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Mechanical stress-activated PKCδ regulates smooth muscle cell migration

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ABSTRACT

Vascular smooth muscle cells (SMCs) are exposed to altered mechanical stress that may contribute to SMC migration in the development of atherosclerosis. Signal transduction pathways in SMCs activated by mechanical stress that instigate cell migration are undefined. Herein, we provide evidence that mechanical stress enhances SMC migration, which is mediated, at least in part, by protein kinase C (PKC) δ. When rat SMCs cultivated on a flexible membrane were subjected to cyclic strain stress (60 cycles/min, 5, 15, or 20% elongation), PKCδ was translocated to the Triton-insoluble fraction, whereas PKCα was translocated to the membrane, which was confirmed by PKC kinase assays. Immunofluorescence and actin staining revealed a cytoskeleton translocation of PKCδ in SMCs stimulated by cyclic strain. PKCδ-deficient SMCs cultivated from PKCδ–/– mice showed an abnormal cytoskeleton structure, which was related to a diminished phosphorylation of paxillin, focal adhesion kinase, and vinculin in response to mechanical stress. Mechanical stress enhanced SMC migration, which was diminished in PKCδ–/– SMCs. Taken together, our data demonstrated that mechanical stress activates PKCδ translocation to the cytoskeleton, which is related to decreased SMC migration and indicates that PKCδ is a key signal transducer between mechanical stress and cell migration.

Key words: mechanical stress • smooth muscle cells • cell migration • cytoskeleton

Vascular smooth muscle cell (SMC) migration is a key event in the development of vascular diseases, including postangioplasty restenosis and spontaneous atherosclerosis (1). It has been identified that growth factors and cytokines, such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), induce SMC migration (2, 3); however, the direct effects of mechanical stress on this process of SMC migration have not been studied. In vivo, the vessel wall is exposed to two main hemodynamic forces or mechanical stress: shear stress, the dragging frictional force created by blood flow, and mechanical stretch, a cyclic strain stress created by blood pressure (4, 5). Shear stress stimulates endothelial cells to release nitric oxide (6) and prostacyclin (7), resulting in vessel relaxation and protection of vascular cells, whereas cyclic strain stress stimulated SMCs (8). In humans, atherosclerotic lesions occur preponderantly at bifurcations and curvatures (9) where hemodynamic force is

disturbed, that is, lower shear stress and higher mechanical stretch (10). Thus, mechanical stress could be a crucial factor in the pathogenesis of atherosclerosis.

Protein kinase C (PKC) comprises a large family of serine/threonine kinases activated by lipid-derived second messengers that differ in substrate preferences, intracellular localization, and activation mechanisms. The PKC family is subdivided into three groups: the conventional or classic PKC, the novel PKC, and the atypical PKC. Classic PKCs (α, β1, β2, γ) contain two conserved areas in their regulatory domains. The novel PKCs (δ, ε, ϕ, η/L, and possibly μ or PKD) have a C1 region and are activated by diacylglycerol or phorbol esters (11–13). Studies have indicated that PKC is involved in mechanical stress-induced gene expression of PDGF and Et-1 in endothelial cells. We demonstrated that mechanical stress results in rapid activation of PDGF receptor in SMCs without the involvement of PDGF ligand binding (14), which has been confirmed by several other groups (15–18). The downstream signal pathways and signaling network in SMCs initiated by mechanical stress seem to include Ras/Raf-1/ERK and Rac-p38MAPK (19). Among the likely signaling networks, different PKC isoforms have been shown to modulate these signal pathways crucial for SMC migration (17, 20, 21). However, direct evidence of any of the PKC isoforms being involved in the signaling pathways during SMC response to mechanical stress leading to cell migration has not been defined. In the present study, we evaluated potential effects of mechanical stress-activated PKCδ on SMC migration. We demonstrated that mechanical stress causes rapid activation of PKCδ in SMCs, which appears to mediate cytoskeleton reorganization and SMC migration.

MATERIALS AND METHODS

Cell culture and cyclic strain stress

PKCδ-deficient mice were generated by gene targeting in our laboratories as described previously (22). SMCs were isolated by enzymatic digestion of mouse aortas (knockout and wild-type mice) as described elsewhere (22, 23) and were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 20% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every 3 days, and cells were passaged by treatment with 0.2% trypsin, 0.02% EDTA solution. For cyclic strain stress experiments, SMCs were plated on silicone elastomer-bottomed and collagen-coated plates, (Flexcell, Meckeesport, PA). Cells achieving 90% confluence were serum-starved for 3 days and subjected to mechanical stress with the computer-controlled Cyclic Stress Unit (Flexcell 4000) placed in a humidified incubator with 5% CO_2 at 37°C. The Cyclic Stress Unit consisted of a controlled vacuum unit and a base plate to hold the culture plates. Vacuum (15 to 20 kPa) was repeatedly applied to the elastomer-bottomed plates via the base plates. Cyclic deformation (60 cycles/min) and 5, 15, or 20% elongation of elastomer-bottomed plates were used (24). This new model of the apparatus generates a homogeneous strain stress on the membrane.

Cell treatment and protein extraction

SMCs growing in silicone elastomer-bottomed culture plates to 90% confluence were serum-starved for 3 days. After strain stress, SMCs were washed twice with cold (4°C) phosphate-buffered saline (pH 7.4) and harvested on ice in buffer A (20 mM HEPES (pH 7.4), 2 mM

EDTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4, 1% Triton, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 100 μM phenylmethylsulfonyl fluoride) and then centrifuged at 13,000 rpm (Eppendorf centrifuge, Osterode, Germany) at 4°C for 30 min. The supernatant was harvest as total cell proteins for Western blot analysis. The membrane and cytosolic protein preparation was similar to that described by Pomerantz et al (25) with a slight modification (26). Briefly, SMCs were washed with cold phosphate-buffered saline (4°C), dislodged in phosphate-buffered saline, pelleted, and resuspended in 500 μl of homogenizing buffer (25 mM HEPES, 1.0 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 100 μM phenylmethylsulfonyl fluoride). Cells were sonicated for 10 s and centrifuged at 2000 rpm for 10 min to remove debris. The supernatant was centrifuged at 55,000 x *g* for 1 h at 4°C. The cytosolic supernatant was harvested as cytosolic protein. The pellet was resuspended in 50 μl of buffer A, sonicated for 10 s, and centrifuged at 55,000 x *g* for 1 h at 4°C. The supernatant was harvested as membrane protein. The pellets were resuspended in buffer A and sonicated for 30 s, which was harvested as Triton-insoluble fraction. The protein concentration was measured by the Bradford assay.

