Activation of Phorbol Ester Responsive Form of Protein Kinase Cζ in Association with Ca^{2+}-Induced Differentiation of Primary Cultured Mouse Epidermal Cells

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ABSTRACT—In primary cultured mouse epidermal cells, protein kinase C (PKC) ζ consists of multiple forms: a low salt-eluted PKCζ (l-PKCζ, 79 and 85 kDa) and a high salt-eluted PKCζ (h-PKCζ, 79 and 85 kDa) by anion-exchange column chromatography (K. Nishikawa et al., Cell. Signal. 7, 491–504, 1995). In the present study, PKC isozyme-specific responses during terminal differentiation of epidermal cells, which were induced by the increase of Ca^{2+}-concentration in culture medium, were examined. After 24 hr-treatment with 1.8 mM Ca^{2+}, 79-kDa l-PKCζ in the particulate fraction was apparently shifted to the 85-kDa form. The phosphatidylserine-dependent kinase activity of this l-PKCζ was increased in association with the shift. These results suggest the pivotal role of l-PKCζ in the particulate fraction in the Ca^{2+}-induced epidermal cell differentiation processes.

Keywords: Protein kinase C, Isozyme, Ca^{2+}-induced differentiation

Protein kinase C (PKC) is a family of phospholipid-dependent protein kinases involved in various signal transduction processes including cell growth, differentiation events and tumor promotion (1, 2). Today, twelve isozymes of PKC have been identified and classified into three groups based on structural and enzymatic properties. Conventional isozymes (cPKC: PKCα, βI, βII, γ) are activated by Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (DG) (1, 2). Novel PKC (nPKC: PKCδ, ε, η, μ, θ), which lacks the Ca^{2+}-binding region C2 in the regulatory domain, is activated by PS and DG/phorbol esters (1, 2). Both cPKC and nPKC have two cysteine-rich zinc-finger motifs in the C1 region that constitute a tumor promoter phorbol ester/DG binding site. On the other hand, atypical PKC (aPKC: PKCι, λ, ζ) lacks the C2 region and has only one cysteine-rich zinc-finger motif in the C1 region (1–3). Since the individual isozymes have different tissue distribution, co-factor requirement and substrate specificity (1), each isozyme may play some specific roles in signal transduction processes.

It is known that cultured epidermal cells are terminally differentiated by increase of extracellular Ca^{2+} concentration from 0.05 mM to above 0.1 mM (4) and that this Ca^{2+}-induced differentiation is associated with increased levels of inositol phosphates (5, 6) and DG (7), which is known as an endogenous PKC activator. It is also reported that the activation of PKC by tumor promoter phorbol esters or DGs stimulates the expression of differentiation markers such as filaggrin, loricrin (8), transglutaminase (9) and cholesterol sulfotransferase (10). These data suggest that the activation of PKC is closely related to Ca^{2+}-induced epidermal cell differentiation. Recently, it has been reported that the translocation of PKCα, δ and ε was stimulated in cultured murine epidermal keratinocytes during Ca^{2+}-induced differentiation (11). Under the same condition, PKCη increased in both soluble and particulate fractions, but PKCζ did not change in its amount and cellular distribution (11). In these studies, however, the identification of PKC isozymes was performed using total cell lysate or preparations after ultracentrifugations.

We have previously shown that PKCα and PKCη are present only in the 105,000 × *g* supernatants and PKCζ is present both in the 105,000 × *g* supernatants and particulate fractions of primary cultured mouse epidermal cells (12). Recently we demonstrated that PKCζ is present as multiple forms: a low salt-eluted PKCζ (l-PKCζ) and a high salt-eluted PKCζ (h-PKCζ) by Resource Q column

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chromatography (13). l-PKCζ and h-PKCζ were detected as a doublet protein of 79 and 85 kDa in the 105,000 × *g* supernatants, but as a 79-kDa protein in particulate fractions (13). In the present sturdy, PKC isozyme-specific responses during terminal differentiation of epidermal cells, which were induced by the increase of Ca^{2+}-concentration in culture medium, were examined.

The sources of materials used in this study were as follows: phorbol 12-myristate 13-acetate (PMA), phenylmethylsulphonyl fluoride (PMSF), PS, from Sigma Chemical Co. (St. Louis, MO, USA); Resource Q (1 ml), from Pharmacia (Piscataway, NJ, USA); [γ-^{32}P]ATP, from ICN Biomedicals Inc. (Irvine, CA, USA); protein kinase C-ε substrate peptide (peptide ε), from Upstate Biotechnology Incorporated (Lake Placid, NY, USA). Anti-PKCα and ζ were purchased from Gibco BRL (Eggenstein, Germany). Anti-PKCη antiserum was raised in rabbits against a synthetic oligopeptide of the sequence 669–683 (CDEFRNFSYVSPELQL) as described previously (12).

