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Cyclic Strain Stimulates Isoform-Specific PKC Activation and Translocation in Cultured Human Keratinocytes

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Abstract Previous studies have demonstrated that cyclic strain induces keratinocyte proliferative and morphological changes. Since protein kinase C (PKC) is known to play an important role in the regulation of keratinocyte growth and differentiation, the objective of this study was to determine the role of the PKC signaling pathway as a mediator of strain modulation of the keratinocyte phenotype. In particular, we tested the following specific hypotheses: (1) cyclic strain stimulates PKC activity and translocation, (2) cyclic strain activates PKC in an isoform-specific manner, and (3) PKC mediates the strain activated proliferative and morphological response in cultured human keratinocytes. To test these hypotheses, keratinocytes were subjected to vacuum-generated cyclic strain (10% average strain), followed by measurement of PKC activity, PKC isoform distribution by Western blot analysis and confocal microscopy, and examination of the effect of PKC inhibitors (calphostin C and staurosporine) on strain induced proliferative and morphological changes. We observed stimulation of PKC activity (62.3 ± 5.1% increase) coupled with translocation of PKC from the cytosolic to the membrane fraction in keratinocytes subjected to acute cyclic strain. Cyclic strain also caused translocation of PKC α and δ, but not ζ isoforms, from the cytosolic to the membrane fraction as demonstrated by both Western blot analysis and confocal microscopy. PKC β was not detected in these cells. PKC inhibitors, calphostin C (10 nM), and staurosporine (5 nM), inhibited strain-induced PKC activation and keratinocyte proliferation, but did not block the effects of strain on cellular morphology or alignment. We conclude that these data support our hypothesis that cyclic strain stimulates PKC activity and translocation in an isoform-specific manner in cultured human keratinocytes. Moreover, our studies with PKC inhibitors support the hypothesis that strain-induced changes in the keratinocyte phenotype may be selectively modulated by PKC. J. Cell. Biochem. 67:327–337, 1997.

Key words: protein kinase C (PKC); keratinocytes; cyclic strain; proliferation; morphology; PKC isoforms

Tissue expansion has been used clinically to gain the extra skin surface area with an expandable balloon, but it remains unclear whether mechanical strain leads to enhanced keratinocyte growth or hypertrophy. We have previously reported the effects of cyclic strain in human epidermal keratinocyte proliferation and morphology. Our data demonstrated that keratinocytes subjected to cyclic strain show significant increases in cell growth and DNA synthesis and also revealed that keratinocytes elongate and align perpendicular to the vector force [Takei et al., 1997].

In the present study, to investigate a possible pathway involved in strain-induced responses, we examined the involvement of protein kinase C (PKC), which has been shown to have an important role in cell proliferation and differentiation [Denning, 1995; Nishizuka 1995, 1988]. In early studies, phorbol esters were identified as cutaneous carcinogens [Berenblum, 1982] and activators of PKC [Fujiki et al., 1989], suggesting the importance of PKC in keratinocyte growth and differentiation [Matsui et al., 1992; O'Driscoll et al., 1994; Le Panse et al., 1994]. Phorbol 12-myristate 13-acetate (PMA) was also reported to translocate PKC activity from the cytosol to the membrane fraction in various cell types [Kazanietz et al., 1992; Rosales et al., 1992a].

To date, five isoforms of PKC (α, δ, ϵ, ν, and ζ) in keratinocytes have been identified [Reynolds et al., 1994]. Each isoform appears to have a different and specific function [Dlugosz et al., 1993], but it is still unclear whether individual isoforms play a specific role in signal transduction. PKC α in both keratinocytes and endothe-

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lial cells has been reported to translocate with PMA stimulation [Reynolds et al., 1994; Rosales et al., 1992a]. However, it is not known whether the application of mechanical load could induce PKC translocation in keratinocytes.

To test the hypothesis that strain-induced proliferation could be mediated through the PKC pathway and that a specific isoform might be involved in this signal transduction, we analyzed PKC activity and isoforms in keratinocytes subjected to strain. Previous studies using different cell types including endothelial cells [Rosales et al., 1992a], smooth muscle cells [Mills et al., 1997], osteoblasts [Carvalho et al., 1994] and cardiac myocytes [Sadoshima et al., 1993; Yamazaki et al., 1995] confirm that PKC is activated by mechanical strain, suggesting that PKC might have a key role in modifying mechanotransduction.

PKC inhibitors have been used to examine the role of PKC in mediating strain-induced activation of keratinocyte proliferation and morphological changes. Various inhibitors block PKC activity by different mechanisms; staurosporine blocks the catalytic domain, while calphostin C inhibits the regulatory domain [Bruns et al., 1991; Tamaoki, 1991]. We used both calphostin C and staurosporine to block the different sites and studied the effects of PKC downregulation on strain-induced proliferation and PKC activation.

METHODS

Cell Cultures

Human keratinocytes were derived from neonatal foreskins as previously described [Eisinger, 1985; Takei et al., in press] and cultured at 37°C in a 5% CO_2 incubator in serum free medium (Keratinocyte SFM, Gibco BRL Products, Grand Island, NY) containing 0.2 ng/ml epidermal growth factor, 30 µg/ml bovine pituitary extract, and 1.02 g/L L-glutamine. Keratinocytes were seeded on flexible membrane culture plates coated with collagen type I (FLEX I, Flexcell, McKeesport, PA), and then incubated for 48 h prior to exposure to strain. At least three different keratinocyte cell lines were used to eliminate variations among each cell line and cells from passage 2–4 were used for these experiments.