Western blot analysis

The procedure used for Western blot analysis was similar to that described previously (26, 27). In short, 30–100 μg of proteins were separated by electrophoresis through a 10 or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto nitrocellulose membranes. The blots were probed with antibodies against PKCδ and α, F-actin, G-actin, vinculin (Santa Cruz Biotechnology, Santa Cruz, CA), α-actin (Sigma, St. Louis, MO), phospho-FAK (Upstate Biotechnology Inc., Lake Placid, NY), phospho-paxilin, phospho-vinculin (New England Biolab). Specific antibody-antigen complexes were detected by using the ECL Western Blot Detection Kit (Amersham Pharmacia Biotech).

PKC Kinase Assays

Protein extracts (1/2 ml) containing 0.5 mg of proteins were incubated with 10 μl of antibodies against mammalian PKCδ and α, respectively, for 2 h at 4°C with rotation. Subsequently, 40 μl of protein G-agarose suspension (Santa Cruz Biotech. Inc.) was added and rotation was continued for 1 h at 4°C. Immunocomplexes were precipitated by centrifugation and washed twice with buffers A, B (500 mM LiCl, 100 mM Tris, 1 mM dithiothreitol, 0.1% Triton X-100, pH 7.6), and C (20 mM Mops, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Triton X-100, pH 7.2), respectively. PKCδ and α activities in the immunocomplexes were measured as described previously (27, 28). Briefly, immunocomplexes were incubated with myelin basic protein (6 μg; Upstate Biotechnology) and [γ-^{32}P]ATP (5 μCi) for 20 min. To stop the reaction, 15 μl of 4X Laemmli buffer was added and the mixture was boiled for 5 min and cooled on ice. Proteins in the kinase reaction were resolved by SDS-polyacrylamide gel electrophoresis (15% gel) and subjected to autoradiography.

Immunofluorescent Staining

The procedure used for immunofluorescent staining was similar to that described previously (29, 30). Briefly, stretch-stressed cells growing in the silicone elastomer-bottomed culture plates were washed with cold phosphate buffered saline (PBS), and fixed with a solution (2% formaldehyde,

0.2% gluteraldehyde in PBS, pH 7.2) for 15 min at room temperature and then treated with 0.02% Triton X-100 in PBS for 2 min. After washing with PBS, cells were blocked with 1% bovine serum albumin (BSA) in PBS. Cells were labeled with a rabbit antibody against PKCδ or α or vinculin for 1 h at room temperature and then washed with PBS. The cells were labeled with swine anti-rabbit Ig conjugated with FITC for 1 h and washed with PBS followed by rhodamine phalloidin (Sigma) staining for actin for 30 min. Cells were mounted with 90% glycerol PBS and examined by confocal microscopy.

Cell spreading assay

SMCs were plated on a slide bottle and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FCS in the presence or absence of Go6976 or Rottlerin (BioMol) at 37°C in a humidified atmosphere of 5% CO_2. Cells were fixed at different time points as shown in Figs. 3 and 8. For actin staining, rhodamine phalloidin was added to the cells and incubated for 30 min. For nuclear staining, cells on the slide were incubated with Sytox-Green (5 μM; Mol. Probe) for 20 min.

Migration assay

For the wound healing assay, SMC were seeded on flexible plates and grown until 90% confluence in DMED supplemented with 20% FCS. Cells were stretched mechanically (15% elongation) for 3 h and incubated at 37°C for 12 h. A line of cells was removed with a sterile pipette tip (~0.7 mm), and the cells were incubated at 37°C for 6 h. The cells were fixed with 2% formaldehyde, and nucleic staining was performed. The uncovered areas were measured under the microscope at different time points. Migration of SMCs was assayed on polycarbonate filters (Nucleopore Corp.) that had 8 μm pores in 48-well chemotaxis chambers (Neuro Probe Inc.; 23). Cultured SMCs were pre-treated with mechanical stretch (15% elongation) for 3 h, incubated at 37°C for 12 h, trypsinised, and suspended at a concentration of 5×10^5 cells/ml in serum-free DMEM. A volume of 50 μl of SMC suspension was placed in the upper chamber, and 30 μl of DMEM containing 100 ng/ml PDGF-BB was placed in the lower chamber. The chambers were incubated at 37°C for 8 h. After incubation, the filters were removed and the SMCs on the upper side of the filter were scraped off. SMCs that had migrated to the lower side of the filter were fixed in methanol, stained with Diff-Quick staining solution (Baxter), and quantified under a microscope. Migration activity was expressed as the mean number of cells that had migrated per ×200 field.

Statistical analysis

ANOVA was performed when more than two groups were compared. An unpaired Student's *t*-test was used to assess differences between two groups. A P value <0.05 was considered statistically significant.

RESULTS

Cyclic strain stress stimulates PKCδ and α translocation and activation

Traditionally, it has been believed that PKC translocates from the cytoplasm to the membrane in response to specific agonists or other stimuli (31). To investigate whether this translocation of PKCδ is altered after mechanical stress, PKCδ in stressed SMCs were separately determined by Western blot analysis. Surprisingly, strain stress treatment (60 cycles/min, 15% elongation) resulted in significant translocation of PKCδ to the cytosol and PKCδ protein levels were increased in the Triton-insoluble fraction that represents cytoskeleton-related proteins (Fig. 1*a*). Kinetic analysis indicates that this response occurred as early as 2 min with maximum translocation achieved after 2 min in the Triton-insoluble fraction and 30 min in the cytosol after treatment then declining thereafter (Fig. 1*a*). In contrast to that, PKCα translocated from the cytosol to the membrane, whereas no PKCα proteins were detectable in Triton-insoluble fractions, indicating a "classic" translocation for PKCα.

