0026-895X/94/050840-11$03.00/0

MOLECULAR PHARMACOLOGY, 46:840–850, 1994

Bryostatin 1 Protects Protein Kinase C-δ from Down-regulation in Mouse Keratinocytes in Parallel with Its Inhibition of Phorbol Ester-Induced Differentiation

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Received May 13, 1994; Accepted August 4, 1994

SUMMARY

Bryostatin 1 and phorbol-12-myristate-13-acetate (PMA) are both potent activators of protein kinase C (PKC), although in primary mouse keratinocytes bryostatin 1 does not induce differentiation and blocks PMA-induced differentiation. We report here that in primary mouse keratinocytes PMA caused translocation of PKC-ε to the Triton X-100-soluble fraction with an approximately 2-order of magnitude higher potency, compared with translocation of PKC-α and PKC-δ. The kinetics of translocation were fastest for PKC-ε, slower for PKC-α, and slowest for PKC-δ. At 5–20 min bryostatin 1 showed potency similar to that of PMA for translocating PKC-α, higher potency for translocating PKC-δ, and lower potency for translocating PKC-ε. At a later time (6 hr), bryostatin 1 was 1–2 orders magnitude more potent than PMA for causing loss of PKC-α, -δ, and -ε from the soluble fraction. Bryostatin 1 was 40-fold more potent than PMA for down-regulating PKC-α and showed a biphasic dose-response curve for down-regulating PKC-δ. Bryostatin 1 at 0.1–1 nM down-regulated PKC-δ to a similar extent as did PMA. Bryostatin 1 at 100 nM to 1 μM, on the other hand, failed to induce down-regulation, and these high (100 nM to 1 μM) doses of bryostatin 1 showed noncompetitive inhibition of PKC-δ down-regulation by 1 μM PMA after coapplication. This protected portion of PKC-δ retained kinase activity. The dose-response curve for bryostatin 1 protection of PKC-δ from down-regulation by PMA correlated with bryostatin 1 inhibition of the effects of PMA on cornified envelope formation (a marker of differentiation) and epidermal growth factor binding. Although PKC-ε was readily translocated by both PMA and bryostatin 1, the PKC-ε originally associated with the particulate fraction showed no down-regulation by either of these agents. We hypothesize that differential regulation of PKC isozymes by PMA and bryostatin 1 may contribute to the different patterns of biological responses that they induce.

PKCs are a growing family of isozymes involved in a wide variety of cellular processes (for review, see Ref. 1). PKCs have been implicated in differentiation for a range of cell lines, including human chronic myelogenous leukemia cells (2), Friend erythroleukemia cells (3), HL-60 human promyelocytic and U937 human histiocytic leukemia cells (4, 5), and, as one of the most studied systems, primary mouse keratinocytes (6). Of the 11 isozymes identified so far, PKC isozymes α, β, δ, ζ, η, and γ (in one study) were reported to be present at the protein level in mouse epidermis (7–9). Of these, PKC-α, -δ, -ε, -ζ, and -η are present in keratinocytes and PKC-β is predominantly in Langerhans cells (10, 11).

Down-regulation of PKC upon phorbol ester treatment first was reported as loss of phorbol ester binding (12) and later was confirmed by detection of loss of PKC activity and immunopositivity (13). The role of down-regulation of PKC is unclear. In several systems this down-regulation is thought to play an inhibitory role by eliminating the active enzyme (for review, see Ref. 14). Alternatively, it has been suggested that down-regulation may lead to a biological response, e.g., hyperplasia in mouse skin, by relieving inhibition exerted by the normally present PKC. Because a frequently detected intermediate in the proteolytic breakdown of PKC is the constitutively active catalytic domain, often termed protein kinase M, in some systems proteolysis may represent an alternative activation pathway (14, 15).

There is significant evidence that the activation of PKC, the only phorbol ester receptor thus far identified in this cell type, is intimately involved in the differentiation process in mouse primary keratinocytes (6). The question of involvement of individual PKC isozymes has not been resolved, however. Bryostatin 1, a potent, non-phorbol ester activator of PKC, failed to induce differentiation in primary mouse keratinocytes and inhibited the differentiation induced by phorbol ester (6,

ABBREVIATIONS: PKC, protein kinase C; PMA, phorbol 12-myristate-13-acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

16). This observation raises the possibility that the activation of only certain PKC isozymes induces differentiation and the activation of others might inhibit it. Clarifying the biochemical mechanisms of the unusual actions of bryostatin 1 is urgent, because bryostatin 1 is currently being evaluated as a potential chemotherapeutic agent for several malignancies, including leukemia and melanoma (17–19).

In this paper we report that PMA and bryostatin 1 differentially regulate PKC-α, -δ, and -ε in primary keratinocytes; the differences are consistent with the differential biological responses induced by these agents, and the inhibition by bryostatin 1 of PMA-induced down-regulation of PKC-δ correlates with the inhibition of cornified envelope formation, a marker of differentiation. We also provide evidence that the inhibitory effect of bryostatin 1 is noncompetitive.

Experimental Procedures

Primary keratinocytes were prepared from newborn BALB/c mice by the trypsin flotation technique and were cultured in low-calcium (0.05 mM) Eagle's minimal essential medium containing 8% fetal calf serum and antibiotics, as described previously (20). PMA was purchased from LC Services (Woburn, MA). Bryostatin 1 was isolated from *Bugula neritina* as described (21).

For Western blot analysis of the PKC isozymes, treatments were started 3 days after the cells were plated. At that time they had attained 80–90% confluency. All compounds were applied in ethanol (0.1% final concentration). Western blot analysis was performed as described previously (22). The cells were harvested into 20 mM Tris·HCl, pH 7.4, containing 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μM leupeptin and were lysed by sonication. Total cell lysates were fractionated by ultracentrifugation. The soluble fraction represents the supernatant after a 1-hr centrifugation at 100,000 × *g* at 4°. The Triton X-100-soluble particulate fraction was prepared by a 1-hr extraction of the pellet with the same buffer containing 1% Triton X-100 and subsequent centrifugation for 1 hr at 100,000 × *g*. The remaining pellet is the Triton X-100-insoluble fraction.

The protein samples were subjected to SDS-PAGE according to the method of Laemmli (23) and were transferred to nitrocellulose membranes. Western blots were stained with 0.1% Ponceau S solution in 5% acetic acid (Sigma Chemical Co., St. Louis, MO) for determination of the protein content in individual lanes. The protein staining was found to be linear up to 30 μg of protein/lane. The Ponceau S staining was removed by several washes with phosphate-buffered saline, pH 7.4; the membranes were blocked with 4% milk in phosphate-buffered saline, pH 7.4, and subsequently immunostained for PKC-α, -δ, -ε, -ζ, and -η.

Monoclonal antibodies against the catalytic domain of PKC-α, the regulatory domain of PKC-β, and the regulatory domain of PKC-γ were purchased from Upstate Biotechnology Inc. (Lake Placid, NY) and used at a 2 μg/ml concentration. Affinity-purified polyclonal antibody against the carboxyl terminus (PKC-δ amino acids 662–673) of PKC-δ was purchased from Research and Diagnostics Antibodies (Berkeley, CA) and applied at a 1/50,000 dilution. Polyclonal antibody (referred to as 53784) against the carboxyl terminus of PKC-ε (PKC-ε amino acids 724–737, H_2N-EFGKFSYFGEDLMP) was raised in our laboratory and applied at a dilution of 1/1000. Polyclonal antibody against the carboxyl terminus of PKC-ζ (PKC-ζ amino acids 480–492) was purchased from Research and Diagnostics Antibodies and applied at a dilution of 1/5000. Polyclonal antibody (referred to as 88078) against the carboxyl terminus of PKC-η (PKC-η amino acids 666–683, H_2N-INQDEFRNFSYVSPELQL-OH) was raised in our laboratory and applied at a dilution of 1/1000. The specificity and lack of cross-reactivity of the primary antibodies for PKC isozymes α, β, γ, δ, ε, and ζ were evaluated by the manufacturers and were verified by us for cloned PKC-α, -β, -γ, -δ, -ε, -ζ, and -η expressed in a baculovirus system (24). Polyclonal antibody against a specific sequence of c-*jun* was purchased from Oncogene Science (Uniondale, NY). The blots were incubated overnight at 4° with the indicated amounts of primary antibody dissolved in 4% milk in phosphate-buffered saline. The PKC isozymes were detected with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Richmond, CA) and quantitated as described previously (22). Densitometric analysis was performed under conditions that yielded a linear response, as analyzed using the National Institutes of Health Image 1.52 program (written by Dr. Wayne Raspband, National Institutes of Health).[1] For quantitation of the absolute levels of PKC isozymes in keratinocytes, cloned PKC-α, -δ, and -ε expressed in the baculovirus system (24) were used as controls. The amount of PKC for the controls was determined by measuring the maximum amount of [^{3}H]phorbol 12,13-dibutyrate bound (24) and assuming a stoichiometry of binding of 1:1.