Exposure to Strain

The Flexercell Stress Unit (Flexcell) was used for our study. This unit uses vacuum to deform a flexible bottom culture plate. The level of vacuum is controlled by solenoid valves monitored by a computer timer program. When flexible membranes were subjected to 150 mm Hg of vacuum, the membrane experiences a 10% average strain. For these experiments, keratinocytes were seeded (10,000 cells/cm^2) and then subjected to strain for 3 s alternating with 3 s of relaxation in the neutral position (i.e., 10 cycles per min [cpm]). Control cells were cultured on identical plates in the same incubator in the absence of strain.

Total PKC Activity

PKC activity was measured using the Pep Tag Assay Kit (Promega, Madison, WI) according to manufacturer's instruction. Initially, keratinocytes were collected and centrifuged at 1,000*g* for 5 min. Pellets were lysed with extraction buffer (25 mM Tris–HCl, 5 mM EGTA, 0.7 mM $CaCl_2$, 1 mM PMSF, 0.1 mM TLCK, 10 µM leupeptin, pH 7.6) and homogenized with a Type A Dounce Homogenizer for 10 strokes on ice. The cell lysates were centrifuged at 8,000*g* for 10 min, and the supernatant was saved as the cytosolic fraction. The pellet was further dissolved with extraction buffer containing 0.3% Triton X-100 (Eastman Kodak Company, Rochester, NY), then centrifuged in the same manner. The supernatant was recovered as the particulate or membrane fraction as described by Bissonnette [1994] and Gopalakrishna [1986]. Samples were mixed with reaction buffer (100 mM HEPES, 6.5 mM $CaCl_2$, 5 mM DTT, 50 mM $MgCl_2$, 5 mM ATP, pH 7.4), activator solution (1 mg/ml phosphatidyl serine) and fluorescent peptide (PKC-specific substrate, 0.4 µg/L, Promega), and incubated for 30 min at 30°C. The phosphorylated peptides were analyzed by separation on a 0.8% agarose (50 mM Tris–HCl, pH 8.0) gel run at 100 volts for 20 min. The phosphorylated bands were localized by ultraviolet (UV) light, cut, and solubilized with glacial acetic acid (Baker, Phillipsburg, NJ) and gel stabilization solution (8 M urea). The phosphorylated peptides were then quantitatively measured with a Fluorescence Spectrophotometer (Perkin-Elmer, Foster City, CA), using 568-nm excitation and 592-nm emission wavelengths. PKC activity is expressed as unit/ml

[nanomolar phosphate transferred by PKC/min/ml] or as the ratio of PKC activity relative to control. PKC activity in the cytosol and membrane fraction is presented as a percentage of total PKC activity (cytosol + membrane).

Effects of PKC Inhibitors on Total PKC Activity, Cell Proliferation, and Morphology

To assess the effects of PKC inhibition on total PKC activity, keratinocytes were exposed to the culture medium with or without calphostin C (10 nM) or staurosporine (5 nM). The culture medium was freshly made and changed every other day. For calphostin C experiments, culture plates were exposed to fluorescent light for 3 h for uniform activation of this compound [Bruns et al., 1991; Gopalakrishna et al., 1992]. Keratinocytes were subjected to cyclic strain for up to 60 min in PKC activity study and 7 days for proliferation study, followed by PKC assay as described above.

For the proliferation study, keratinocytes were subjected to cyclic strain with or without PKC inhibitors for up to 7 days; then, the cell number was determined on day 1, 3, 5, and 7, using a Coulter Counter ZM (Coulter, Miami, FL). The culture medium without PKC inhibitors contained 0.01% dimethyl sulfoxide (DMSO, Sigma Chemical Co., St Louis, MO), which served as a vehicle for PKC inhibitors. Control cells were cultured under stationary condition in the same incubator. These measurements were obtained in five separate experiments and the data are presented as mean ±SEM.

For morphological assessment, keratinocytes were subjected to strain for 3 days with or without PKC inhibitors, fixed with 3.7% formalin for 10 min and stained with 1% crystal violet (Sigma) for 5 min.

Immunocytofluorescence of PKC Isoforms With Confocal Microscopy

Immunocytofluorescence of PKC isoforms has been previously described in endothelial cells [Rosales et al., 1992b]. We used PKC isoform-specific antibodies, anti-PKC α monoclonal antibodies (Upstate Biotechnology, Lake Placid, NY) and anti-PKC β, δ, ζ polyclonal antibodies (Gibco BRL, Gaithersburg, MD) to track the intracellular localization of each isoform. Anti-PKC α antibodies were prepared from rabbit brain cytosol. Anti-PKC β, δ, ζ antibodies derived from the cDNA sequences were purified from rabbit serum [Makowske et al., 1988; Ono et al., 1988]. Both antibodies were specific to respective human PKC isoforms as confirmed by Western blotting [Jaken et al., 1989; Leach et al., 1988].

