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PKCα induces differentiation through ERK1/2 phosphorylation in mouse keratinocytes

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Abbreviations: DAG, diacyglycerol; ERK, extracellular signal-regulated kinase; JNK, c-JUN N-terminal kinase; MAPK, mitogen-activated protein kinase; PBS, phosphate buffered saline; PKC, protein kinase C

Abstract

Epidermal keratinocyte differentiation is a tightly regulated stepwise process that requires protein kinase C (PKC) activation. Studies on cultured mouse keraitnocytes induced to differentiate with Ca^{2+} have indirectly implicated the involvement of PKCα isoform. When PKCα was overexpressed in undifferentiated keratinocytes using adenoviral system, expressions of differentiation markers such as loricrin, filaggrin, keratin 1 (MK1) and keratin 10 (MK10) were increased, and ERK1/2 phosphorylation was concurrently induced without change of other MAPK such as p38 MAPK and JNK1/2. Similarly, transfection of PKCα kinase active mutant (PKCα-CAT) in the undifferentiated keratinocyte, but not PKCβ-CAT, also increased differentiation marker expressions. On the other hand, PKCα dominant negative mutant (PKCβ-KR) reduced Ca^{2+}-mediated differentiation marker expressions, while PKCβ-KR did not, suggesting that PKCα is responsible for keratinocyte differentiation. When downstream pathway of PKCα in Ca^{2+}-mediated differentiation was examined, ERK1/2, p38 MAPK and JNK1/2 phosphorylations were increased by Ca^{2+} shift. Treatment of keratinocytes with PD98059, MEK inhibitor, and SB20358, p38 MAPK inhibitor, before Ca^{2+} shift induced morphological changes and reduced expressions of differentiation markers, but treatment with SP60012, JNK1/2 inhibitor, did not change at all. Dominant negative mutants of ERK1/2 and p38 MAPK also inhibited the expressions of differentiation marker expressions in Ca^{2+} shifted cells. The above results indicate that both ERK1/2 and p38 MAPK may be involved in Ca^{2+}-mediated differentiation, and that only ERK1/2 pathway is specific for PKCα-mediated differentiation in mouse keratinocytes.

Keywords: Ca^{2+}-mediated differentiation; ERK1/2; mouse keratinocytes; p38 MAPK; PKCα

Introduction

Keratinocytes provide an excellent *in vitro* model for the study of normal cell differentiation (Green *et al.*, 1977; Eckert, 1989). Keratinocytes, the normal epidermis progress through four phenotypic stages as they migrate from the basement membrane to the surface of the skin. Specific markers of differentiation have been identified, including spinous cell keratin (MK) 1 and 10, granular cell proteins filaggrin, loricrin, and SPR-1, and transglutaminases TGK and TGE (Rice *et al.*, 1992; Fuchs *et al.*, 1994; Kartasova *et al.*, 1996; Kim *et al.*, 1996; Kim and Bae, 1998). Earlier reports suggest that activation of PKC regulates the expression of genes involved in the terminal stages of epidermal differentiation (Yuspa *et al.*, 1990; Dlugosz *et al.*, 1994; Dlugosz *et al.*, 1994). When keratinocytes are grown at calcium concentration below 0.05 mM, they continue to proliferate by either failure or slow development of intercellular contacts, stratify little if at all, and fail or are slow to form cornified envelopes.

In cultured keratinocytes, elevation of extracellular Ca^{2+} concentration increases phosphatidylinositol turn-

over, resulting in increased DAG levels in keratinocytes (Jaken *et al.*, 1988; Punnonen *et al.*, 1993; Tang *et al.*, 1993; Min DS *et al.*, 2002), and induces phosphorylation of a subset of proteins, similar to pharmacological PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) (Wirth *et al.*, 1987). Activation of PKC by TPA or DAG stimulates cornified envelope formation (Lichti *et al.*, 1988) and causes the transition of spinous expression to granular layer differentiation markers: the expression of filaggrin and loricrin is induced, whereas the expression of MK1 and MK10 is suppressed (Yuspa *et al.*, 1983). Although PKC activation can not explain all of the effects of calcium on keratinocyte differentiation, it appears to clearly play a major role. However, the study of PKC in differentiation is complicated due to the large number of PKC isozymes (α, δ, ε, η and ζ). Sudden increase of the exracellular calcium concentration above 0.12 mM leads to a number of acute changes, including redistribution of protein kinase Cα (PKCα) to the membrane (Sheu *et al.,* 1989; Denning *et al.*, 1995) at least in mouse keratinocytes, and PKCα antisense treatment inhibits Ca^{2+} mediated keratinocytes differentiations (Lee *et al.*, 1997). However, the mechanism by which extracelluar free Ca^{2+} triggers differentiation is not well understood.