Kinase activity of immunoprecipitated PKC-δ was measured as follows. Primary keratinocytes were treated with 500 nM bryostatin 1 or vehicle for 24 hr. The cells were washed twice in ice-cold phosphate-buffered saline and scraped into immunoprecipitation lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The lysates were centrifuged in a microcentrifuge for 5 min, and PKC-δ in the supernatant (0.75–1.5 mg of protein) was immunoprecipitated for 2.5 hr with 1 μl of anti-PKC-δ antibody (Calbiochem, San Diego, CA) and 50 μl of Protein A-Sepharose. The immunoprecipitates were washed once with lysis buffer and twice with 50 mM Tris·HCl, pH 7.4, and were resuspended in 25 μl of Tris·HCl with 5 mM 2-mercaptoethanol. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into PKC substrate peptide [Ser^{25}]PKC(19–31) (GIBCO-BRL, Gaithersburg, MD) as described by Nakadate *et al.* (25), in the absence of Ca^{2+}, using 20%/80% phosphatidylserine/phosphatidylcholine vesicles. After completion of the reaction, the beads were centrifuged, 25 μl of supernatant were spotted on a phosphocellulose membrane, the membrane was washed, and radioactivity was determined in a scintillation counter. The PKC-δ bound to the beads was boiled in SDS sample buffer and quantitated by SDS-PAGE Western immunoblotting. These values were used to normalize the kinase assay.

Cornification was measured by the cross-linked protein assay of Hough-Monroe and Milstone (26), with minor modifications. Cells were treated with the indicated amount of PMA and/or bryostatin 1 for 48 hr. The attached cells were trypsinized, pooled with the floating cells, and centrifuged at 1000 × *g* for 5 min. The medium was removed and the cell pellet was solubilized by heating at 90° for 10 min in a 2% SDS/20 mM dithiothreitol solution. Aliquots were spotted onto RC60 cellulose filters (Schleicher and Schuell, Keene, NH) and washed two or three times with 2% SDS/20 mM dithiothreitol. The filter paper was air dried, incubated in 7.5% trichloroacetic acid at 80° for 30 min, and subsequently stained with 1% Coomassie blue G-250 in 7% acetic acid for 15 min at 50°. The membrane was washed with 7% acetic acid at 50° until background staining was removed. The stained cross-linked protein remaining on the filter was eluted into 60% methanol/1% NH_3OH and quantitated by measuring absorbance at 600 nm.

The ^{125}I-EGF binding assay was performed as described before (6).

Results

PKC isozyme distribution in control cells. In subconfluent control primary mouse keratinocyte cultures the following PKC isozyme concentrations were measured: PKC-α, 3.0 ± 0.3 fmol/μg of protein; PKC-δ, 1.2 ± 0.16 fmol/μg of protein; PKC-ε, 0.7 ± 0.05 fmol/μg of protein. We did not determine

[1] The program is available electronically from zippy.nimh.nih.gov by anonymous FTP via Internet or from Library 9 of the MacApp forum on CompuServe and is available on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161.

the absolute amount of PKC-ζ because PKC-ζ does not bind phorbol ester and we normalized the amount of recombinant PKC isozymes based on their [^{3}H]phorbol 12,13-dibutyrate binding. We did not detect any PKCβ or -γ. We did not detect any full size PKC-η either, although both PKC-η antibodies that were used (see Experimental Procedures) recognized several bands at lower molecular weight, presumably degradation products of this isozyme (data not shown). The regulation of this isozyme, especially the absence of the full size form despite measurable levels of PKC-η transcripts in these cells (11), is currently under investigation. The absolute distribution of the individual PKC isozymes among the fractions obtained by centrifugation was determined based on the protein levels measured in these fractions (the soluble fraction contains 58 ± 5%, the Triton X-100-soluble particulate fraction contains 10 ± 3%, and the Triton X-100-insoluble fraction contains 30 ± 5% of the total protein). The distribution of the individual isozymes among the soluble, Triton X-100-soluble, and Triton X-100-insoluble fractions was 95%/5%/0% for PKC-α, 65%/35%/0% for PKC-δ, 65%/35%/0% for PKC-ϵ, and 75%/25%/0% for PKC-ζ.

Translocation and down-regulation of PKC-α. PMA caused rapid translocation of PKC-α from the soluble fraction to the Triton X-100-soluble particulate fraction (Figs. 1 and 2A). PMA at 1 μM induced faster translocation than did an equal concentration of bryostatin 1. Complete translocation was induced within 2 min by 1 μM PMA and between 5 and 10 min by 1 μM bryostatin 1 (Fig. 2).

The dose-response curves for translocation were determined using the amounts of isozyme remaining in the soluble fraction. Because of the difference in the kinetics of translocation induced by the two agents, we determined the dose-response curves at two time points, 5 min and 20 min. In *in vitro* systems, at 5 min the binding of PMA to its receptor is maximal (27) and indirect measurements suggest that at 20 min the binding of bryostatin 1 reaches saturation (28). The equivalent amount of PKC-α disappearing from the soluble fraction could be detected in the Triton X-100-soluble particulate fraction at these shorter time points (data not shown). In addition, no down-regulation was observed in the total fraction up to 20 min. At 5 min bryostatin 1 did not induce complete translocation, and at 20 min the potencies of bryostatin 1 and PMA for translocating PKC-α were very similar (Fig. 3, A and B) (ED_{50} for bryostatin 1, 24 nM for translocation at 20 min; for PMA, 65 nM for translocation at 20 min).

The significant shift in the dose-response curves for the translocation of PKC-α suggested that even more PKC-α could be removed and subsequently activated with increasing incubation times. Indeed, the dose-response curves derived from the amount of PKC-α remaining in the soluble fraction continued to shift with time until 6 hr (Fig. 3, A and B), when the amount of PKC-α reached its lowest level in the soluble fraction at all of the concentrations examined (0.1 nM to 1 μM for PMA and 1 pM to 1 μM for bryostatin 1) (data not shown). At later time points these curves do not give a precise measurement of translocation, because after 20 min the amount of isozyme in the total fraction significantly decreases; therefore, it is impossible to monitor whether the isozyme lost from the soluble fraction can be recovered in the Triton X-100-soluble particulate fraction. Interestingly, at 6 hr bryostatin 1 was about 2 orders of magnitude more potent than PMA for removing PKC-α from the soluble fraction.