Keratinocytes were subjected to cyclic strain at 10 cpm for 10 min, fixed with 4% formaldehyde, permeabilized with 0.3% Triton X-100 for 30 min, and incubated with 50 mM ammonium chloride for 15 min at room temperature. Keratinocytes were then incubated with 1% bovine serum albumin (BSA) diluted with PBS (1% BSA/PBS) for 60 min and further incubated with PKC antibodies diluted with 1% BSA/PBS (1 : 20) for 2 h. The preparations were washed with 1% BSA/PBS three times, followed by incubation with Texas-Red (Molecular Probes, Eugene, OR) conjugated secondary antibodies diluted with 1% BSA/PBS (1 : 100) for 1 h and placed on glass slides with 50% glycerol. Each preparation was observed using a BioRad MRC-600 Confocal Imaging System with a 40× objective on a Zeiss Axiovert microscope. Aperture, gain, and black level for imaging acquisition were maintained constant. Background autofluorescence was not visible.

Detection of PKC Isoforms by Western Blotting

PKC isoform-specific antibodies were used to identify PKC α, β, δ, ζ in keratinocytes as described above for the confocal studies. Keratinocytes were subjected to strain for 2, 5, or 10 min, then homogenized and fractionated as described above in the total PKC assay protocol. Protein concentration was determined by the Bradford method [Bradford, 1976] using the BioRad protein assay system (Bio Rad, Hercules, CA), with BSA (Pierce, Rockford, IL) as the standard. Samples (10 g protein) were boiled with sampling buffer (60 mM Tris HCl, 20% glycerol, 2% SDS, 10 mM mercaptoethanol, bromophenol blue) with a 1 : 1 dilution for 5 min. Electrophoresis was performed with a 7.5% SDS polyacrylamide gel [Laemmli, 1970], followed by blotting onto a nitrocellulose sheet as previously described [Towbin et al., 1979]. Nonspecific antibody binding was prevented by incubating the blots in Tris-buffered saline with Tween 20 (TBST; 50 mM Tris HCL, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat milk for 30 min at room temperature. The blots were incubated for one h with primary antibodies (PKC isoform-specific antibodies), and for 1 h with peroxidase linked secondary antibodies

(donkey antirabbit IgG, Amersham, Arlington Height, IL) with washings per the manufacturer's directions. The immunoreactive bands were identified by the ECL system (Amersham) detected on X-OMAT AR film, and developed by using X-OMAT AR processor (Kodak, Rochester, NY). Each band was quantitated by a GS 300 scanning densitometer (Hoeffer Scientific Instruments, San Francisco, CA).

Statistical Analysis

The results are presented as the mean ±SEM. Significance was assessed by one-way analysis of variance (ANOVA) with a multiple comparison method. Post hoc testing was performed by Bonferroni analysis using a commercially available statistical package (SigmaStat, Jandel Scientific, San Rafael, CA). $P < 0.05$ was considered significant.

RESULTS

PKC Activity Assay

Particulate PKC activity was progressively stimulated in keratinocytes exposed to strain in a monophasic manner. By 5 min, we detected a 2.7 ± 0.2-fold increase as compared to unstretched controls (Fig. 1a). This was mirrored by a progressive decrease of PKC activity (39.4 ± 4.8% decrease at 5 min) in the cytosolic fraction. Total PKC activity, or the sum of the membrane and cytosolic pools was mildly stimulated, peaking to a 27 ± 3.5% increase after 5 min of cyclic strain.

Basal distribution of total PKC activity in unstimulated keratinocytes was 68.2 ± 1.3% in the cytosolic fraction and 31.8 ± 1.4% in the membrane fraction, as shown in Figure 1b. After 5 min of exposure to cyclic strain, PKC in the membrane fraction increased to 67.7 ± 2.7% of total activity, whereas the cytosolic fraction significantly decreased to 32.3 ± 2.8% (Fig. 1b).

As shown in Figure 2, cyclic strain induced a gradual increase in PKC activity that peaked at 10 min and remained activated for 60 min. The levels of PKC activity at later times (1 day and longer) were restored to control levels (data not shown). As expected, both calphostin C (Fig. 2a) and staurosporine (Fig. 2b) prevented strain-induced stimulation of PKC activity.