In the present study, we observed that PKCα was involved in Ca^{2+}-mediated keratinocyte differentiation through ERK1/2 phosphorylation in mouse system.

Materials and Methods

Cell culture

Primary mouse epidermal keratinocytes were isolated from BALB/c mice and were grown in Eagle's minimal essential medium with 8% Chelex-treated fetal calf serum, 0.2% penicillin/streptomycin solution (Gibco, BRL, Gaithersburg, MD), and 0.05 mM Ca^{2+} to maintain a basal cell-like population of undifferentiated cells (Henning *et al.*, 1980). To induce terminal differentiation, $CaCl_2$ was added directly to culture media.

Chemicals and antibodies

PD98059 (inhibitor of MEK) and SB203580 (inhibitor of p38 MAPK) were purchased from Calbiochem (La Jolla, CA) and SP600125 (inhibitor of JNK) was from TOCRIS (Ballwin, MO). Anti-PKCα, –β, –δ and anti-β-tublin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-MAPK, anti-phospho-MAPK (P202/Y204), anti-JNK, anti-phospho-JNK1/2, anti-p38 MAPK and anti-phospho-p38 MAPK polyclonal antibodies were from New England BioLabs (Beverly, MA). The keratinocyte markers MK1, MK10, MK14, loricrin, and filaggrin were from Babco (Richmond, CA).

Vector construction

MFG retroviral vector by replacing the *GFP* sequence of MFG.GFP.IRES.puro (Park *et al.*, 2000) was used for gene transfection. The MFG.GFP.IRES.puro itself was used as a negative control throughout the experiment. The retroviral plasmids were introduced into 293gpg retrovirus packaging cell line by transient transfection with Lipofectamine (Gibco/BRL). After 72 h, the supernatants were harvested and used for retroviral infection. The virus titers, measured in NIH3T3 cell line by puromycin-resistant colony formation, were between 10^5 and 5×10^5/ml. The infection and selection of the target cells by puromycin were performed as described previously (Ory *et al.*, 1996). PKC-KR expression MFGpuro retroviral vectors were generated by ligating full length open reading frames of PKC isoforms with a K⟶R point mutation at the ATP binding site. PKC-CAT expression MFGpuro retroviral vectors were generated by ligating cDNA fragments encoding only the CAT domain of isoforms. All the cDNA fragments of PKC mutants were generated by PCR and were analyzed to confirm their sequences with an automated DNA sequencer (Lee *et al.*, 2002). The eukaryotic expression vectors, rat ERK2-KR, under the control of the cytomegalovirus promoter were produced by cloning the inserts from the respective NpT7-5 clones into pCMV5 (Kortenjann *et al.*, 1994). The catalytically inactive dominant-negative JNK-2 (Lee *et al.*, 2003) was amplified with sense (5'-aaaatctagactgccatggcatacccatacgacgtcc-3') and anti-sense (5'-aaaaggatcctcatcgacagccttaagg-3') primers using High fidelity *taq* polymerase (GIBCO/BRL). The PCR products were digested with *Nco*-1 and *Bam*H1 restriction enzymes, and cloned into the corresponding sites in the MFG retroviral vector by replacing the GFP sequence of MFG.GFP.IRES.puro. Adenovirus mediated PKCα and -δ were kindly donated by Dr. T. Kuroki (Ohba *et al.*, 1998). The infection of adenovirus was carried out in serum-free medium containing 2.5 mg of polybrene (Sigma, St. Louis, MO) per ml at 50 PFU/cell for BCE cells for 30 min at room temperature. Fresh serum containing medium was added thereafter. Adenoviral-CMV-β-gal was generated by the method described previously (Park *et al.*, 2003).