A 2-order of magnitude difference in potency was also evident in the dose-response curves for down-regulation of PKC-α from the total fraction (Fig. 3C) (ED_{50} for bryostatin 1, 0.8 nM for removal from the soluble fraction and 0.3 nM for down-regulation; for PMA, 40 nM for removal from the soluble fraction and 44 nM for down-regulation). The potencies of PMA and bryos-

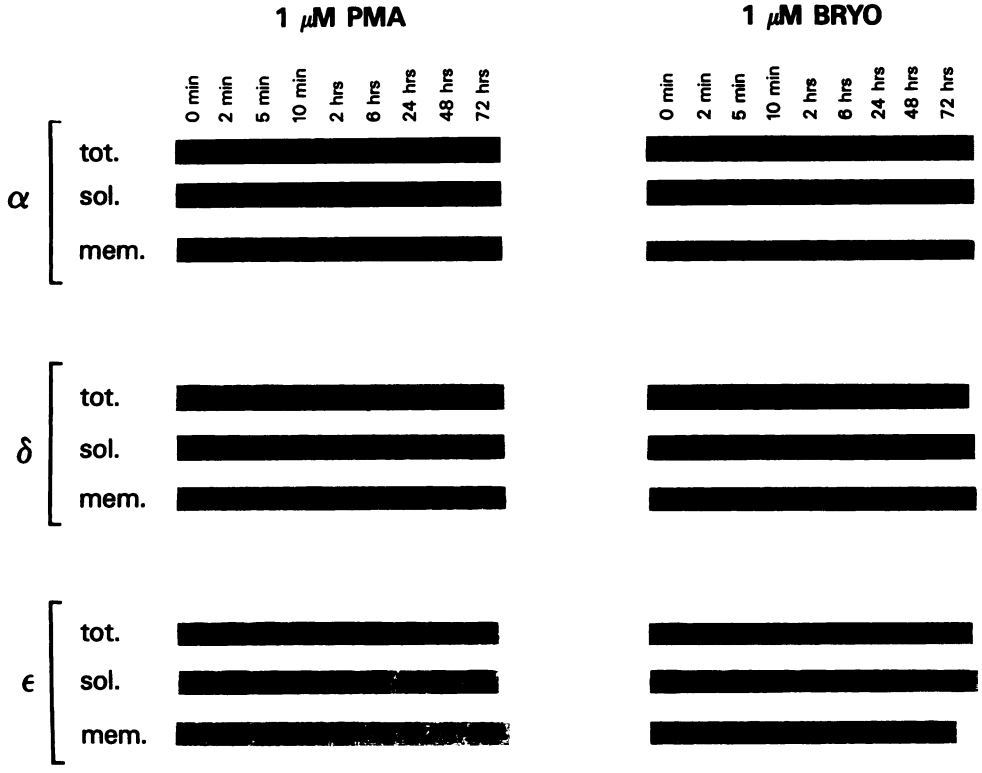

Fig. 1. Translocation and down-regulation of PKC isozymes induced by 1 μM PMA and 1 μM bryostatin 1 (*BRYO*). Primary mouse keratinocytes were treated as indicated for 2, 5, or 10 min or 2, 6, 24, 48, or 72 hr. Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. Equal amounts of proteins were loaded in each lane for the total (10 μg of protein/lane), soluble (10 μg of protein/lane), and Triton X-100-soluble (5 μg of protein/lane) fractions. *tot.*, total fraction; *sol.*, soluble fraction; *mem.*, Triton X-100-soluble fraction. Identical results were obtained in two more independent sets of experiments.

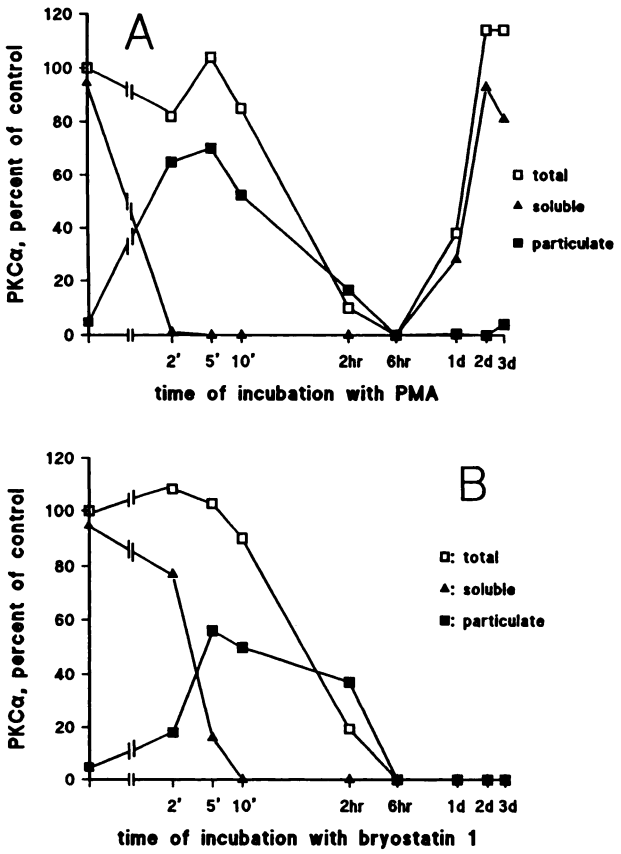

Fig. 2. Translocation and down-regulation of PKC-α induced by 1 μM PMA (A) or 1 μM bryostatin 1 (B). Primary mouse keratinocytes were treated with 1 μM PMA or bryostatin 1 for 2, 5, or 10 min or 2, 6, 24, 48, or 72 hr. Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of enzyme in the total (□), soluble (▲), and Triton X-100-soluble particulate (■) fractions was quantitated by densitometry and expressed as percentage of the total amount of isozyme present in control cells. Time is plotted on a logarithmic scale. Two more experiments yielded almost identical results.

tatin 1 for down-regulation were quantitated at 6 hr, the time giving maximal down-regulation, using the amount of PKC-α remaining in the total fraction. PKC-α remained down-regulated by high doses of bryostatin 1 up to 72 hr, the longest time point examined. In contrast to this, 24 hr after PMA treatment the amount of PKC-α in the soluble fraction started to increase and by 72 hr after treatment the original level of the isozyme was restored at all of the concentrations examined (Figs. 1 and 2A). This was probably due to the metabolism of PMA, because readdition of PMA to the culture induced rapid translocation and down-regulation of the isozyme (data not shown).

Translocation and down-regulation of PKC-δ. High doses of PMA and bryostatin 1 translocated PKC-δ from the soluble fraction to the Triton X-100-soluble fraction very rapidly, within 2–5 min (Figs. 1 and 4, A and B). The amount of PKC-δ was monitored in the total, soluble, and Triton X-100-soluble particulate fractions at all time points (2, 5, 10, and 20 min and 2, 6, 24, 48, and 72 hr) for a wide range of concentrations of PMA (0.1 nM to 1 μM) and bryostatin 1 (1 pM to 1 μM). The dose-response curves for translocation were determined at 5 min and 20 min after treatment, using the amount of PKC-δ remaining in the soluble fraction. (At these time points the amount of PKC-δ removed from the soluble fraction could be recovered from the Triton X-100-soluble particulate fraction.) At both time points bryostatin 1 was about 10-fold more potent than PMA for translocating PKC-δ (Fig. 5, A and B). PKC-δ reached its lowest level in the soluble fraction at 6 hr after treatment at all of the doses examined. The amount of PKC-δ disappearing from the soluble fraction initially appeared in the Triton X-100-soluble particulate fraction, followed by rapid down-regulation. As at shorter time points, bryostatin 1 was more potent than PMA for removing PKC-δ from the soluble fraction at 6 hr (Fig. 5, A and B). Both bryostatin 1 and PMA were more potent for translocating PKC-δ than PKC-α (ED_{50} for bryostatin 1, 2.6 nM at 5 min, 1.5 nM at 20 min, and 0.055 nM at 6 hr; for PMA, 28 nM at 5 min, 15 nM at 20 min, and 0.8 nM at 6 hr). Similarly to PKC-α, the amount of PKC-δ in the soluble fraction started to increase 24–48 hr after PMA treatment, and by 72 hr after treatment the amount of PKC-δ was restored to 50–100% of the original level of the isozyme, depending on the concentration examined.

Both the portion of PKC-δ translocated by PMA from the soluble fraction to the Triton X-100-soluble particulate fraction and the portion originally present in the Triton X-100-soluble particulate fraction were down-regulated upon PMA treatment. Down-regulation was maximal at 24 hr after PMA treatment both in the total fraction and in the Triton X-100-soluble particulate fraction, with an ED_{50} of 1 nM for the total fraction and 0.7 nM for the Triton X-100-soluble particulate fraction (Fig. 5, C and D).