To further establish the relationship between mechanical strain stress and PKCδ translocation to Triton-insoluble fractions, a tensile strength-response analysis of mechanical stress-induced PKCδ translocations was performed. As shown in Fig. 1*b*, SMCs were stretched with elongations of 5, 15, and 20% of original size, respectively. The increase of PKCδ translocation to Triton-insoluble fractions corresponded to the increase in magnitude of stretch stress from 5–20%. A decline of PKCδ proteins in the membrane fraction coincided with increased amounts of PKCδ proteins in the cytosol in SMCs stimulated by increasing intensities of stretch (Fig. 1*b*). Again, no PKCα proteins were detectable in Triton-insoluble fractions, although the membrane translocation could be observed (Fig. 1*b*, lower panel). Because of the novel response where PKCδ proteins translocated to Triton-insoluble fractions, we performed kinase assays for both PKCδ and PKCα causing specific antibodies. A marked increase in kinase activities of PKCδ immunoprecipitated from Triton-insoluble fractions was observed, but no kinase activity for PKCα was detected after cyclic strain stress (Fig. 1*c*).

To directly confirm PKCδ translocation, double-staining for PKCδ and the cytoskeleton was used for SMCs stimulated by mechanical stress. Fig. 2 shows PKCδ translocation in SMCs in response to stretch. There was no significant change in the distribution patterns of PKCδ, that is, localization in the cytoplasm before and after mechanical stress (Fig. 2*a* vs 2*b*). However, a double-positive staining (yellow) for PKCδ proteins (green) and cytoskeleton (red) was observed in stressed SMCs (Fig. 2*c*), indicating translocation to the cytoskeleton.

Alterations in cytoskeleton rearrangement in PKCδ–/– SMCs

To further investigate whether PKCδ has any influence on cytoskeleton re-arrangement, SMCs were isolated from the aortic media of PKCδ-deficient mice generated in our laboratories. During cell spreading, wild-type SMCs showed that actin fibers mainly distributed on the edge of the cell 1 h after seeding, and rearranged in the cytoplasm by 6 h (Fig. 3*a* and *b*). Interestingly, PKCδ–/– SMCs had a different pattern of actin filament distribution (Fig. 3*c* and *d*), indicating a loss of normal actin reorganization.

Because cytoskeleton rearrangement requires actins and related enzymes or proteins, we examined several types of actins and actin-related proteins by Western blot analysis. The data shown in Fig. 4 indicate no difference in F-actin, G-actin, and α-actin between PKCδ–/– and PKCδ+/+ SMCs in response to mechanical stress. FAK, paxilin, and vinculin phosphorylation was markedly induced in PKCδ+/+ SMCs stimulated by cyclic strain, but much less in PKCδ–/– SMCs. Interestingly, paxillin protein levels were significantly lower in both PKCδ–/– and PKCα–/– SMCs compared with PKCδ+/+ SMCs (Fig. 4). To further scrutinize the effects of PKCd on phosphorylation of FAK, paxilin, and vinculin, SMCs were treated with PKC inhibitors Go6976 and Rottlerin and, subjected to Western blot analysis. Data shown in Fig. 5 indicate that pre-treatment of SMCs with PKCδ inhibitor significantly diminished phosphorylation of FAK, paxillin, and vinculin and influenced cytoskeleton re-arrangement, which was not affected by PKCα inhibitor Go6976. These findings further suggest the role of PKCδ in cytoskeleton reorganization. To visualize the distribution of actin-related proteins, double-staining for vinculin and actin fibers of PKCδ+/+ and PKCδ–/– SMCs was performed. In response to mechanical stress, vinculin proteins were re-localized from even to cluster patterns in wild-type SMCs, whereas such changes in vinculin distribution in PKCδ–/– SMCs were significantly diminished (Fig. 6), confirming the above findings of abnormal functioning actin-related proteins.

The impact of PKCδ in SMC migration

Because of the observed PKCδ-dependent alterations in the cytoskeleton, it would be interesting to investigate the effects of PKCδ on SMC migration, a key event in the pathogenesis of vascular diseases. After the cell layer was disrupted by scraping, SMC migration was evaluated at 0 and 24 h. PKCδ deficiency markedly reduced SMC migration resulting in slower closure of the wound (Fig. 7). Pre-treatment with cyclic strain stress significantly enhanced SMC migration, that is, complete closure of the wound in wild-type SMCs, but less effects on PKCδ-deficient SMCs (Fig. 7). In addition, PKCδ–/– SMCs showed a similar ability of proliferation to that of wild-types in the culture (data not shown). To confirm the PKCδ-dependent decrease in cell migration, a classic method for the assessment of cell migration was performed by using a Boyden Chamber. As shown in Fig. 8, the number of migrated PKCδ–/– SMCs was significantly lower than the wild-type controls. Importantly, pre-treatment of wild-type SMCs with mechanical stress resulted in enhanced migration, but less effects on PKCδ–/– SMCs, suggesting that mechanical stress-induced SMC movement is mediated, at lest in part, by PKCδ.

DISCUSSION

Migration of vascular SMCs plays an important role in the pathogenesis of vascular diseases (2, 3). In the present study we provide the first evidence that mechanical stretch-enhanced SMC migration is mediated, at least in part, by PKCδ activation. We demonstrate that PKCδ translocates to the cytoskeleton which is abnormal in PKCδ–/– SMCs. Because cell migration is a coordinated process consisting of signaling and cytoskeleton rearrangement, our data suggest that PKCδ could be a link between mechanical stress and actin fiber structuring during cell migration. Thus, these findings could be crucial to better understand the molecular mechanisms of SMC migration and to find new targets for therapeutic intervention.

Previous work has established that members of the classic PKC family, for example, PKCα, translocate to the cell membrane in response to TPA and other stimuli (32, 33). Concerning novel PKC, recent data indicate that PKCδ translocates into the mitochondria (34) and the nucleus (35) in U937 cell lines in response to TBA. Recent studies demonstrate that the mechanical treatment of SMCs is associated with translocation of PKCδ to the cytoskeleton, whereas PKCα is found in the membrane. These findings have been confirmed by cell fractionation, kinase assays, and immunofluorescence studies. The results indicate a diversity of translocation mechanisms for PKCδ in response to different stimuli, which may be related to different functions. In fact, Majumder et al. (34) provided evidence that the mitochondrial translocation of PKCδ is associated with cytochrome *c* release and apoptosis. We found that a proportion of PKCδ proteins also translocates to the mitochondria in response to mechanical stress (data not shown) as implicated by an increase of PKCδ in the cytosol (Fig. 1). Importantly, a proportion of PKCδ proteins appears in the cytoskeleton of SMCs stimulated by mechanical stress, indicating the presence of multiple translocations and functions for PKCδ in a variety of cell types.