Polyacrylamide gel electrophoresis and Western blot

The cells were washed twice with ice-cold PBS, scraped into SDS sample buffer, boiled, and run immediately on 8.5% polyacrylamide gels. Proteins were transferred electrophoretically to nitrocellulose, and the membranes were blocked in 5% milk. For

detection of PKC isozymes, the membranes were incubated with specific antibodies. Proteins were detected with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA), and specific bands were visualized by chemiluminescence (ECL, Amersham International). Autoradiographs were recorded onto X-Omat AR films (Eastman Kodak Co.).

Results

Ca^{2+} shift to 0.12 mM induced differentiation in mouse keratinocytes

Healthy mouse keratinocytes culture in medium containing low Ca^{2+} (0.05 mM) proliferate and express basal keratinocyte markers. However, the cells can be induced to differentiate by elevating the Ca^{2+} concentration above 0.1 mM (Figure 1A), and differentiation markers, including loricrin, filaggrin, and MK10, also increased 48 h after the Ca^{2+} shift (Figure 1B).

PKCα activation was responsible for phosphorylation of ERK1/2 and JNK1/2, as well as differentiation markers

Earlier reports suggested that PKCα is a key molecule involved in Ca^{2+} mediated differentiation. To elucidate the relationship between PKCα activation and induction of differentiation, adenoviral PKCα was overexpressed in mouse keratinocytes. PKCδ using adenoviral vector in low Ca^{2+} media was also transfected to compare functions of PKCα and PKCδ in keratinocyte differentiation. Adenoviral PKCα or PKCδ overexpression induced increased kinase activity (Figure 2B) as well as protein level (Figure 2A) of both PKCα and PKCδ. Downstream activation by PKCα overespression is phospho-ERK1/2, while the level of this protein phosphorylations was not changed in the case of PKCδ. When downstream pathways were examined, PKCα activation induced phospho-ERK1/2 and phospho-JNK1/2 expressions, while p38 phosphorylation was not changed. When PKCδ was overexpressed, no difference in phosphorylations of ERK1/2, p38 MAPK, and JNK1/2 was found (Figure 2C). When differentiation marker expressions were also examined, PKCα overexpression induced MK1, MK10, filaggrin and loricrin, similar to the level of high Ca^{2+} shift. The observation that PKCδ overexpression slightly affected the late maker expressions such as loricrin and filaggrin, but not the early differentiation markers, suggests a possibility of PKCα involvement in late differentiation marker regulation (Figure 2D). Similar result was obtained, when using retroviral catalytic active PKCα vector (CAT-PKCα) in low Ca^{2+} media (Figure 3B). When dominant negative PKCα (PKCα-KR) was transfected, the expression of differentiation markers by high Ca^{2+} media was blocked, while PKCβ-KR did not affect Ca^{2+}-mediated differentiation (Figure 3C).

Ca^{2+} shift to 0.12 mM induced ERK1/2, p38 and JNK phosphorylation

To elucidate the mechanism of Ca^{2+} mediated differentiation, phosphorylation of ERK1/2, p38 MAPK, and JNK1/2 was examined. As shown in Figure 4, ERK1/2 activation was bi-phasic at 30 min and 24 h after Ca^{2+} shift, and peak activation was at 24 h after the Ca^{2+} shift. In the case of p38 MAPK and JNK1/2, phosphorylation was increased from 15 min after Ca^{2+} shift and they lasted until 48 h.

ERK1/2 activation is responsible for Ca^{2+}-mediated differentiation

To elucidate downstream pathway of Ca^{2+}-mediated differentiation, PD98059 (MEK1/2 inhibitor), SB203580 (p38 MAPK inhibitor) and SP600125 (JNK1/2 inhibitor)