We completed time-response curves (2 min to 72 hr) over a wide range of bryostatin 1 concentrations (10 pM to 1 μM). The down-regulation by bryostatin 1 of PKC-δ in the Triton X-100-soluble particulate fraction followed a biphasic dose-response curve. Maximal down-regulation was achieved by the application of 0.1–1 nM bryostatin 1, whereas 100 nM to 1 μM or 1–10 pM bryostatin 1 failed to down-regulate PKC-δ. The amount of PKC-δ in the Triton X-100-soluble particulate fraction reached its peak 2–5 min after treatment with bryostatin 1. Subsequently the amount of isozyme steadily decreased, leading to nearly complete down-regulation by 1 nM bryostatin 1 at 24 hr (Figs. 4C and 6A). The biphasic down-regulation of PKC-δ at 24 hr in the total and Triton X-100-soluble particulate fractions is shown in Fig. 5, C and D. Note that the amount of PKC-δ remaining in the total fraction after 24 hr of treatment with high doses (100 nM to 1 μM) of bryostatin 1 is approximately equal to the portion originally associated with the Triton X-100-soluble particulate fraction.

Translocation and down-regulation of PKC-ε. PKC-ε, similarly to PKC-α and PKC-δ, was quickly translocated from the soluble fraction to the Triton X-100-soluble particulate fraction. Two minutes after treatment with 1 μM PMA or the same dose of bryostatin 1, no isozyme was left in the soluble fraction (Fig. 1). We determined the dose-response curves for translocation at 5 min after treatment. PMA was significantly more potent than bryostatin 1 for translocation at this time point (Fig. 7, A and B) (ED_{50} for bryostatin 1, about 2.2 nM; for PMA, about 0.4 nM). We also determined the potency of both ligands to remove PKC-ε from the soluble fraction at a later time point (6 hr), when the amount of the isozyme reached its lowest level at all of the concentrations examined. At this time bryostatin 1 was about 10-fold more potent than PMA (ED_{50} for bryostatin 1, about 50 pM; for PMA, about 0.50 nM). Upon translocation, the amount of PKC-ε in the Triton X-100-

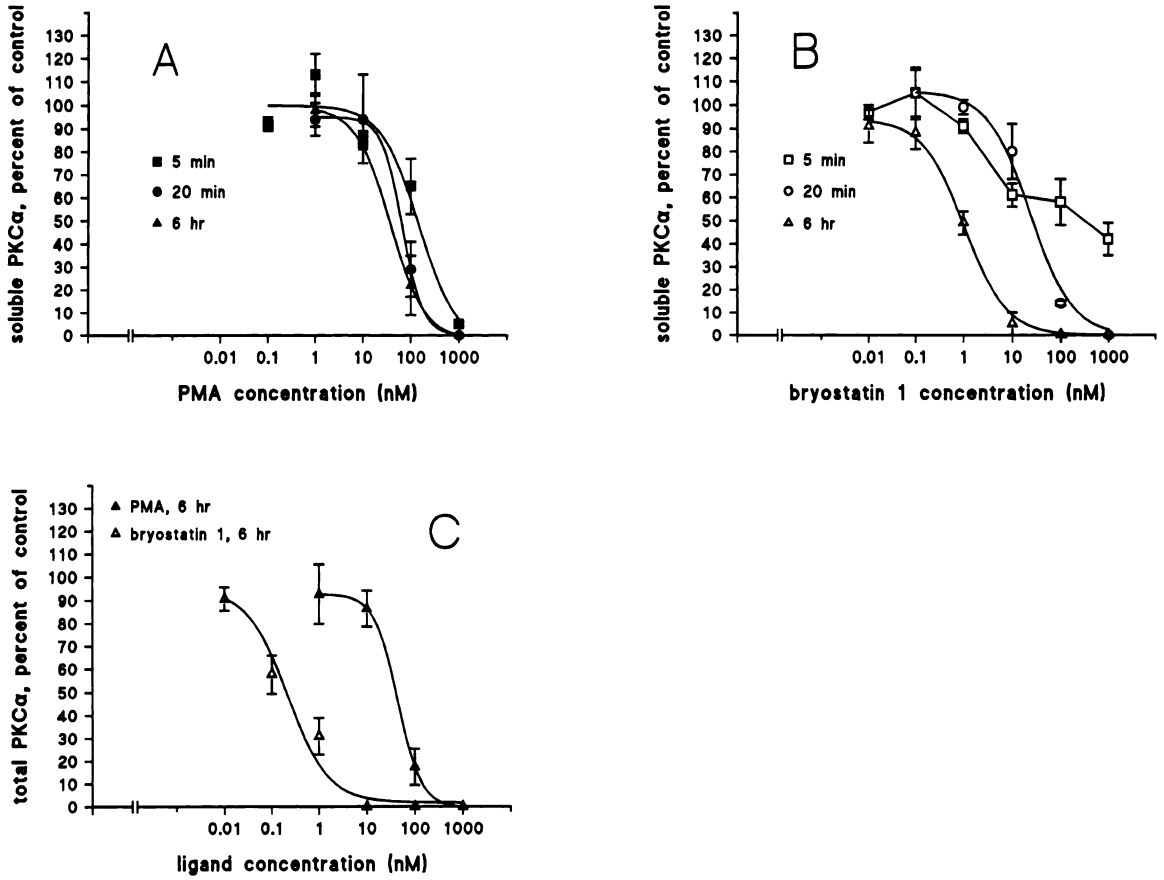

Fig. 3. PMA- and bryostatin 1-induced changes in levels of PKC-α in the soluble (A and B) and total (C) fractions of primary mouse keratinocytes. Primary mouse keratinocytes were treated with the indicated doses of PMA or bryostatin 1 for 5 min, 20 min, or 6 hr. Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of enzyme was quantitated by densitometry and expressed as percentage of the amount of isozyme present in the soluble fraction (A and B) or in the total fraction (C) in control cells. ■, PMA at 5 min; ●, PMA at 20 min; ▲, PMA at 6 hr; □, bryostatin 1 at 5 min; ○, bryostatin 1 at 20 min; △, bryostatin 1 at 6 hr. Each *point* is the average ± standard error of three independent sets of experiments.

soluble particulate fraction increased (Fig. 7C), reached its peak between 5 and 10 min, and subsequently decreased to about 50–60% of the original level by 6 hr after treatment with PMA or by 1–2 days after treatment with bryostatin 1. In either case the kinetics suggested that a significant portion of the Triton X-100-soluble particulate fraction-associated PKC-ϵ was resistant to PMA- or bryostatin 1-induced down-regulation. In the case of PKC-ζ, neither translocation nor down-regulation was observed in response to any of the treatments applied (data not shown).

Coapplication of bryostatin 1 with PMA. The coapplication of 0.1 nM to 1 μM bryostatin 1 and 1 μM PMA translocated PKC-α, PKC-δ, and PKC-ϵ in a fashion similar to treatment with 1 μM PMA (data not shown). The same was true for the down-regulation of PKC-α and PKC-ϵ (data not shown). In sharp contrast, in the Triton X-100-soluble particulate fraction increasing doses of bryostatin 1 inhibited down-regulation of PKC-δ. When high doses (100 nM to 1 μM) of bryostatin 1 were coapplied with 1 μM PMA, after an initial increase the amount of PKC-δ returned to the original level by 24 hr (Figs. 4D and 6A). The inhibitory effect of bryostatin 1 on the PMA-induced down-regulation of PKC-δ from the Triton X-100-soluble particulate fraction was dose dependent, with an ED_{50} of 5 nM (Fig. 8A). As expected, in the total fraction bryostatin 1 inhibited the down-regulation of about 45% of the total amount of PKC-δ (which roughly equals the original amount of the Triton X-100-soluble particulate fraction-associated isozyme per total cell mass). The ED_{50} of bryostatin 1 for this inhibition in the total fraction was 25 nM (Fig. 8B). When a 100-fold lower dose of PMA (10 nM) was used instead of 1 μM, the dose-response curves for inhibition by bryostatin 1 of the down-regulation of PKC-δ showed practically no shift, thus strongly arguing that the inhibitory effect of bryostatin 1 on the PMA-induced down-regulation of PKC-δ was not competitive (Fig. 8A).

Role of protein synthesis in protection of PKC-δ by bryostatin 1. To determine the role that new protein synthesis plays in the protection of PKC-δ by bryostatin 1, new protein synthesis was blocked by the addition of 20 μg/ml cycloheximide. After 30 min the cells were treated for 6 hr with 1 μM PMA, 1 nM or 1 μM bryostatin 1, or the combination of 1 μM PMA and 1 μM bryostatin 1. The amounts of PKC-δ remaining in the different fractions were identical to those observed without the addition of cycloheximide, i.e., high doses of bryostatin 1 inhibited the down-regulation of PKC-δ from the Triton X-100-soluble particulate fraction (Fig. 6B), whereas the induction of c-Jun, the product of one of the genes swiftly up-regulated by PMA or bryostatin 1, was completely inhibited (data not shown). Therefore, the PKC-δ protected from down-regulation by high doses of bryostatin 1 is not the result of enhanced synthesis of the enzyme.