Primary cultured mouse epidermal cells were prepared as described previously (14). The epidermal cells were cultured in Ca^{2+}-free minimum essential medium supplemented with 10% Chelex-treated (Ca^{2+}-deprived) fetal calf serum and 50 μM $CaCl_2$ (final concentration). Epidermal cells equivalent to two 100-mm plastic dishes were treated with or without 1.8 mM Ca^{2+} for the indicated time periods. The cells were homogenized at 4℃ in buffer A (20 mM Tris-HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 2 mM PMSF, 10 μM sodium orthovanadate). Thereafter, 105,000 × *g* supernatants and particulate fractions were prepared as described previously (14). These fractions were loaded onto an anion exchange column, Resource Q, equilibrated with buffer B (buffer A sup-

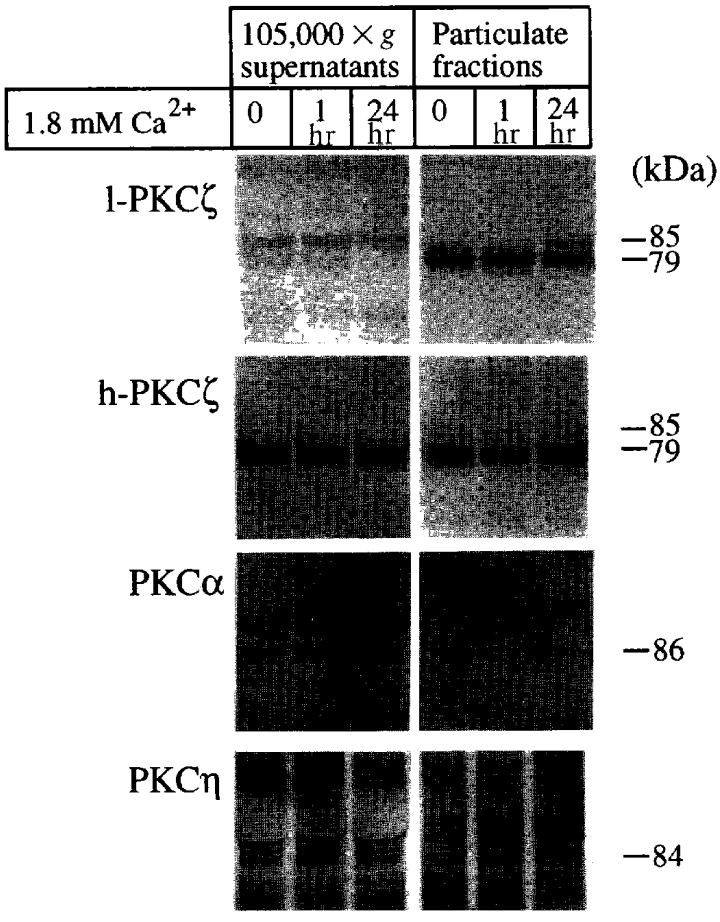

Fig. 1. Effects of 1.8 mM Ca^{2+}-treatment on the intracellular distribution and forms of PKC isozymes in epidermal cells. Epidermal cells equivalent to two 100-mm plastic dishes were treated with 1.8 mM Ca^{2+} for the indicated time periods. After treatment, 105,000 × *g* supernatants and particulate fractions were prepared and separated by Resource Q column chromatography. Fractions no. 3 to 5 were mixed and used for Western blot analysis using anti-PKCζ antibody (detection of l-PKCζ). Similarly, fractions no. 9–12, 10–12 and 11–12 were used for Western blot analysis of h-PKCζ, PKCα and PKCη, respectively. The results are representative of three separate experiments.

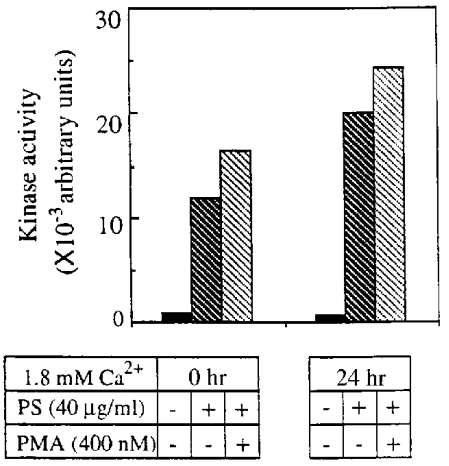

Fig. 2. Effects of 1.8 mM Ca^{2+}-treatment of epidermal cells on the kinase activity of l-PKCζ obtained from particulate fractions. Epidermal cells equivalent to two 100-mm plastic dishes were treated with 1.8 mM Ca^{2+} for 24 hr. After treatment, particulate fractions were prepared and separated by Resource Q column chromatography as described in the legend to Fig. 1. Fractions no. 3 to 5 were mixed, and an aliquot (50 μl) of the resultant mixture was used for the kinase assay. Kinase activity was determined either in the absence or presence of PS (40 μg/ml) or PS plus PMA (400 nM). The results are representative of three separate experiments.

plemented with 50 mM 2-mercaptoethanol and 0.02% Triton X-100). Proteins were eluted with a 40-ml linear gradient of 0–500 mM NaCl at a flow rate of 2.0 ml/min. Fractionation was started from 0 mM NaCl. Each fraction consisted of 2 ml of eluate. Western blot analysis was performed for each fraction using anti-PKC isozyme-specific antibodies as described previously (12). The specificities of the detected proteins were confirmed by the absorbing experiment with the corresponding specific peptides and by kinase assay after immunoprecipitation (12, 13).