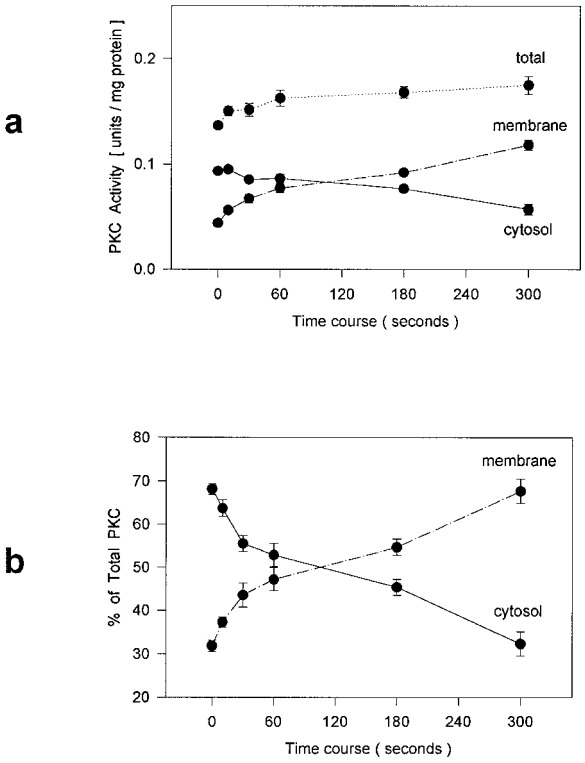

Fig. 1. Effects of cyclic strain on PKC activity. **a:** Keratinocytes were subjected to strain for up to 300 s, PKC activity of cytosolic or membrane fraction was determined as described in Methods. PKC activity in membrane fraction continuously increased, while that of cytosolic fraction decreased during the time course of strain. Total PKC activity (cytosol + membrane fraction) increased to 27.0 ± 3.5% after 300 s of strain. **b:** Keratinocytes were subjected to strain in the same manner as in a. The percentage of each fraction relative to total PKC is presented here. Most PKC activity remains in cytosolic fraction (68.2 ± 1.3%) compared to membrane fraction (31.8 ± 1.4%) under stationary condition. Once keratinocytes are subjected to strain, PKC activity translocated to the membrane fraction (67.7 ± 2.7%) at 300 s; cytosolic fraction decreased to 32.3 ± 2.8% correspondingly. Similar results were obtained in three independent experiments. Values are presented as mean ±SEM.

Cell Proliferation and Morphology With PKC Inhibitors

Calphostin C (5–50 nM) inhibited static keratinocyte proliferation in a dose- and time-dependent manner, as shown in Figure 3a. At 5 and 10 nM calphostin C, the ability of cyclic strain to significantly stimulate keratinocyte proliferation was prevented. The strain-induced proliferative response was completely ablated at a concentration of 50 nM calphostin C. As shown in Figure 3b, staurosporine inhibited keratinocyte proliferation in a similar pattern to calphostin C.

Keratinocytes subjected to strain showed elongation and alignment after a 3-day expo-

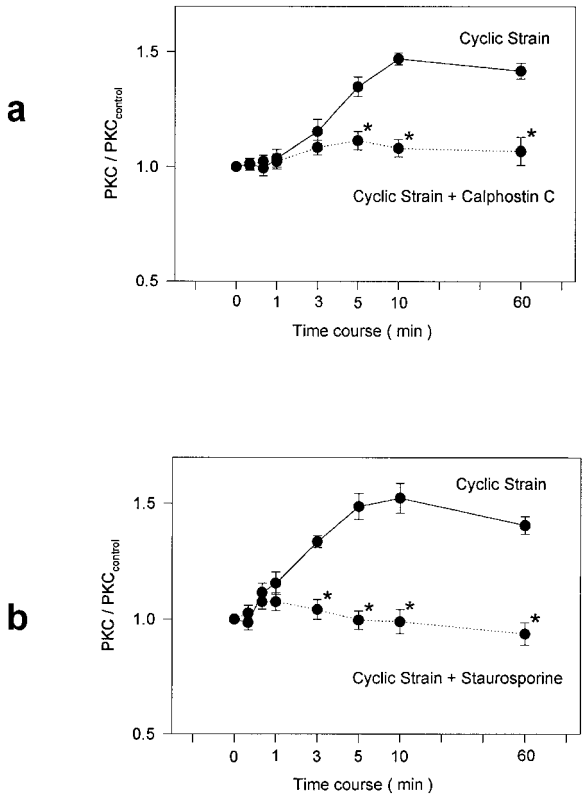

Fig. 2. Effects of PKC inhibitors on total PKC activity. **a:** Keratinocytes were treated with or without calphostin C (10 nM) for 1, 5, 10, and 60 min under cyclic strain, then PKC activity was assayed as described in Methods. Each data point is expressed as the ratio of each activity to control (time zero point). Calphostin C significantly inhibited PKC activity in keratinocytes subjected to strain at 5 min. **b:** Keratinocytes were treated with or without staurosporine (5 nM) as described in Material and Methods. Staurosporine significantly blocked strain-induced PKC activation at 3 min which is earlier than that of calphostin C. Results of five independent experiments were presented as mean SEM. *$P < 0.05$ compared to strain.