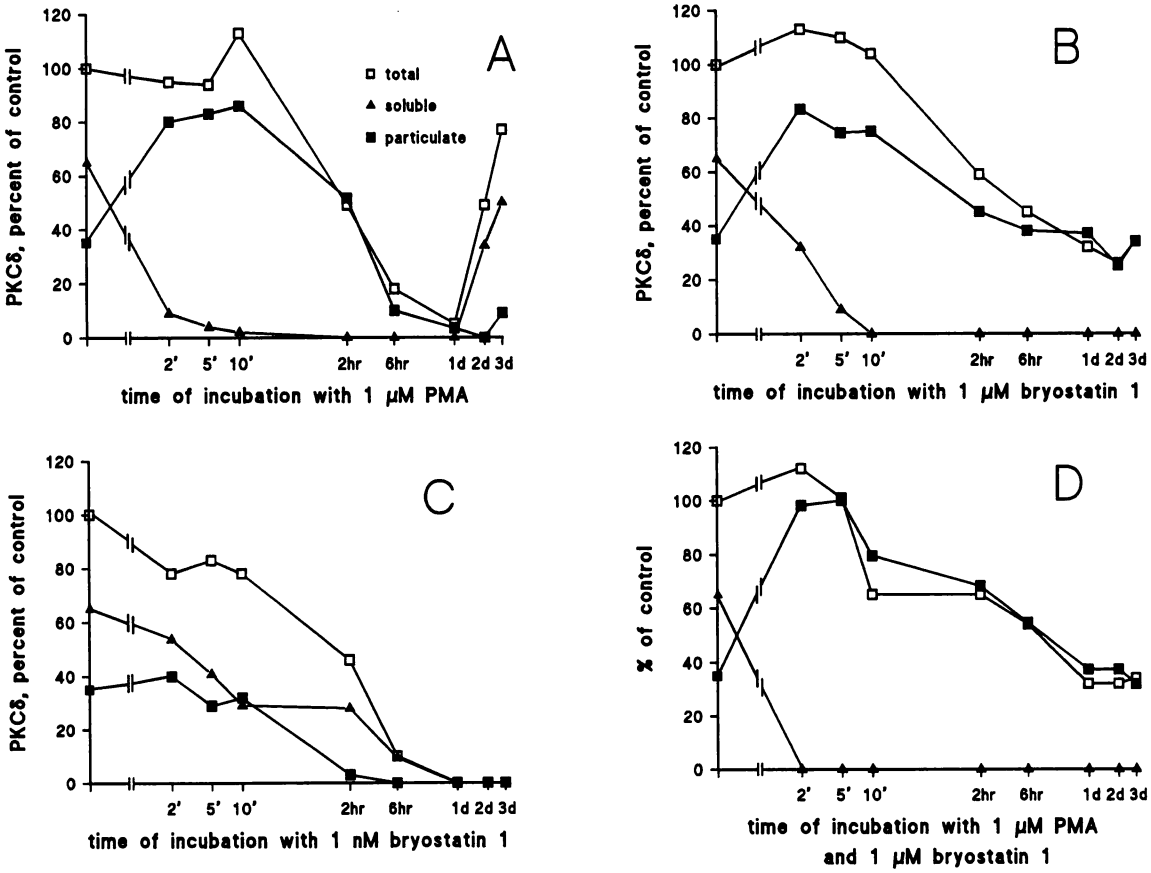

Fig. 4. Translocation and down-regulation of PKC-δ induced by 1 μM PMA (A), by 1 μM (B) or 1 nM (C) bryostatin 1, or by the coapplication of 1 μM bryostatin 1 and 1 μM PMA (D). Primary mouse keratinocytes were treated with 1 μM PMA, 1 μM or 1 nM bryostatin 1, or 1 μM PMA and 1 μM bryostatin 1 for 2, 5, or 10 min or 2, 6, 24, 48, or 72 hr. Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of enzyme in the total (□), soluble (▲), and Triton X-100-soluble particulate (■) fractions was quantitated by densitometry and expressed as percentage of the total amount of isozyme present in control cells. Time is plotted on a logarithmic scale. A second experiment yielded almost identical results.

Correlation between protection of PKC-δ by bryostatin 1 and inhibition of the biological effects of PMA. Differentiation of the primary keratinocytes was monitored by measuring the increase in the amount of SDS-insoluble cross-linked proteins, which is linearly proportional to the number of cornified envelopes (26). Coapplication of bryostatin 1 inhibited PMA (1 μM)-induced cornification in a dose-dependent manner, with an ED_{50} of approximately 7 nM (Fig. 8C). This value correlates closely with the inhibition of PMA-induced down-regulation of PKC-δ in the Triton X-100-soluble particulate fraction. Likewise, bryostatin 1 inhibited the PMA-induced loss of EGF binding with a dose-response curve very similar to that for inhibition of differentiation (ED_{50}, approximately 6 nM) (Fig. 8D).

Enzymatic activity of the PKC-δ protected from down-regulation. We tested whether the portion of PKC-δ protected from down-regulation by high doses of bryostatin 1 shows kinase activity. Activity was measured using the PKC substrate peptide [Ser^{25}]PKC(19–31), a modified form of the pseudosubstrate region of PKC-α. PKC-δ was immunoprecipitated from keratinocytes that had been treated with 500 nM bryostatin 1 or vehicle for 24 hr, kinase activity was assayed, and the results were normalized for the amount of PKC-δ present in the reaction mixture. PKC-δ protected from down-regulation by a high dose of bryostatin 1 showed similar, albeit slightly lower, kinase activity (1900 ± 650 cpm of incorporated ^{32}P/unit of PKC-δ), compared with that from controls (2800 ± 300 cpm of incorporated ^{32}P/unit of PKC-δ)

Discussion

In this paper we have measured the translocation and down-regulation of individual PKC isozymes and the induction of differentiation in primary mouse keratinocytes upon PMA and/or bryostatin 1 treatment. The results can be summarized in the following conclusions. (a) PKC isozymes α, δ, and ε showed unique, isozyme-specific regulation upon PMA and/or bryostatin 1 treatment in mouse keratinocytes. (b) The amount of PKC-δ in the Triton X-100-soluble particulate fraction upon bryostatin 1 and/or PMA treatment was inversely proportional to the extent of differentiation of the keratinocytes, as assessed by cornified envelope formation. (c) At 6 hr bryostatin 1 was 1–2 orders of magnitude more potent than PMA for removing PKC-α, PKC-δ, and PKC-ε from the soluble fraction and down-regulating PKC-α, PKC-δ, and the soluble fraction-associated portion of PKC-ε (Table 1). These findings are very similar to our earlier observations in NIH/3T3 fibroblasts (22) and probably reflect the greater potency of bryostatin 1 for binding to PKC (28). (d) However, in the case of PKC-α and PKC-ε, bryostatin 1 showed markedly slower kinetics for translocation than did PMA. (e) Both bryostatin 1 and PMA exhibited an up to 2-order of magnitude greater potency for translocating