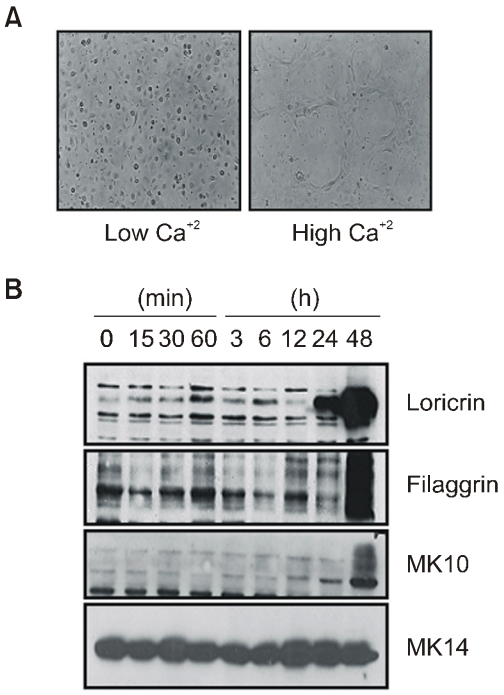

Figure 1. Cellular morphology in 0.12 mM Ca^{2+} shifted normal keratinocytes. (A) Primary mouse keratinocytes were grown in media containing 0.05 mM Ca^{2+} concentration, and then switched to 0.12 mM Ca^{2+}-containing media. After 48 h, photomicrographs were examined. (B) At indicated times of 0.12 mM Ca^{2+} shift, whole SDS lysates were prepared, and protein samples were subjected to PAGE and transferred to nitrocellulose membranes, and keratinocyte marker proteins were detected with specific antibodies. The results represent one of three independent experiments.

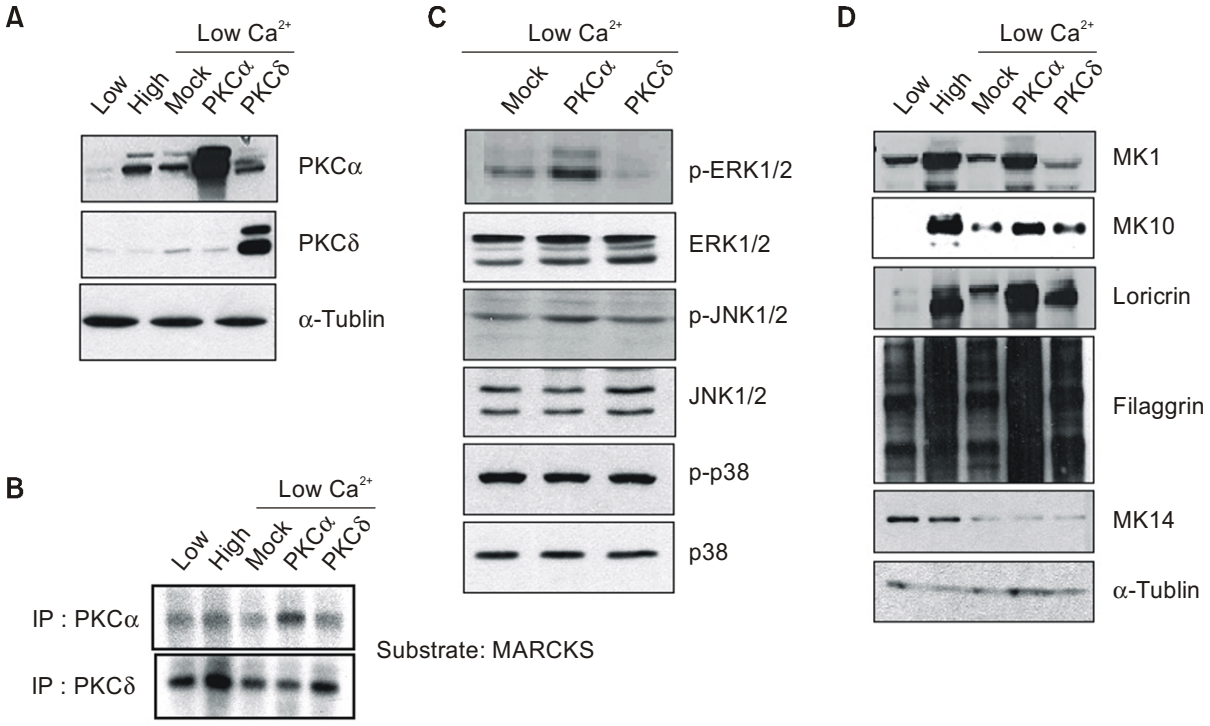

Figure 2. PKCα overepxression in 0.05 mM Ca^{2+} induced ERK1/2 phosphorylation and differentiation marker expressions. (A, C) Primary mouse keratinocytes were grown in media containing 0.05 mM Ca^{2+} concentration, and then adenoviral vectors of PKCα -β and control β-gal were transfected. At 24 h of transfection, protein lysis were prepared, subjected to PAGE and Western blotting. (B) Cellular proteins were extracted by lysis with PKC extraction buffer. Proteins from 300 μg of cell extracts were immunoprecipitated by using an anti-PKC antibody and protein G-Sepharose. Immune complex kinase reactions were performed in the presence of GST-MARCKS substrate and [γ-^{32}P]ATP. The results represent one of three independent experiments. (D) Whole SDS lysates were prepared, protein samples subjected to PAGE and transferred to nitrocellulose membranes, and keratinocyte marker proteins were detected with specific antibodies. The results represent one of three independent experiments.