Cell migration is a complex process involving receptor-mediated adhesion, membrane protrusion, and the formation of discrete cell-matrix adhesion sites linked to a reorganization of the actin cytoskeleton (36–38). The modulation of proteins by PKC-dependent phosphorylation and by phosphoinositide binding is critically involved in the regulation of these phenomena (39–41). For instance, vinculin, an adaptor protein between actin fibers and focal adhesions, is found to be a target of PKC phosphorylation during junctional assembly (42, 43). In the present study, we provide direct evidence that mechanical stress results in phosphorylation of vinculin, FAK, and paxillin, which are diminished in PKCδ–/– SMCs. Liu et al. showed that the association of paxillin with integrins markedly enhanced the rates of integrin-dependent phosphorylation of FAK and cell migration (44). Our finding that PKCδ–/– SMCs have significantly lower levels of phosphorylated paxillin might explain the diminished phosphorylation of downstream kinases, such as FAK, in those cells. Because the amount of cytoskeleton proteins, for example, F-actin. G-actin and α-actin, does not change in PKCδ–/– and PKCδ+/+ SMCs, the effects of PKCδ on actin structure could be due to PKCδ-mediated changes of actin-related proteins. These results suggest that PKCδ directly or indirectly influences phosphorylation of FAK, paxilin, and vinculin, which are essential for actin fiber rearrangement.

How does mechanical stress lead to SMC migration? Our hypothesis is schematically illustrated in Fig. 9. Two main signal pathways link mechanical stress to cell migration. One is the PDGF receptor-MAPK-MMP pathway that is responsible for cell detachment from matrix proteins. The other involves a PKCδ paxillin-cytoskeleton pathway essential for cell movement. Supporting this model is our previous finding that mechanical stress can directly activate tyrosin kinase-coupled receptors, including PDGF receptor (14), which is followed by PI_3K and MAPK activation (19). There is also evidence indicating that mechanical stress activates the transcription factor AP-1 in SMCs in vivo and in vitro (14, 45), which leads to production of matrix metalloproteinases, for example, collagenase (46–48). Furthermore, previous studies established that mechanical stress influences the structure of the cytoskeleton in endothelial cells and SMCs (49–51). In the present study, we demonstrated the crucial role of PKCδ in mediating the actin fiber re-arrangment that influences SMC migration. These findings close the gap between mechanical stress and cytoskeleton alterations and, therefore, the model formulated in

Fig. 7 could provide a better understanding for the molecular mechanisms of signaling involved in SMC migration.