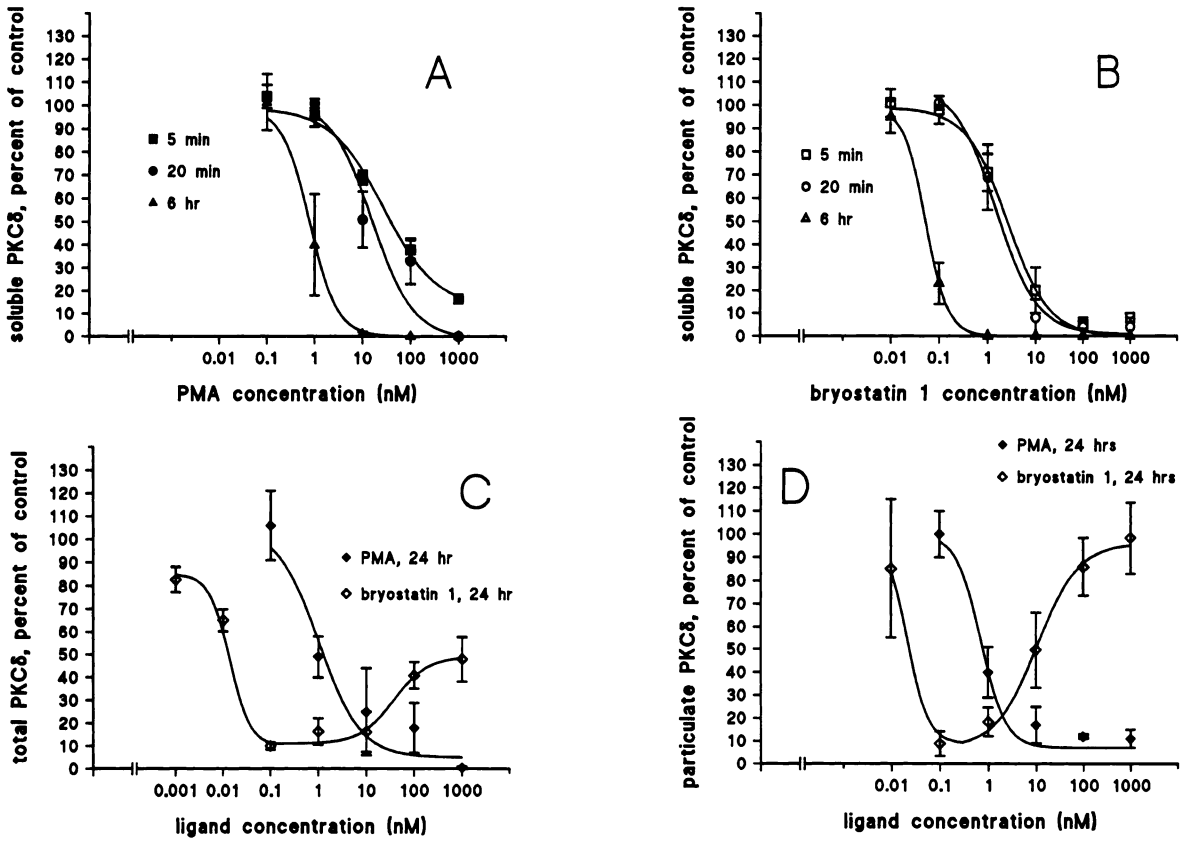

Fig. 5. PMA- and bryostatin 1-induced changes in levels of PKC-δ in the soluble (A and B), total (C), and Triton X-100-soluble particulate (D) fractions of primary mouse keratinocytes. Primary mouse keratinocytes were treated with the indicated doses of PMA or bryostatin 1 for 5 min, 20 min, or 6 hr (A and B) or 24 hr (C and D). Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of enzyme was quantitated by densitometry and expressed as percentage of the amount of isozyme present in the soluble fraction (A and B), in the total fraction (C), or in the Triton X-100-soluble particulate fraction (D) of control cells. ■, PMA at 5 min; ●, PMA at 20 min; ▲, PMA at 6 hr; ◆, PMA at 24 hr; □, bryostatin 1 at 5 min; ○, bryostatin 1 at 20 min; △, bryostatin 1 at 6 hr; ◇, bryostatin 1 at 24 hr. Each *point* is the average ± standard error of three independent sets of experiments.

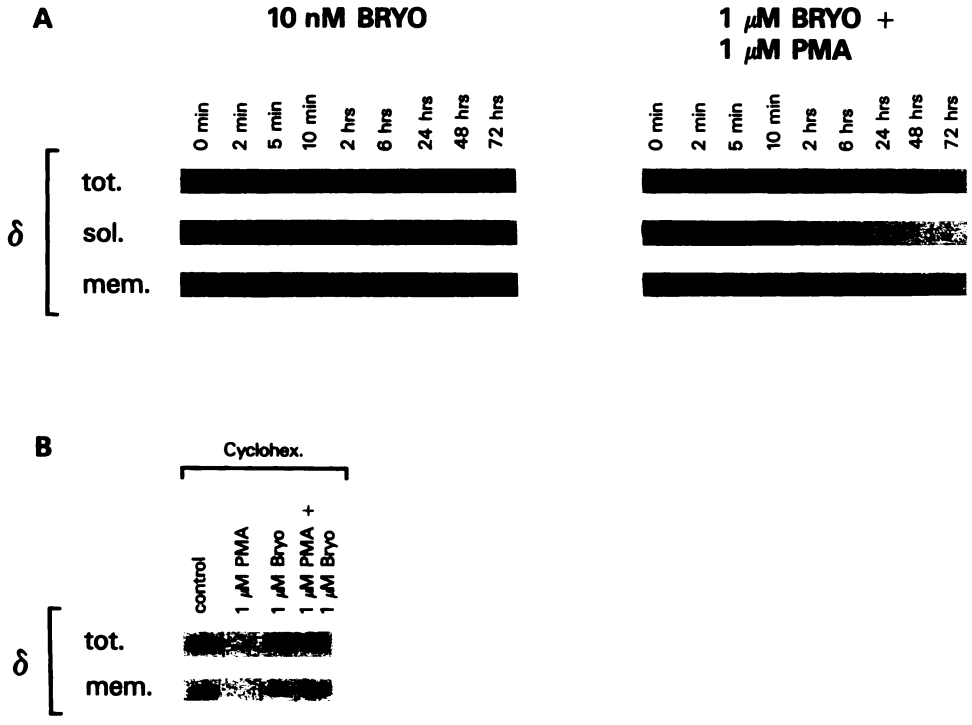

Fig. 6. Translocation and down-regulation of PKC-δ induced by 10 nM bryostatin 1 (*BRYO*) or the coapplication of 1 μM PMA and 1 μM bryostatin 1. Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. Equal amounts of proteins were loaded in each lane for the total (10 μg of protein/lane), soluble (10 μg of protein/lane), and Triton X-100-soluble (5 μg of protein/lane) fractions. *tot.*, total fraction; *sol.*, soluble fraction; *mem.*, Triton X-100-soluble fraction. Identical results were obtained in two more independent sets of experiments. A, Primary mouse keratinocytes were treated as indicated for 2, 5, or 10 min or 2, 6, 24, 48, or 72 hr. B, Primary mouse keratinocytes were pretreated with 20 μg/ml cycloheximide (*Cyclohex.*) for 30 min and then 1 μM PMA, 1 μM bryostatin 1, or the combination of the two compounds was added for 6 hr.