After 1 hr-treatment with 1.8 mM Ca^{2+}, no marked change was observed in all of these PKC isozymes (Fig. 1). After 24-hr treatment, PKCα was slightly translocated from the 105,000×*g* supernatant to the particulate fraction (Fig. 1). PKCγ and h-PKCζ in both the 105,000×*g* supernatants and particulate fractions and also l-PKCζ in the 105,000×*g* supernatants were not affected. In contrast, 79-kDa l-PKCζ in the particulate fractions decreased with the concomitant appearance of 85-kDa l-PKCζ. The amounts of l-PKCζ in the particulate fractions (79-kDa l-PKCζ plus 85-kDa l-PKCζ) determined by a laser scanner (Model DNA 35 scanner; PDI, Inc., Santa Clara, CA, USA) were not significantly altered (data not shown). After 48 hr, the content ratio of 85-kDa l-PKCζ to 79-kDa l-PKCζ was much larger than that at 24 hr (data not shown). These results indicate that 79-kDa l-PKCζ shifts to the 85-kDa form by treatment with 1.8 mM Ca^{2+}. The time course of these changes was similar to that of the expression of Ca^{2+} induced differentiation marker proteins such as keratin K1, K10, filaggrin and loricrin (11). In our previous study, it was demonstrated that PMA or other PKC activators cause the apparent shift of 79-kDa l-PKCζ to the 85-kDa form in the particulate fractions and that this apparent shift results at least in part from the phosphorylation of 79-kDa l-PKCζ (13). Therefore, it is highly possible that the shift induced by 1.8 mM Ca^{2+}-treatment also results from the phosphorylation of 79-kDa l-PKCζ.

In our previous study, it was found that the kinase activity of l-PKCζ in particulate fractions is increased by the apparent shift of 79-kDa l-PKCζ to the 85-kDa form induced by PMA-treatment (13). We examined whether the shift of 79-kDa l-PKCζ to the 85-kDa form induced by the treatment with 1.8 mM Ca^{2+} for 24 hr results in the increase of its kinase activity.

Protein kinase activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide ε as described previously (13). The reaction mixture consists of 20 mM Tris-HCl (pH 7.5), 0.01% Triton X-100, 20 mM 2-mercaptoethanol, 1 mM PMSF, 1.12 mM EGTA, 1.12 mM EDTA, 6.12 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (5 μCi), 25 μg/ml peptide ε and 50 μl of enzyme fraction in a total volume of 150 μl. PS and PMA were added as indicated in each experiment. After 5 min incubation at 30℃, 100 μl of the reaction mixture was immediately applied to phosphocellulose paper. The papers were placed in 10% acetic acid and washed twice for 10 min. The radioactivity on the paper was quantified by BAS-2000 (Fuji Photo Film Co., Ltd., Tokyo) as described previously (14).

As shown in Fig. 2, the kinase activity of l-PKCζ obtained from non-treated cells was markedly stimulated by PS but not so much enhanced by PMA, consistent with our previous observations (13). This kinase activity was increased by about 1.8-fold by treating the cells with 1.8 mM Ca^{2+} for 24 hr (Fig. 2), corresponding to the shift of 79-kDa l-PKCζ to the 85-kDa form (see Fig. 1). These results indicate that l-PKCζ is also activated by the shift from the 79-kDa to the 85-kDa form in response to Ca^{2+}-induced differentiation of intact epidermal cells.

Although the shift of 79-kDa l-PKCζ to the 85-kDa form is induced within 1 hr by PMA (13), the shift induced by Ca^{2+}-treatment was observed after 24 hr. The precise mechanism by which such differences arise is not known at present. In Balb/MK keratinocytes, it has been reported that elevation of extracellular Ca^{2+} concentration, which induces terminal differentiation of these cells, stimulates the production of DG by threefold after 12 hr (15). It is also reported that PKCα translocates from the soluble to the particulate fractions following elevation of extracellular Ca^{2+} concentration in cultured murine

keratinocytes (11). Furthermore, in our present study, the partial translocation of PKCα was observed after the treatment of cells with 1.8 mM Ca^{2+} for 24 hr. These facts indicate that the shift of 79-kDa l-PKCζ to the 85-kDa form induced by Ca^{2+}-treatment may be mediated by the activation of PKCα or other kinase(s) such as Ca^{2+}-calmodulin-dependent protein kinases. Our previous observation that autophosphorylation of 79-kDa l-PKCζ produced only phosphorylated 79-kDa l-PKCζ but not phosphorylated 85-kDa l-PKCζ (13) supports this contention. Further studies must be performed about the molecular mechanisms of this shift of 79-kDa l-PKCζ to the 85-kDa form.

All of these results raise the possibility that the shift of 79-kDa l-PKCζ to 85 kDa and resultant activation of this kinase in particulate fractions caused by Ca^{2+}-treatment may play significant roles in the signal transduction processes of Ca^{2+}-induced epidermal cell terminal differentiation.

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