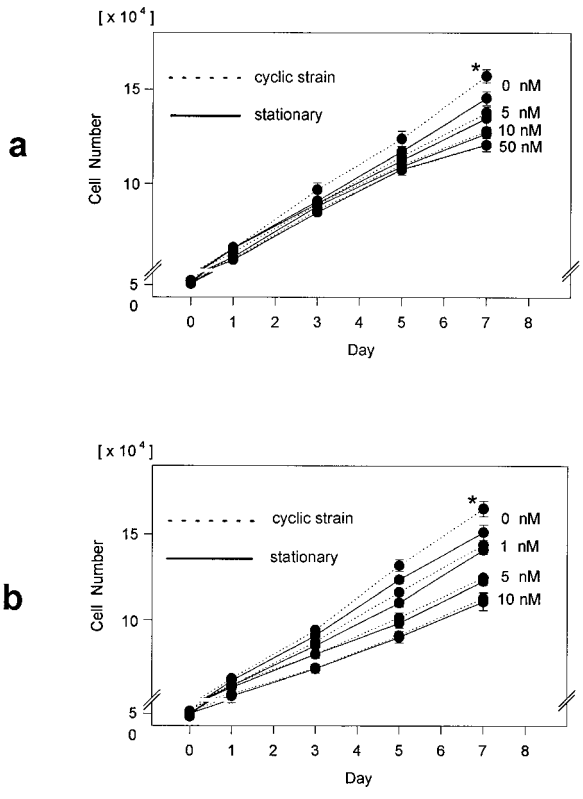

Fig. 3. Effects of PKC inhibitors on keratinocyte proliferation. **a:** Keratinocytes were subjected to cyclic strain or stationary condition with calphostin C (0, 5, 10, and 50 nM) for up to 7 days. Cell numbers were determined as described in Methods. Keratinocyte proliferation was downregulated by calphostin C in a dose-dependent manner. Calphostin C (>10 nM) significantly inhibited proliferation under stationary condition (5 nM for cyclic strain) on day 7. **b:** Keratinocytes were treated with staurosporine (0, 1, 5, and 10 nM) in the same regimen as in a. Staurosporine (>5 nM) significantly inhibited proliferation under both in strain and stationary conditions on day 7. Similar results were obtained in three independent experiments; values are presented as mean ±SEM, *$P < 0.05$ compared to control (stationary).

sure of strain, as shown in Figure 4A. The PKC inhibitors, calphostin C (10 nM) and staurosporine (5 nM), did not block the strain-induced effects on elongation and alignment (Fig. 4B and 4C, respectively).

Detection of PKC Isoforms by Western Blotting and Immunocytofluorescence of PKC Isoforms With Confocal Microscopy

As measured by densitometric analysis, PKC α and δ gradually decreased (68.1 ± 8.5 and 25.1 ± 4.6%, respectively) in the cytosolic fraction, and increased (82.7 ± 10.3 and 139 ± 21.6%, respectively) in the membrane fraction in keratinocytes subjected to 5 min of cyclic strain, respectively (Fig. 5). There was no significant change in the distribution or content of PKC ζ (5.7 ± 1.4% changes by densitometry) in cytosolic or membrane fractions (Fig. 5G,H) and β isoform failed to exhibit a corresponding band (Fig. 5C,D). As shown in Figure 6, PKC α in control keratinocytes exhibited a predominantly cytosolic distribution. After 10 min of cyclic strain, keratinocytes showed a strong perinuclear signal associated with PKC α (Fig. 6). As found with PKC α, PKC δ displayed a shift from the cytosolic region to the perinuclear zone in keratinocytes subjected to cyclic strain, as compared to unstretched controls. By contrast, PKC β isoform failed to display immunofluorescence in either control or strain-exposed keratinocytes. Signals of PKC