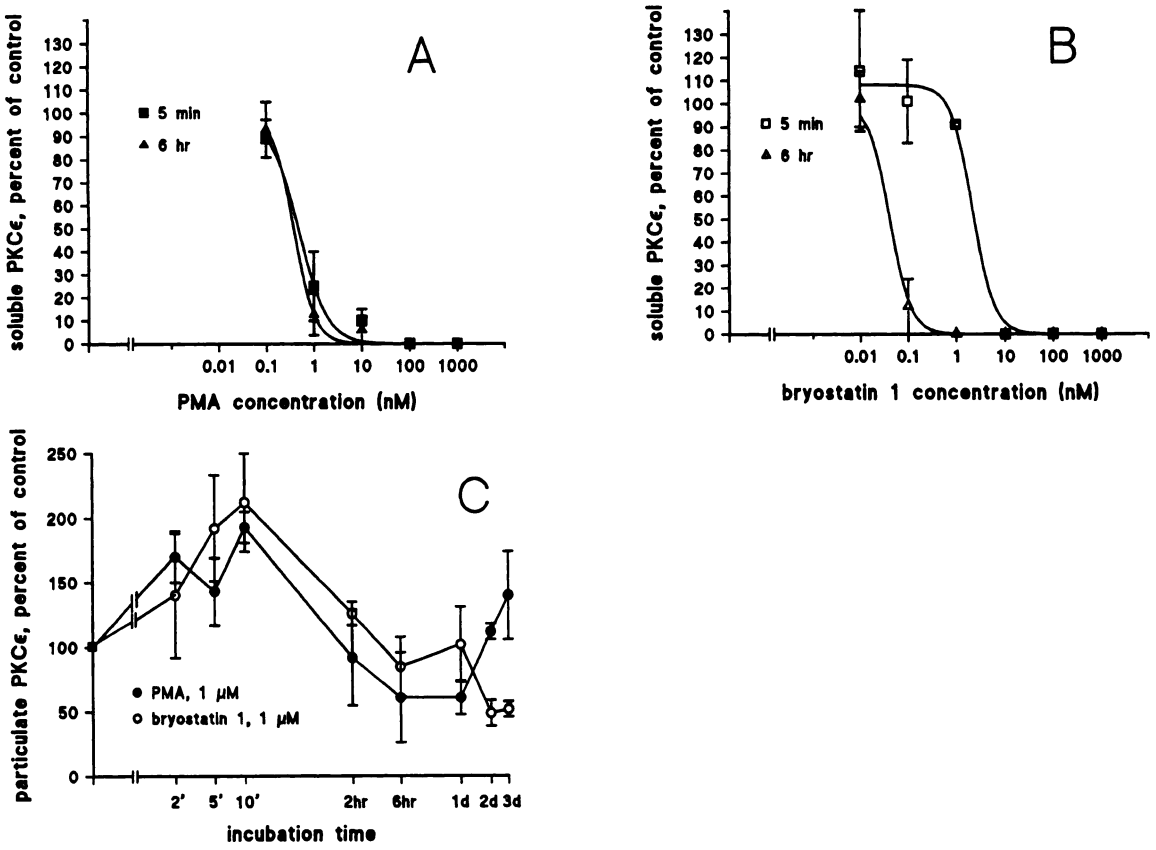

Fig. 7. PMA- and bryostatin 1-induced changes in levels of PKC-ε in the soluble (A and B) and Triton X-100-soluble particulate (C) fractions of primary mouse keratinocytes. Primary mouse keratinocytes were treated with the indicated doses of PMA or bryostatin 1 for 5 min or 6 hr (A and B) or with 1 μM PMA or 1 μM bryostatin 1 for 2, 5, or 10 min or 2, 6, 24, 48, or 72 hr (C). Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of enzyme was quantitated by densitometry and expressed as percentage of the amount of isozyme present in the soluble fraction (A and B) and in the Triton X-100-soluble particulate fraction (C) of control cells. ■, PMA at 5 min; ▲, PMA at 6 hr; □, bryostatin 1 at 5 min; △, bryostatin 1 at 6 hr; ●, PMA; ○, bryostatin 1. Each *point* is the average ± standard error of three independent sets of experiments.

PKC-δ and PKC-ε than translocating PKC-α. They also showed a 20–40-fold higher potency for down-regulating PKC-δ than down-regulating PKC-α. These differences are probably due to the complex interactions between the PKC isozyme, ligand, and microenvironment, e.g., membrane lipid composition, because analysis of *in vitro* binding to the recombinant PKC isozymes expressed in the baculovirus system showed similar binding affinities for PKC-α, -β, -γ, -δ, -ε, and -η (24, 29) and because PMA showed far less isozyme selectivity for translocating the same PKC isozymes in NIH/3T3 fibroblasts (22) than in keratinocytes.

PKC-δ in the Triton X-100-soluble particulate fraction showed a unique biphasic pattern of down-regulation by bryostatin 1 in mouse primary keratinocytes, as was the case in NIH/3T3 fibroblasts (22). Higher concentrations of bryostatin 1 inhibited the down-regulation of this isozyme by up to 1 μM PMA. This inhibitory effect of bryostatin 1 is clearly noncompetitive, as shown in the present study by the nonshifting inhibitory dose-response curves against 100-fold different concentrations of PMA. A similar conclusion was suggested by earlier work from our laboratory, using Friend erythroleukemia cells and a narrower range of PMA concentrations (3). Further experimentation is required to determine whether bryostatin 1 binds to a second binding site on PKC (30) or to a nonrelated receptor or whether the lack of competition reflects the slow rate of release of bryostatin 1 from PKC and the consequent lack of equilibrium conditions.

As was the case for fibroblasts (22), the regulatory pattern for PKC-δ showed excellent correlation with the unique biological effects of bryostatin 1, in this case the inhibition of cornification and inhibition of loss of EGF binding induced by PMA (Table 2). The PKC-δ in the Triton X-100-soluble particulate fraction protected by high doses of bryostatin 1 showed kinase activity, suggesting that PKC-δ plays an active role in the biological responses induced by bryostatin 1. Experiments are now in progress to determine whether the excellent correlation between the down-regulation pattern for PKC-δ and the biological effects of bryostatin 1 reflects an active role of this isozyme in these cellular functions.

Similarly to our observations, in erythroleukemia cells PKC-δ was suggested to be specifically involved in differentiation; erythroleukemia cells resistant to chemically induced differentiation expressed significantly less PKC-δ, and reversion of the phenotype of the resistant cell line to sensitivity for chemically induced differentiation was accompanied by the restoration of the PKC-δ level to that characteristic of the parental cell line (31). We previously showed that bryostatin restores the sensitivity to differentiation in phorbol ester-treated erythroleukemia cells (3). In keratinocytes, it is also likely that the prolonged down-regulation of PKC-α by bryostatin 1 contributes to the resistance to differentiation. Repeated treatment of mouse ker-