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Fig. 1

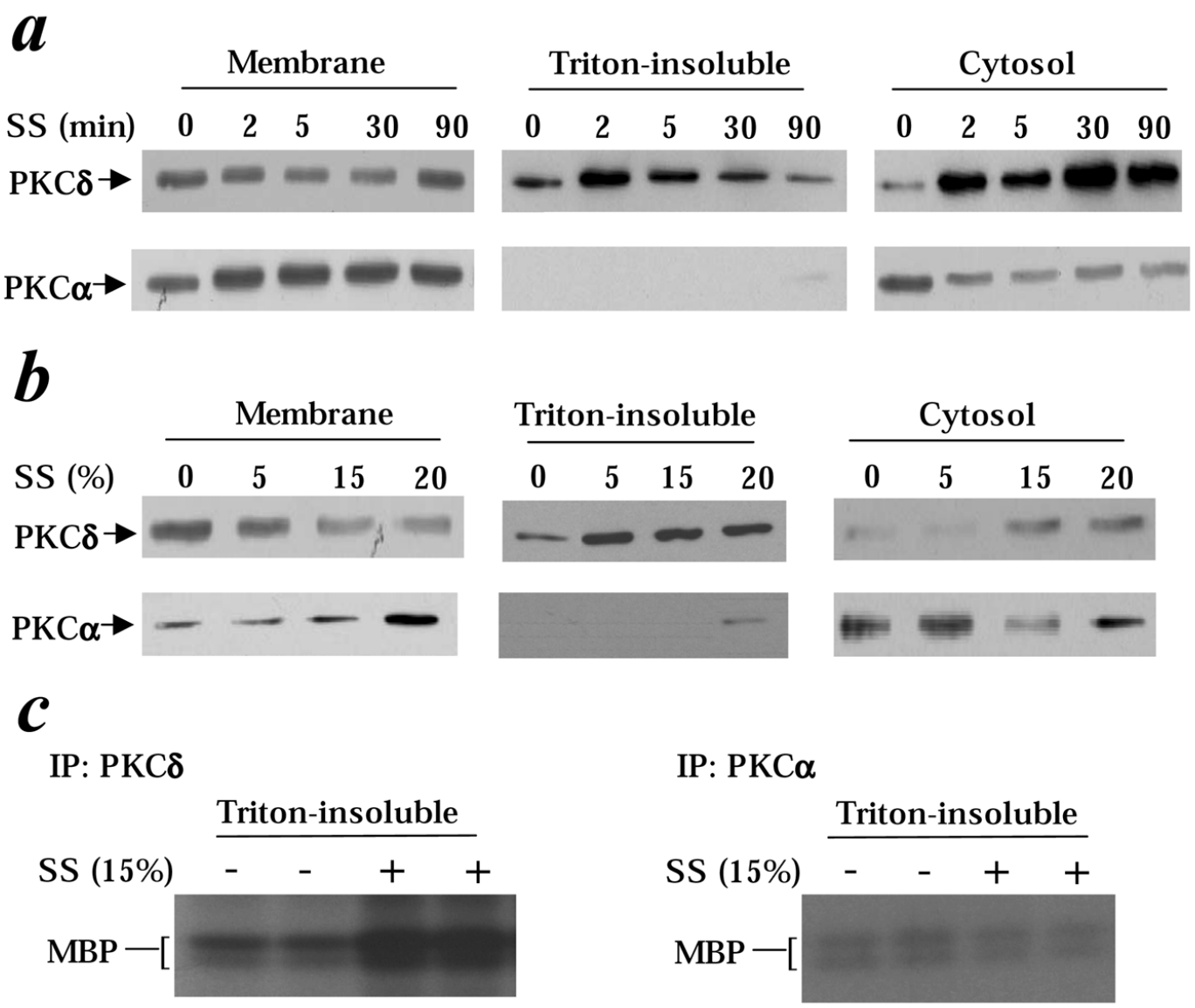

Figure 1. PKC translocation in SMCs exposed to mechanical stress. Serum-starved SMCs were treated with cyclic strain stress for the indicated times and were harvested. Protein extracts were prepared from the cell membrane, the Triton-insoluble fraction, and cytosol as described in Materials and Methods. Protein extracts were separated on 10% SDS-polyacrylamide gel, transferred to membranes, and probed by using an antibody to PKCδ and PKCα, respectively. ***a*)** SMCs were stressed with 15% elongation and 60 cycles/min. ***b*)** SMCs were treated for 2 min with 60 cycles/min. ***c*)** PKC kinase assays. SMCs were stressed with 15% elongation for 2 min. PKCδ and PKCα were immunoprecipitated from the protein extracts of Triton-insoluble fractions by using specific antibodies. Their kinase activities were measured based on the phosphorylation of a myelin basic protein (MBP) substrate. The data represent similar results from three independent experiments. SS indicates stretch stress.

Fig. 2

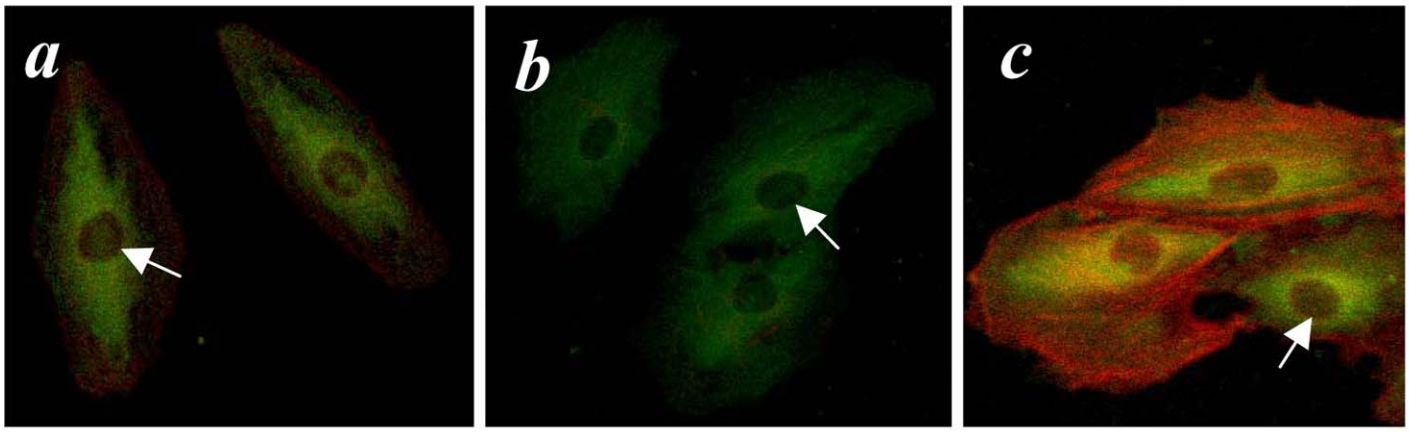

Figure 2. Double staining for PKCδ and actin fibers. SMCs were serum-starved for 3 days and treated with (***b***, ***c***) or without (***a***) strain stress (60 cycles/min) for 2 min. SMCs were fixed, incubated with rabbit anti-PKCδ antibodies for 1 h, and visualized with swine anti-rabbit Ig conjugated with FITC (***a–c***). After washing, cells were stained with rhodamine phalloidin for 30 min (***a***, ***c***). Photographs were taken with a confocal microscope, original magnification ×400.

