrane Fraction	Soluble Fraction	Membrane Fraction
PKC-α	100	6.1	40	70	17	36	78	38	0.2	11
PKC-ε	100	34	0.2	112	0.2	47	0.2	155	0.2	61

[a] Values were obtained by densitometry of Western blots as shown in **Fig 3**, and representative of three experiments that yielded similar results.
[b] Values are relative to soluble PKC-α or -ε, which were set to a value of 100.

human keratinocytes and fibroblasts [42], Ca^{++}-independent isoenzymes are still present. Thus, long-term pretreatment of keratinocytes or fibroblasts with TPA or bryostatin-1 cannot be utilized to identify PKC-independent responses in these (and likely other) cell types, because levels of Ca^{++}-independent PKCs remain relatively unchanged.

TPA induces growth arrest and terminal differentiation in cultured human keratinocytes, whereas bryostatin-1 does not [9,18]. In other cell types, divergent responses to TPA and bryostatin-1 have been associated with selective translocation of PKC-βII to the nucleus [21] or differences in the rate of downregulation of membrane-associated PKC [22,23]. We, found no difference, however, between TPA and bryostatin-1 in induction of PKC isoenzyme translocation or downregulation in human keratinocytes. These data indicate that differential translocation and activation of PKC isoenzymes *per se* are not likely to account for differences in responses of keratinocytes to TPA and bryostatin-1. However, inhibition of TPA-induced growth arrest and terminal differentiation by bryostatin-1, which downregulates PKC-α and -ε, suggests that prolonged activation of PKC-α or -ε, may be required for these processes. In cultured human fibroblasts, TPA and bryostatin-1 do not significantly affect growth or differentiation, but do induce MARCKS protein phosphorylation [38] and also induce translocation of PKC-α and -ε. MARCKS is a prominent cellular PKC substrate, which may function to cross-link filamentous actin to the plasma membrane [43]. In fibroblasts, therefore, PKC may function to regulate cellular functions such as cytoskeletal organization and cell/matrix interactions [44,45] that were not addressed in this study. However, the demonstrated differential expression, subcellular distribution, and modulation by TPA and bryostatin-1 of PKC isoenzymes in keratinocytes and fibroblasts is consistent with the concept that PKC isoenzyme may possess functional specificity in human keratinocytes and fibroblasts.

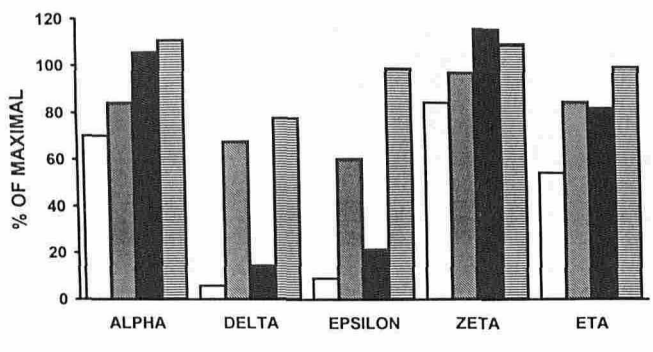

Figure 4. Dose dependent activation of PKC isoenzymes *in vitro* by TPA and bryostatin-1. Partially purified, recombinant human PKC-α (ALPHA), PKC-δ (DELTA), PKC-ε (EPSILON), PKC-ζ (ZETA), and PKC-η (ETA) were assayed for activity in the presence of phosphatidylserine plus 1 nM TPA (□), 50 nM TPA (▨), 1 nM bryostatin-1 (■), or 50 nM bryostatin-1 (▤), as described in *Materials and Methods*. Data are displayed as percent of net 1,2-DAG–stimulated activity for PKC-α, -δ, -ε, and -η, or net phosphatidylserine-stimulated activity for PKC-ζ. The range of net 1,2-DAG–stimulated specific activities (μmol/min/mg) for each isoenzyme was: PKC-α, 45.8–47.4; PKC-δ, 2.2–2.8; PKC-ε, 10.0–11.0; PKC-ζ, 1.0–1.4; PKC-η, 4.0–4.5. Results are representative of three experiments performed in duplicate.

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