were applied at 30 min before Ca^{2+}-shift. As seen in Figure 5A, morphological changes indicated that pretreatment of the cells with PD98059 and SB203480 blocked Ca^{2+}-medaited differentiation, the effect being more prominent in SB203580 pretreated cells, while, Ca^{2+}-mediated differentiation was not changed in the case of SP600125 pretreated cells (Figure 5A). When differentiation markers were examined, PD98059 and SB203580 blocked both early differentiation (MK1 and MK10) and late differentiation (loricrin and filaggrin) marker expressions, while SP600125 pretreatment did not change Ca^{2+}-mediated differentiation marker expressions (Figure 5B). We further confirmed differentiation marker expressions using dominant-negative mutants of ERK1 (ERK1-KR), JNK1 (JNK1-KR) and p38 MAPK (p38-KR). Similar to the effects of pharmacological drugs, ERK1-KR and p38-KR blocked Ca^{2+}-mediated differentiation marker expressions, while no difference was shown in the case of JNK1-KR (Figure 5C).

Discussion

The existence of multiple PKC isozymes, having distinct cofactor requirements, tissue distribution, and substrate specificities, suggests that the individual PKC isozymes have specific roles in cellular physiology. The role of PKC in keratinocyte differentiation is well established (Hawley-Nelson *et al.*, 1982; Yuspa *et al.*, 1982; Dlugosz *et al.*, 1993). Although PKC activation can induce differentiation at low calcium concentrations, its effects are potentiated by calcium (Yuspa *et al.*, 1983). Five PKC isozymes have been identified in mouse (Dlugosz *et al.*, 1992) and human keratinocytes (Fisher *et al.*, 1993; Reynolds *et al.*, 1994), and only one of the five, PKCα, is activated by calcium. In the mouse keratinocytes, PKCα has been implicated in the induction of the late differentiation markers by calcium (Denning *et al.*, 1995; Lee *et al.*, 1997). Studies using pharmacological PKC inhibitors (Lee *et al.*, 1998) and PKCα antisense oligonucleotides (Lee *et al.*, 1997) have suggested that PKCα activation regulates the granular cell stage