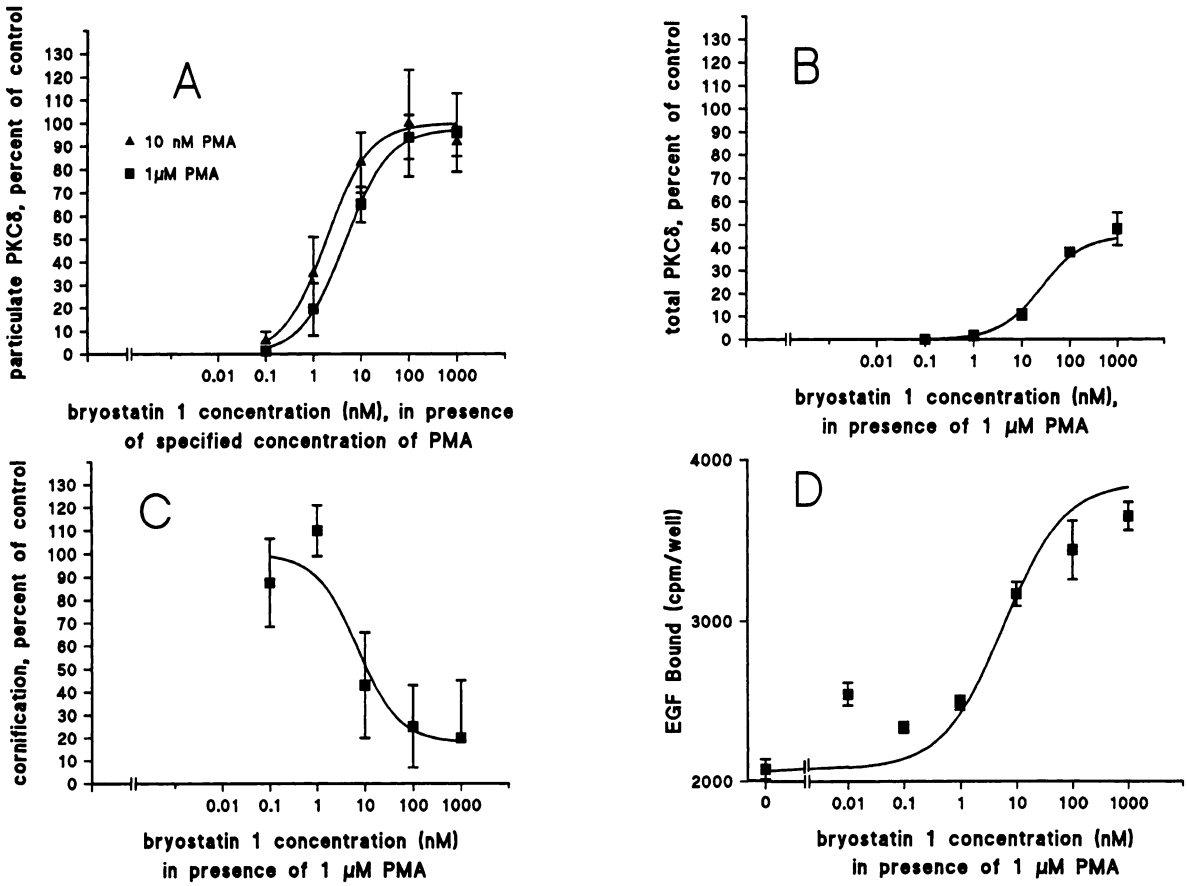

Fig. 8. PMA/bryostatin 1 cotreatment-induced changes in the levels of PKC-δ in the Triton X-100-soluble particulate fraction (A) and total fraction (B), in the level of cornification (C), and in the level of inhibition of EGF binding (D). Cells were treated with 1 μM PMA (unless otherwise indicated) and different doses (0.1 nM to 1 μM) of bryostatin 1 for 24 hr (A, B, and C) or 6 hr (D). A and B, Samples for SDS-PAGE were prepared and Western immunoblotting was performed as described in Experimental Procedures. The amount of enzyme was quantitated by densitometry and expressed as percentage of the total amount of isozyme present in control cells. Each *point* is the average ± standard error of three independent sets of experiments. A, ■, Cotreatment with 1 μM PMA; ▲, cotreatment with 10 nM PMA. C, The level of cornification was measured as described in Experimental Procedures; 100% represents the level of cornification induced by 1 μM PMA and 0% represents the level induced by vehicle alone. Each *point* is the average ± standard error of three samples. One more experiment yielded almost identical results. D, EGF binding was measured as described in Experimental Procedures. Each *point* is the average ± standard error of triplicate dishes. Two additional experiments yielded similar results.

TABLE 1

Potency of PMA and bryostatin 1 for translocating, removing from the soluble fraction, and down-regulating PKC isozymes α, δ, and ε in primary mouse keratinocytes

ED_{50} values for translocating or removing the isozymes from the soluble fraction and down-regulating them from the total fraction were determined as described in the text.

Isozyme	Time	ED_{50} for translocation or removal from the soluble fraction		ED_{50} for down-regulation	
		PMA	Bryostatin 1	PMA	Bryostatin 1
		nM		*nM*	
PKC-α	5 min	160 ± 6	Not complete	NA[a]	NA
PKC-α	20 min	65 ± 2	24 ± 2	NA	NA
PKC-α	6 hr	40 ± 1.6	1.0 ± 0.05	44 ± 3	0.22 ± 0.01
PKC-δ	5 min	30± 3	2.6 ± 0.1	NA	NA
PKC-δ	20 min	15 ± 2	1.5 ± 0.1	NA	NA
PKC-δ	6 hr	0.8 ± 0.02	0.055 ± 0.002	NA	NA
PKC-δ	24 hr	ND[b]	ND	1 ± 0.04	0.013 ± 0.001 (30 ± 3 for restoration)
PKC-ε	5 min	0.5 ± 0.02	2.2 ± 0.08	NA	NA
PKC-ε	6 hr	0.5 ± 0.02	0.05 ± 0.002	NA	NA

[a] NA, not applicable because down-regulation is not complete.
[b] ND, not determined.

TABLE 2

Potency of bryostatin 1 for inhibiting PMA-induced biological responses and PMA-induced down-regulation of PKC-δ.

ED_{50} values for the bryostatin 1-induced inhibition were determined as described in the text.

	Bryostatin 1 ED_{50}
	nM
Inhibition of down-regulation of PKC-δ induced by 1 μM PMA (total fraction)	25 ± 3
Inhibition of down-regulation of PKC-δ induced by 1 μM PMA (Triton X-100-soluble particulate fraction)	4.6 ± 0.2
Inhibition of down-regulation of PKC-δ induced by 10 nM PMA (Triton X-100-soluble particulate fraction)	1.9 ± 0.07
Inhibition of cornification induced by 1 μM PMA	7 ± 0.4
Inhibition of down-regulation of EGF binding induced by 1 μM PMA	6 ± 0.01

atinocytes with PMA, leading to permanent down-regulation of PKC, inhibited the increase of transglutaminase activity induced by a single PMA treatment (32).

The markedly slower kinetics of translocation of PKC-α and PKC-ε by bryostatin 1 may also contribute to the lack of induction of biological responses, although the kinetics of *in vivo* activation of PKC have not yet been related to biological responses. In another study in preparation,[2] we could clearly show that the dose-response curves for translocation of PKC-α and the dose-response curves for induction of ornithine decarboxylase are identical. The dose-response curve for induction of ornithine decarboxylase by bryostatin 1 (16) fits well with the dose-response curve for translocation at 20 min but not the dose-response curve for down-regulation, suggesting that the short term translocation dose-response curves better characterize the potency of bryostatin 1 for inducing biological responses.

There are two more implications of the slower kinetics of bryostatin 1-induced translocation. First, the lack of bryostatin-induced translocation in studies in which only early time points are examined could be due to the slow on-rate of the ligand (33). Second, at certain concentrations bryostatin might translocate and activate PKC with a rate equal or very close to the rate of down-regulation; therefore, it might inhibit PKC-associated responses by down-regulation with a very low level of effective activation. Experiments are underway to test this possibility.

The regulation of PKC-ε by PMA is cell type specific. PMA completely down-regulates PKC-ε in Swiss 3T3 cells (34), NIH/3T3 fibroblasts (22), and rat pituitary tumor GH_4C_1 cells (35) but fails to down-regulate the particulate fraction-associated form of PKC-ε in murine thymocytes (36), SH-SY5Y human neuroblastoma cells (37), monoblastoid U937 cells (38), and rat basophilic RBL-2H3 cells (39). Primary keratinocytes seem to belong to this latter group, because the Triton X-100-soluble fraction-associated portion of PKC-ε was not down-regulated by either PMA or bryostatin 1. This observation is even more exciting because the very potent antitumor promoter 12-deoxyphorbol-13-phenylacetate (40) completely down-regulated PKC-ε in the same primary keratinocytes.[2]

The lack of detection of intact PKC-η in nondifferentiating primary keratinocytes shows good correlation with the observation that no PKC-η was detected in the basal layer of epidermis, whereas it was abundant in the suprabasal, especially the granular, layers (41). The sensitivity of our system is about 0.1 fmol of PKC/μg of protein.

PKC has been implicated in the differentiation process for keratinocytes (6). The primary evidence is that PKC activators and inhibitors effectively modulate the differentiation of these cells. Unfortunately, because of a lack of adequate selectivity, none of these agents permitted the question of whether any of the PKC isozymes present in the keratinocytes were preferentially involved in differentiation to be addressed. Preferential involvement of certain PKC isozymes over others in physiological processes has been shown previously, for example in rat basophilic leukemia cells, where PKC-β and -δ showed significantly greater potency for reconstituting the secretory response than did PKC-α or -ε (39) and, conversely, PKC-α and -ε, but not PKC-β or -δ, inhibited antigen-induced hydrolysis of inositol phospholipids (42). Here we suggest that PKC-δ may play an important role in keratinocyte differentiation. Comparison of the dose-response curves for translocation and down-regulation of the individual PKC isozymes and the dose-response curves for different biological responses induced by PKC activators can provide insight into the specific involvement of PKC isozymes in the given responses, e.g., there is excellent correlation between the dose-response curves for loss of EGF binding induced by PMA (6), for inhibition by bryostatin 1 of PMA-induced loss of EGF binding, and for down-regulation of PKC-δ. On the other hand, dose-response curves for induction of ornithine decarboxylase by PMA or bryostatin show good correlation with the dose-response curves for translocation and consequent down-regulation of PKC-α induced by the same agents.[2]

In animal experiments and phase I and II clinical trials, bryostatin 1 showed promising results with otherwise untreatable melanoma (18, 19). The only specific biochemical effect of bryostatin 1 determined thus far is the specific translocation of PKC-β_{II} to the nuclei of HL-60 promyelocytic leukemia cells (43). Here we add the unusual regulation of PKC-δ and the markedly different kinetics of translocation of PKC-α and PKC-ε. Experiments are underway to determine whether the specific biochemical effects induced by bryostatin 1 can be exploited to interfere with pathological processes in human keratinocytes, such as skin cancer and psoriasis.

Acknowledgments

We thank Erin K. Williams for her technical assistance.

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