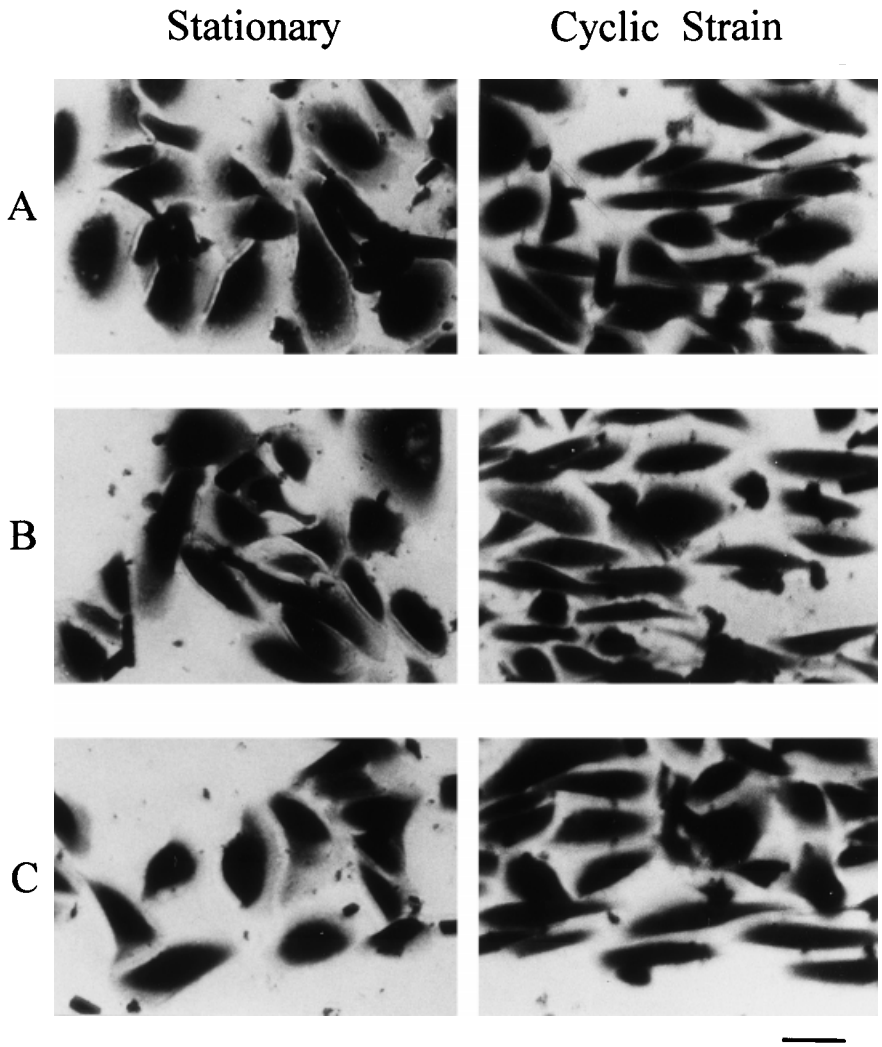

Fig. 4. Effects of PKC inhibitors on keratinocyte morphology under strain or stationary condition. **A:** Keratinocytes were cultured without PKC inhibitors under stationary or strain condition for 3 days. Photographs were taken from the periphery of the culture well under microscopic magnification (×250). Keratinocytes subjected to strain were elongated and aligned perpendicular to the force vector. The upper side is the center of the well. **B:** Keratinocytes were treated with calphostin C (10 nM) for 3 days in the same manner as in A. Keratinocytes in the periphery of the well were still elongated and aligned. Calphostin C did not block the strain-induced morphological features. **C:** Keratinocytes were treated with staurosporine (5 nM) as described in Material and Methods. Cyclic strain induced cellular alignment as seen in Figure 4 A and B under strain condition. Staurosporine (5 nM) did not inhibit the strain-induced morphological changes. Crystal violet staining, magnification ×250, bar scale = 50 μm.

ζ after strain appeared to be high in the perinuclear area, but that of control (stationary) was also noted to be high.

DISCUSSION

This study provides the first observation that a mechanical force stimulates PKC activity and translocation in human keratinocytes. Although the demonstration of strain-induced activation of PKC activity and translocation in keratinocytes is novel, the characteristics of the response have some similarity to previously described stimulation of keratinocytes by chemical agonists such as phorbol esters. First, the magnitude of strain-induced PKC activation in keratinocytes (184% maximal increase) was slightly less than the 200% increase previously reported for stimulation of PKC activity by PMA [Snoek et al., 1987]. Second, the onset of strain-induced activation of PKC (5 min) is comparable to that previously described for PMA (5 min, Reynolds et al., 1994]. Third, strain-

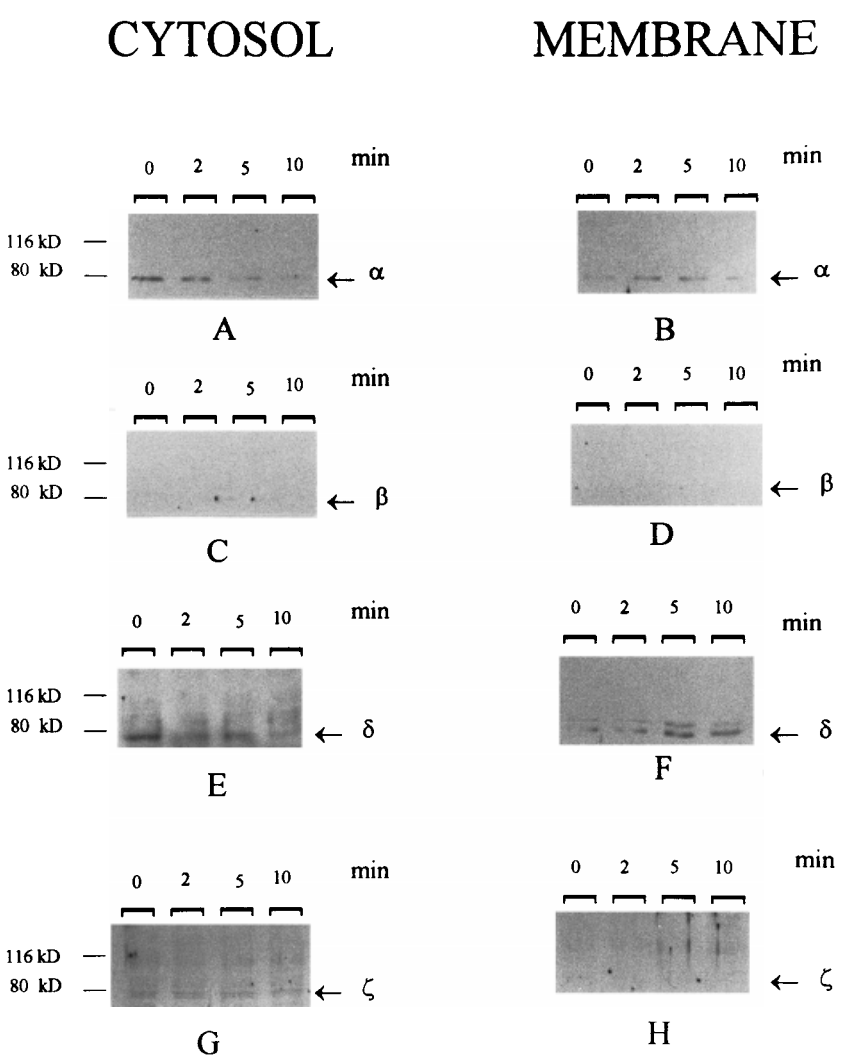

Fig. 5. PKC isoforms by Western blotting. Keratinocytes were subjected to cyclic strain for 2, 5, and 10 min, then lysed and fractionated as described in Methods. The same amount of protein (10 μg) was loaded into each lane and separated by 7.5% SDS-PAGE, followed by blotting on nitrocellulose paper, then detected by isoform-specific antibodies. Western blot analysis of PKC isoforms revealed that PKC α, δ gradually decreased in the cytosolic fraction (68.1 ± 8.5, 25.1 ± 4.6% decrease), while that of membrane fraction increased (82.7 ± 10.3, 139 ± 21.6% increase), respectively, at 5 min. There was no significant change in either PKC β, ζ in cytosolic, or membrane fraction, as shown in C, D, G, H (n = 6).

induced translocation of PKC from the cytosol to the particulate fraction in a manner analogous to that reported with PMA stimulation [Snoek et al., 1987]. Thus, the similarity between the effects of mechanical strain and chemical agonists on PKC signaling would suggest some similarities in distal transduction events.