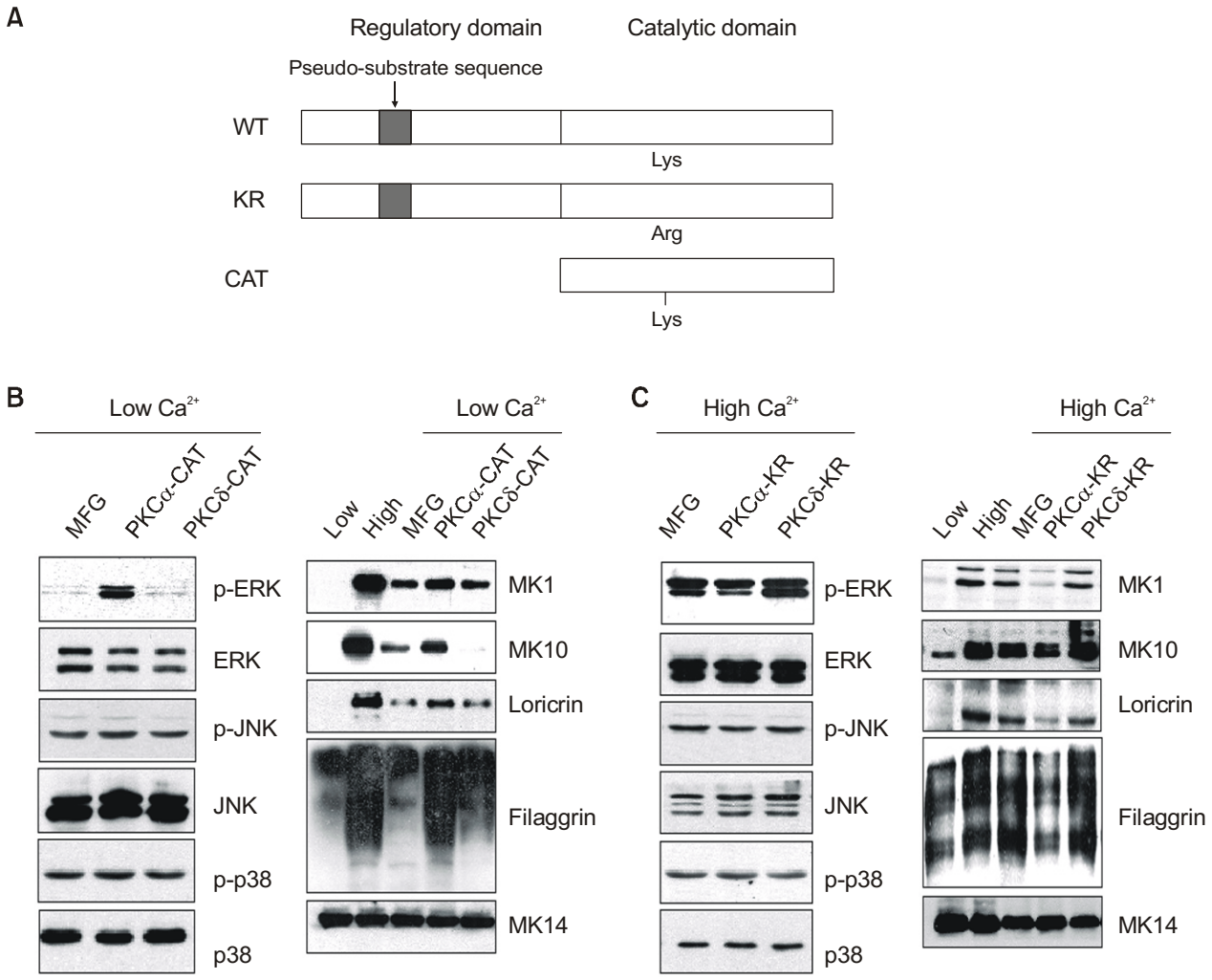

Figure 3. PKCα-CAT in 0.05 mM Ca^{2+} induced ERK1/2 phosphorylation and differentiation marker expressions, but PKCα-KR in 0.12 mM Ca^{2+} inhibited. Control vector (MFG) and kinase active types of PKCα and PKCβ (PKCα-CAT and PKCβ-CAT) in 0.05 mM Ca^{2+} media (A) or dominant negative mutants of PKCα and PKCβ (PKCα-KR and PKCβ-KR) in 0.12 mM Ca^{2+} (B) were transfected and after 24 h, whole SDS lysates were prepared, protein samples subjected to PAGE and Western blotting. The results represent one of three independent experiments.

of epidermal differentiation by enhancing expression of loricrin, profilaggrin, and TGk mRNA. In the present study, we more addressed this issue rigorously by overexpression of PKCα using adenoviral and retroviral vector system.

When Ca^{2+} was shifted to 0.12 mM, the expressions of keratinocyte differentiation markers such as loricrin, filaggrin and MK10 were induced from 24 h of Ca^{2+}-shift and peaked at 48 h, while the expression of basal cell marker MK14 was unaffected. Morphological changes also indicated that 0.12 mM Ca^{2+} induced differentiation in mouse keratinocytes, compared to the cells in low-Ca^{2+} media (Figure 1). Furthermore, direct correlation between PKCα and keratinocyte differentiation was examined: When PKCα or -δ was overexpressed, both PKCα and -δ kinase activities were increased, suggesting that both PKC isozymes were activated in both PKC isozyme overexpression systems. However, keratinocyte differentiation markers were expressed only in PKCα overexpressed cells, but not in PKCδ overexpressed cells, indicating that PKCα is essential for keratinocyte differentiation. Similar effects were also observed when catalytic active PKCα retroviral vector was overexpressed (Figure 2). Reversely, transfection of dominant negative PKCα (PKCα-KR) in high Ca^{2+} media inhibited differentiation, further indicating that PKCα is essential for keratinocyte differentiation. When PKCα downstream molecules such as ERK1/2, JNK1/2 and p38 MAPK were studied, ERK1/2 phosphorylation was found to have changed by PKCα; adenoviral PKCα and PKCα-CAT increased and