Fig. 3

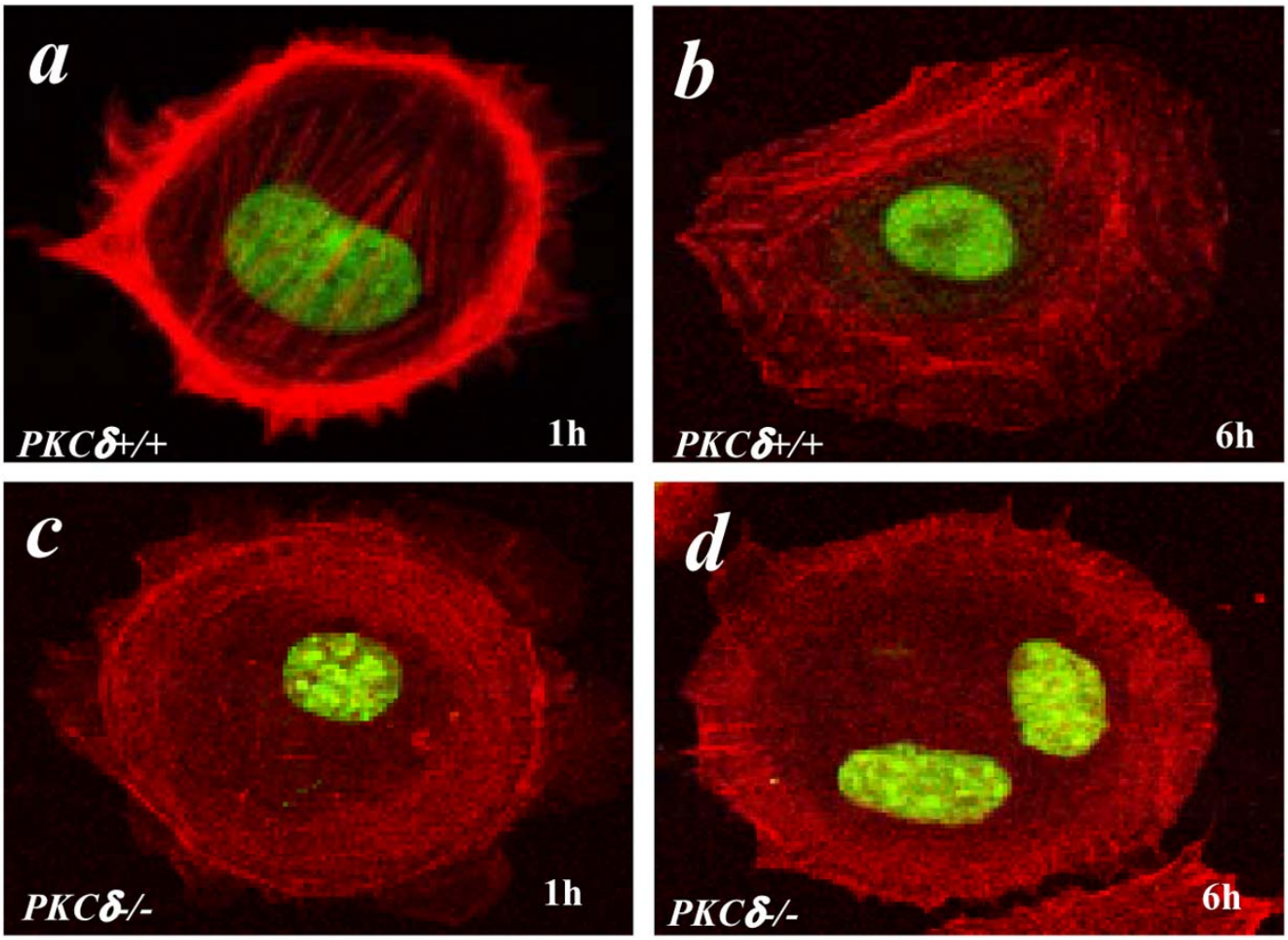

Figure 3. SMC spreading assays. PKCδ+/+ and PKCδ–/– SMCs were harvested with trypsin and EDTA solution, suspended in DMEM medium containing 20% FCS, and seeded onto a slide bottle. Cells were fixed at the indicated times and stained with rhodamine phalloidin for 30 min and Cytox-Green for 20 min. Photographs were taken with a confocal microscope, original magnification ×1000.

Fig. 4

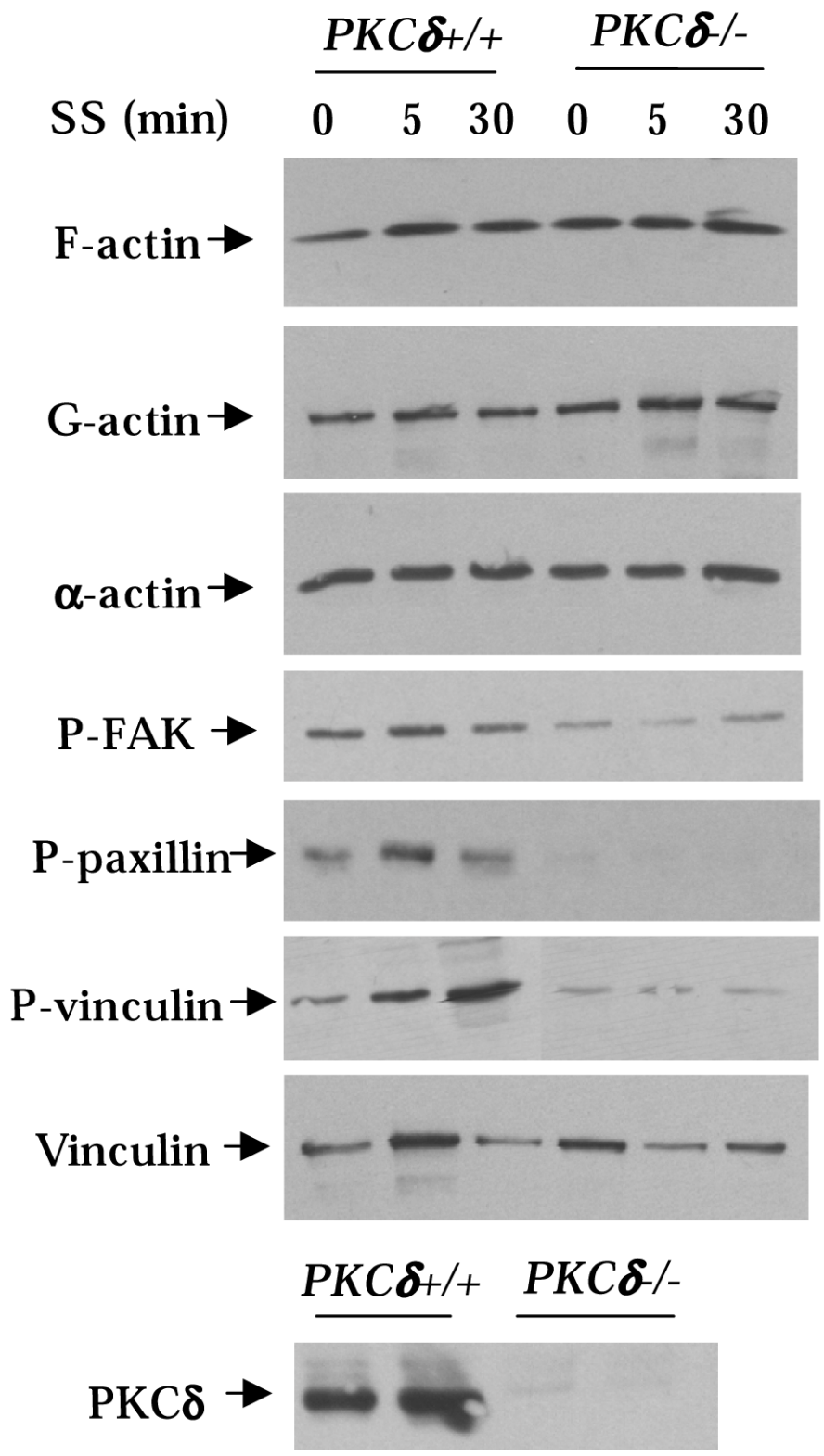

Figure 4. Western blot analysis for cytoskeleton and related proteins. Quiescent SMCs were treated with stretch stress (15% elongation, 60 cycles/min) for the indicated times. Cells were harvested for protein extracts. The results of Western blot analysis are shown for actins and cytoskeleton-related proteins. The lower panel indicates no PKCδ protein detected in PKCδ-deficient SMCs. Data represent similar results of two independent experiments. SS indicates stretch stress, and P, phosphate.