However, the effect of mechanical strain and PMA on keratinocyte morphology are markedly different. In response to strain, keratinocytes elongate and align perpendicular to the strain vector. In contrast, chemical stimulation with PMA or Ca^{2+} for 48 h causes flattened phenotype and multiple fragmented nuclei [O'Driscoll et al., 1994]. These findings suggest that there could be common processes involved in mechanical and chemical stimulation of PKC activation and translocation, but distinct pathways involved in altering keratinocyte morphology. The strain-induced changes in morphology were most prominent in the periphery of the well. This is directly attributable to the heterogeneous nature of the strain, since the well periphery is a high-strain area as opposed to the low-strain center of the well. Thus, strain-induced morphological alteration of keratinocytes is proportionate to the magnitude of strain [Banes et al., 1990, 1985; Sumpio et al., 1988].

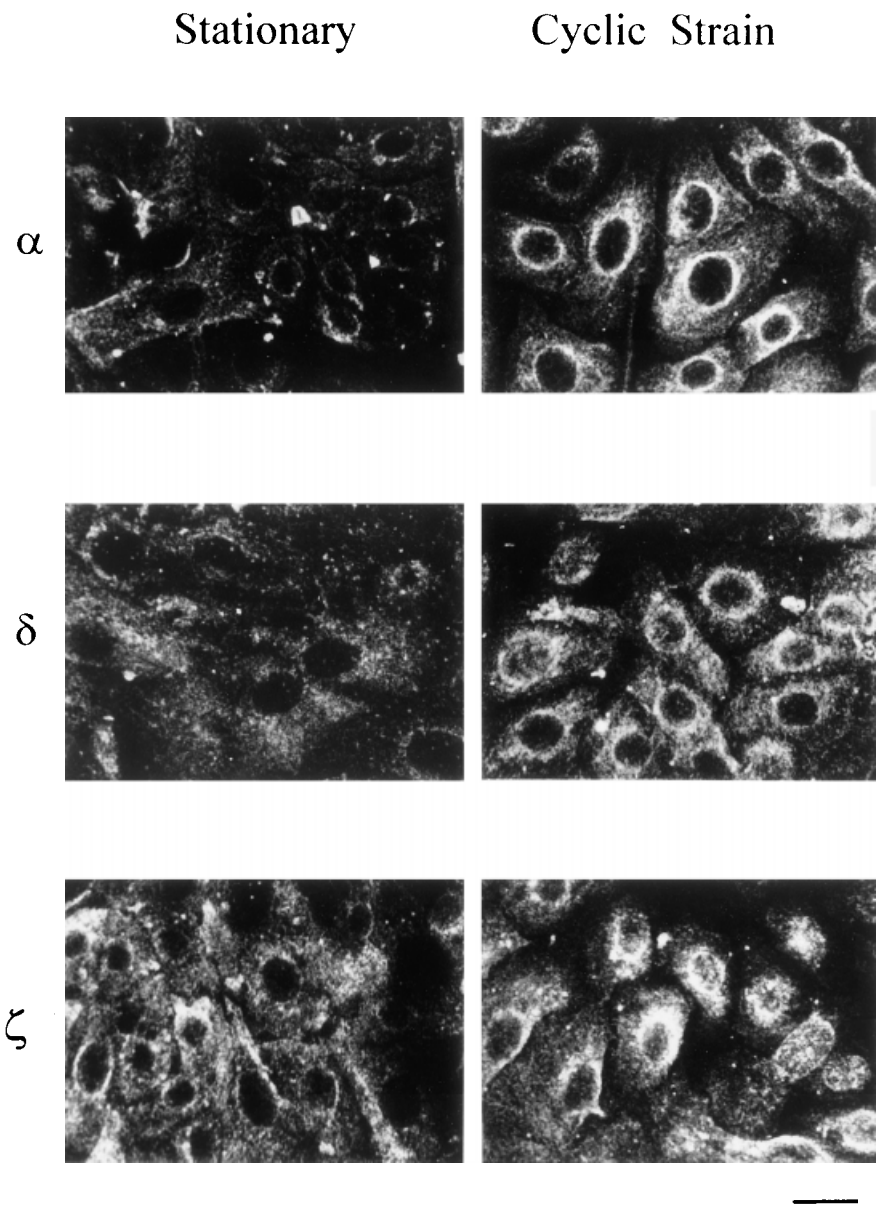

Fig. 6. PKC isoforms with confocal microscopy. Keratinocytes were subjected to cyclic strain or stationary condition for 10 min, then preparations were processed as described under Methods. Strong signals of PKC α and δ were observed in the perinuclear area under strain condition and that of PKC ζ also demonstrated a slight increase in fluorescent signal in the perinuclear area. Under stationary condition, PKC isoforms remained in the cytosol and showed a nonspecific intracellular distribution (bar scale = 25 μm).

The failure to replicate the earlier morphological findings observed in response to chemical stimulation with mechanical strain is supported by our studies with the PKC inhibitors, calphostin C and staurosporine. Exposure to either inhibitor failed to prevent elongation and alignment in keratinocytes subjected to cyclic strain. These findings suggest that the morphological changes induced by cyclic strain are independent of the PKC signaling pathway.