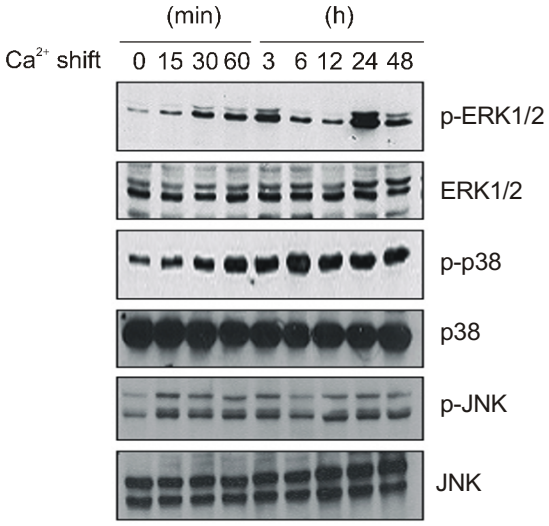

Figure 4. Ca^{2+} shift increased MAPK phosphorylation. Primary mouse keratinocytes were grown in media of 0.05 mM Ca^{2+} concentration, and then switched to 0.12 mM Ca^{2+}-containing media. At indicated times of 0.12 mM Ca^{2+} shift, protein lysates were prepared, subjected to PAGE and Western blotting. The results represent one of three independent experiments.

PKCα-KR reduced ERK1/2 phosphorylation, suggesting ERK1/2 phosphorylation as PKCα downstream in keratinocyte differentiation.

Since phosphorylation of ERK1/2, JNK1/2 and p38 MAPK was increased after Ca^{2+} shift (Figure 4), employing inhibitors of ERK1/2, p38 MAPK, and JNK1/2, we examined morphological changes with inhibition of differentiation marker expressions after Ca^{2+} shift, and observed that PD98059 and SB203580, the morphological changes while SP600125 potentiated, suggesting that ERK1/2 and p38 MAPK may be involved in Ca^{2+}-mediated differentiation (Figure 5). In our data, ERK1/2 phosphorylation was increased in biphasic pattern. We do not know exactly how biphsic expression of phopho-ERK1/2 was regulated in keratinocyte differentiation. However, our preliminary data indicated that both biphasic expression of phospho-ERK1/2 was necessary for keratinocyte differentiation (data not shown) and we are doing further study. When we used dominant negative ERK1/2, JNK1/2 or p38 MAPK to establish direct correlation in Ca^{2+}-mediated differentiation, both dominant negative ERK1/2 and p38 MAPK inhibited differentiation