Fig. 5

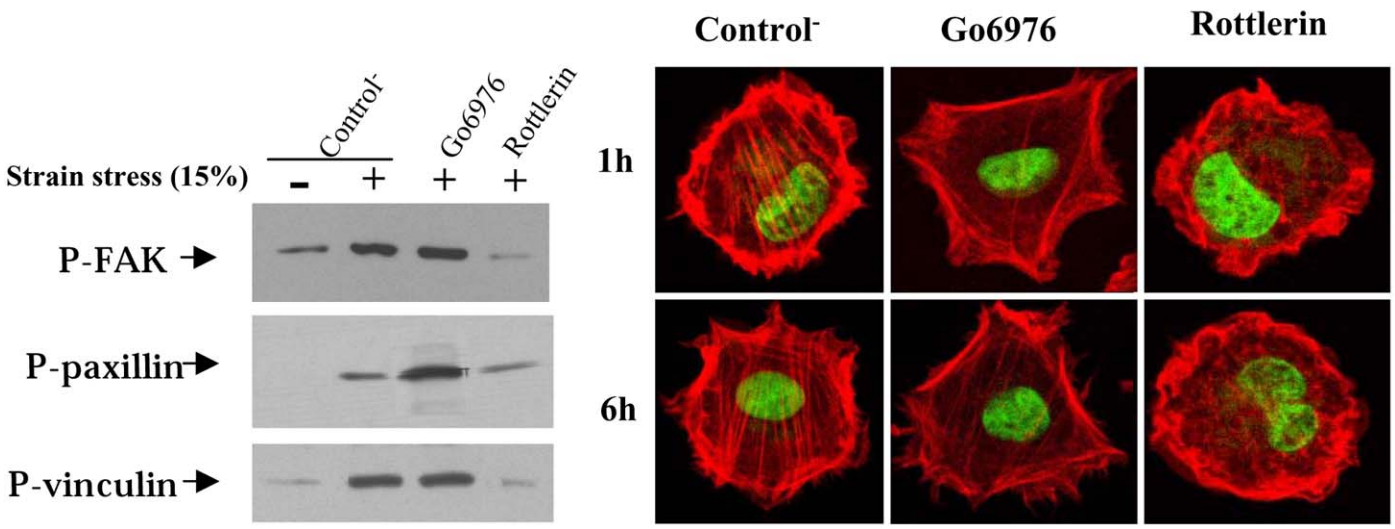

Figure 5. Effects of mechanical strain on cytoskeleton-related proteins. Left panel: Quiescent SMCs were treated with stretch stress (15% elongation, 60 cycles/min) for 10 min in the presence of G06976 (10 nM) or Rottlerin (10 μM) or the absence of the inhibitor (Control⁻). Cells were harvested for protein extracts. The results of Western blot analysis are shown for phorsphorylated-FAC, -paxillin and -vinculin. Right panel*:* SMC spreading assays. SMCs were harvested with trypsin and EDTA solution, suspended in DMEM medium containing 20% FCS in the presence of G06976 (10 nM) or Rottlerin (10 μM) or the absence of the inhibitor (Control⁻), and seeded onto a slide bottle. Cells were fixed at the indicated times and stained with rhodamine phalloidin for 30 min and Cytox-Green for 20 min. Photographs were taken with a confocal microscope, original magnification ×1000.