Strain-induced stimulation of PKC activity appears to play a more important role in the strain-induced stimulation of keratinocyte proliferation than for morphology. Unlike that described for strain-induced morphological changes, PKC blockade by calphostin C and staurosporine significantly blocked strain-induced proliferation. The relevance of PKC in mediating strain-induced proliferation in keratinocytes is bolstered by the different mecha-

nisms by which calphostin C and staurosporine inhibit PKC activity (i.e., calphostin C affects the regulatory domain [Bruns et al., 1991]), while staurosporine blocks the catalytic domain [Tamaoki, 1991]). Strain-induced increases in cell proliferation were subtle, and both PKC inhibitors blocked the strain-induced changes as well as the basal rate of proliferation. This finding is probably caused by one of the mechanical properties of strain apparatus that flexible membrane experiences the average 10% deformation during our experiments [Sumpio et al., 1988; Awolesi et al., 1995].

Western blot analysis demonstrated that the major isoforms responsible for mechanical strain-induced PKC translocation form the cytosol to the membrane were PKC α and δ. These findings were confirmed by immunofluorescence studies using confocal microscopy. Strong signals were observed in the perinuclear area, confirming earlier studies performed in endothelial cells [Rosales et al., 1992b]. The membrane fraction, also known as particular fraction, is a detergent-extractable fraction that contains the cell membrane, nuclear membrane and cytoskeleton [Bissonnette et al., 1994; Gopalakrishna et al., 1986]. Therefore, the shift of these isoforms to the perinuclear area is in agreement with our results of translocation.

PKC isoforms are classified into different groups depending on the Ca^{2+} requirement for maximal activation, PKC α, β, and μ are Ca^{2+} dependent while PKC δ, ϵ, ν, θ, μ, ζ, and λ isoforms are Ca^{2+} independent [Nishizuka, 1988, 1995]. Strain-induced translocation could be attributed to PKC α and PKC δ, which included both Ca^{2+} dependent and independent isoforms, implying that strain-induced responses are not mediated through Ca^{2+} dependent pathways.

Although the exact role of PKC isoform translocation is unknown in keratinocytes, several hypotheses are feasible including a role in cell adhesion and proliferation. Liu et al. [1995] reported that PKC α translocation to the cell membrane facilitates binding to adhesion proteins in melanoma cells, suggesting a role for PKC α as a cell adhesion modulator. A definitive role for PKC δ has not been delineated, but current studies have revealed that PKC δ is closely associated with epidermal growth factor receptor [Denning et al., 1995]. It is possible that strain-induced proliferation of keratinocytes could be mediated by PKC δ through a similar mechanism. Additionally, the cytoskeleton was also reported to be associated with EGFR [Denning et al., 1995], implying that the cytoskeleton might play a key role in mediating the physiologic signals of PKC δ. Although these findings are very promising for further investigations, the role of PKC δ as a growth modulator is still hypothetical. More studies need to be performed to confirm this hypothesis.

PKC α and δ appear to be the major isoforms involved in translocation in keratinocytes. Previous studies conducted in endothelial cells also showed PKC α translocation by cyclic strain, revealing that PKC α is commonly involved in translocation [Rosales et al., 1992a]. Whether PKC δ translocation is unique to keratinocytes is unclear, as this isoform was not examined in the earlier study. There exist some divergence among cell lines with respect to isoforms. For example, α and ϵ translocated in IIC9 fibroblasts, whereas PKC α, β, and ζ were the major isoforms involved in blood platelets [Nishizuka, 1995; Dekker, 1994]. However, PKC α showed a common translocation pattern between different cells, suggesting that some degree of conservation of the individual isoforms might exist among different cell types. Since the β isoform of PKC was undetectable in keratinocytes by Western blot analysis, it remains uncertain whether cyclic strain could modify the expression of PKC β. As shown by Reynolds et al. [1994], the PKC β isoform is hardly detectable in human keratinocytes.

In addition, similarities among different cell lines have also been supported by published observations. Sadoshima [1993] reported that PKC was activated by mechanical strain in rat cardiac myocytes, which could initiate a hypertrophic response of cardiac myocytes. In osteoblastes, PKC activity was increased by mechanical strain [Carvalho et al., 1994], implying that PKC appears to be involved in signal transduction induced by mechanical stimulation in various cell types. Ryan [1989] suggested that PKC could act as mechano-receptor, based on the facts that PKC was required for binding of actin-containing microfilament bundles to cell membrane, and streochemical configuration of PKC, which is sensitive to physical forces.

With regard to other physical forces [Vandenburgh, 1992; Osol, 1995], shear stress has been

extensively investigated, which is normally generated by blood flow and modulates endothelial cell growth and orientation [Davies et al., 1995; Langille et al., 1986]. These findings could suggest that mechanical perturbation of cells might induce various physiologic responses; however, the difference between each mechanical stimulus needs to be determined.

In summary, mechanical strain induced PKC activation and translocation, which involved PKC α and δ isoforms, as demonstrated by Western blot analysis and immunofluorescence studies using confocal microscopy. Our studies also provide supporting evidence that strain-induced changes in keratinocyte phenotype is differentially regulated by PKC, namely a role in strain-induced alterations in proliferation, but not in morphology.

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