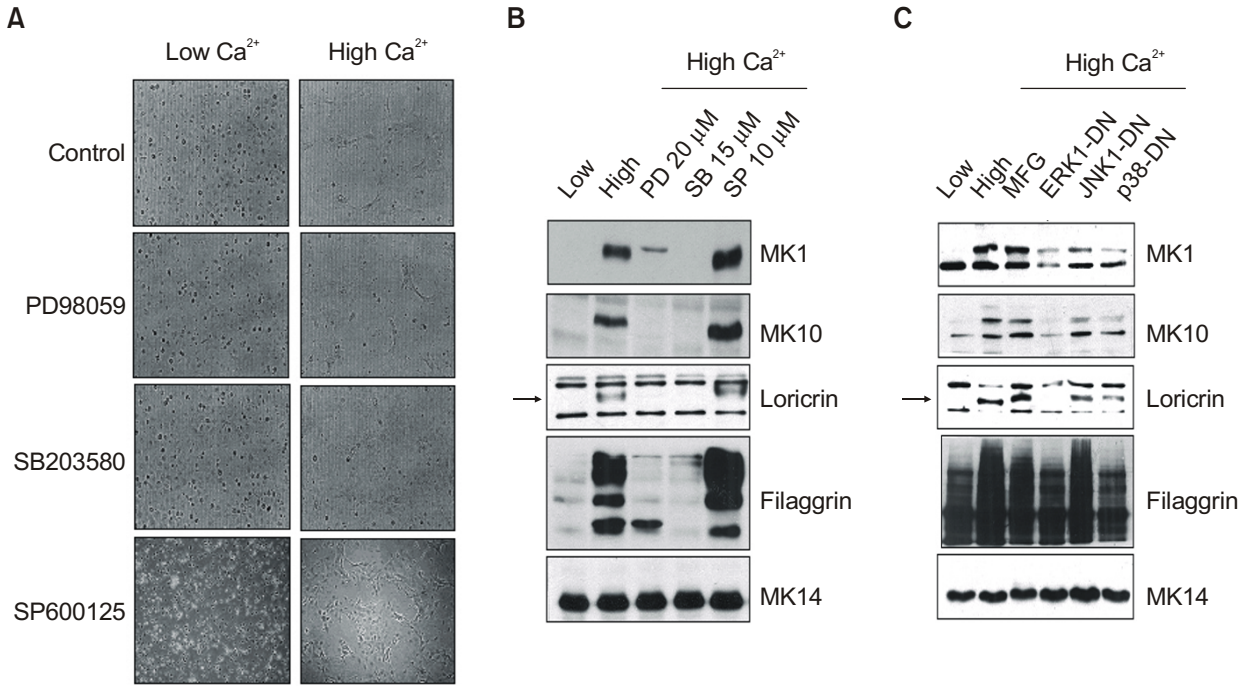

Figure 5. ERK1/2 and p38 MAPK phosphorylation was involved in Ca^{2+} mediated differentiation. (A) Primary mouse keratinocytes were grown with the media of 0.05 mM Ca^{2+} concentration, and then switched to 0.12 mM Ca^{2+}-containing media with or without pretreatment of PD98059, SB203580, and SP600125. After 48 h of Ca^{2+} shift, photomicrographs were examined. (B) Whole SDS lysates were prepared, protein samples subjected to PAGE and transferred to nitrocellulose membranes, and keratinocyte marker proteins were detected with specific antibodies. (C) Primary mouse keratinocytes were grown with the media of 0.05 mM Ca^{2+} concentration, and then switched to 0.12 mM Ca^{2+}-containing media with or without transfection of dominant negative mutants of ERK1, p38 MAPK and JNK1 (ERK1-KR, p38-KR and JNK1-KR). After 48 hr of Ca^{2+} shift, whole SDS lysates were prepared, protein samples subjected to PAGE and transferred to nitrocellulose membranes, and keratinocyte marker proteins were detected with specific antibodies. The results represent one of three independent experiments.

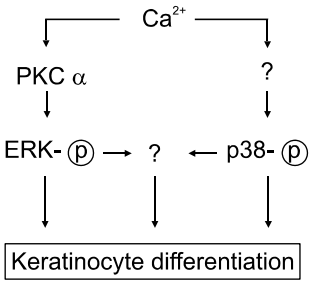

Figure 6. Hypothetical scheme of Ca^{2+} mediated differentiation in mouse keratinocytes

marker expression; the effect of ERK1/2 was more prominent. Based on the results, we propose that Ca^{2+}-mediated differentiation marker expression is dependent on both ERK1/2 and p38 MAPK pathways (Figure 6).

Tropical application of TPA on mouse skin increases epidermal thickness accompanied with inflammation: the phenotype similar to psoriatic skin (Furstenberger *et al.*, 1985). Recent studies disclosed that the expression of involucrin and cystatin A, two constituents of cornified cell envelope, are regulated by PKC- MAPK pathway (Efimove *et al.*, 1998). Although increased ERK and JNK expressions in psoriatic epidermis was reported (Takahashi *et al.*, 2002) and p38 MAPK is also involved in calcium-induced differentiation (Efimova *et al.*, 2003), the expression and activities of MAPKs in keratinocyte differentiation has not been fully elucidated.

In conclusion, we examined whether Ca^{2+}-mediated differentiation is induced by PKCα and downstream of PKCα is ERK1/2. In the present study, we could not exclude possible involvement of p38 MAPK pathway, because Ca^{2+} induced both ERK1/2 and p38 MAPK phosphorylation and inhibition of ERK1/2 and p38 MAPK blocked differentiation marker expression. Nevertheless, since PKCα activation does not directly induce p38 MAPK phosphorylation, another Ca^{2+} response signaling which may respond to Ca^{2+} may also be involved in Ca^{2+}-mediated keratinocyte differentiation.

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