Fig. 6

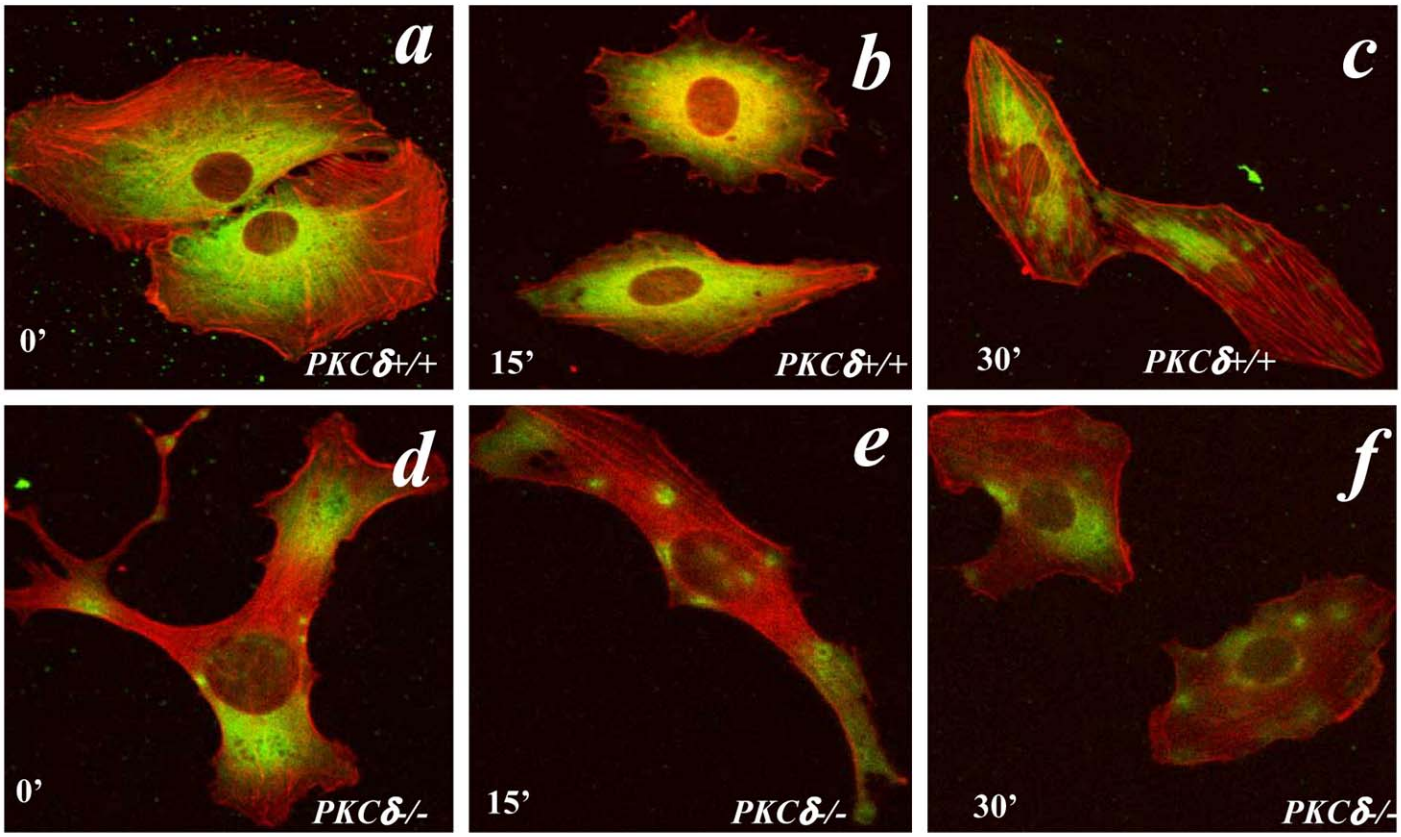

Figure 6. Double staining for vinculin and actin fibers. SMCs were serum-starved for 3 days and treated with strain stress (60 cycles/min, 15% elongation) for the indicated times. SMCs were fixed, incubated with rabbit anti-vinculin antibodies for 1 h, and visualized with swine anti-rabbit Ig conjugated with FITC. After washing, cells were stained with rhodamine phalloidin for 30 min. Photographs were taken with a confocal microscope, original magnification ×400.

Fig. 7

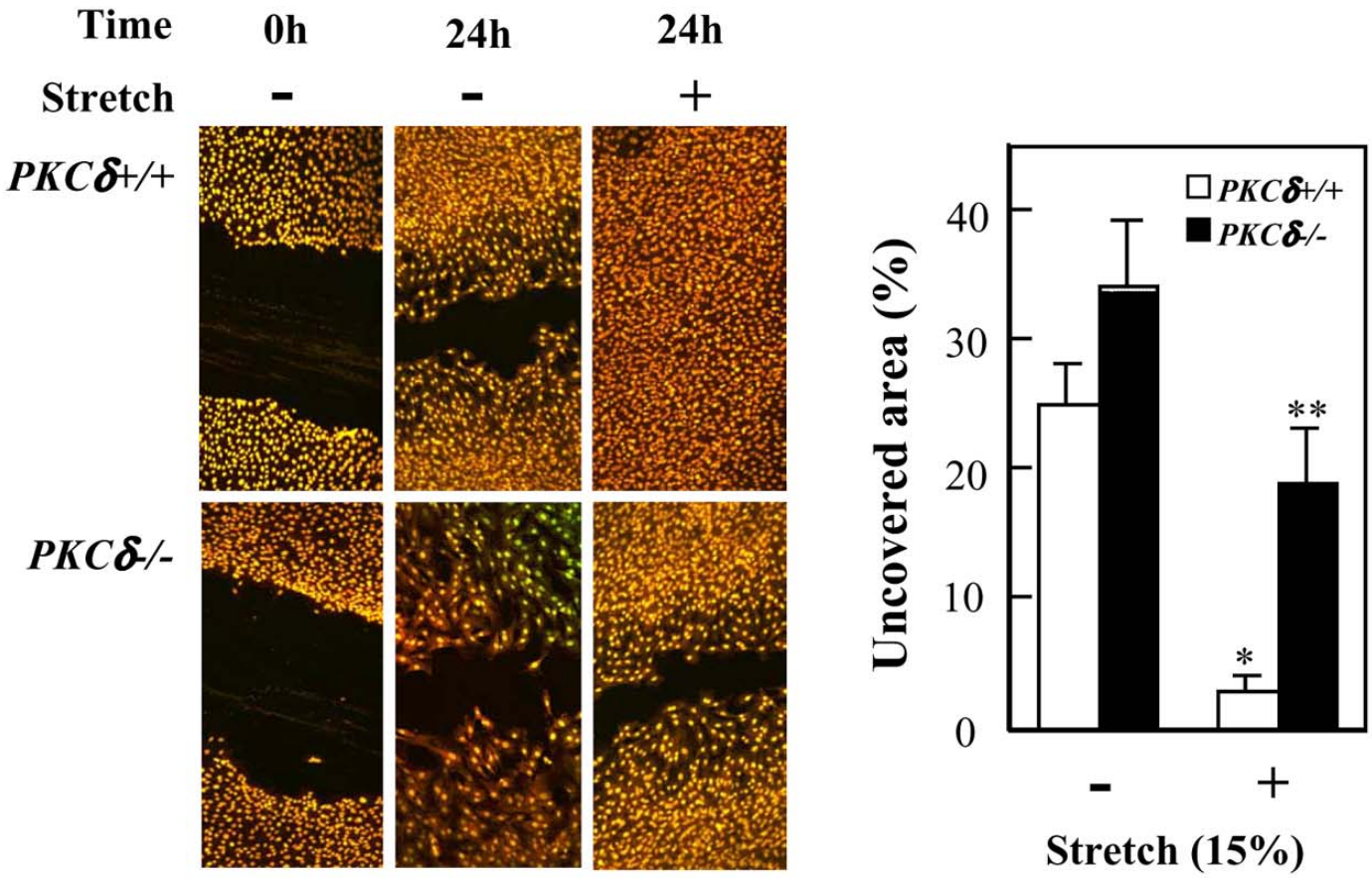

Figure 7. SMC migration or wound healing assays. PKCδ+/+ and PKCδ–/– SMCs were treated with stretch stress (15% elongation, 60 cycles/min) for 3 h and damaged with a pipette tip. Cellular nuclei were stained with propidium iodide after 0 and 24 h. Uncovered areas of the wound were measured under the microscope. The graph shows statistical data indicating a significant difference between treated and untreated cells, **P*<0.01 compared with untreated cells, ***P*<0.05 compared with PKCδ+/+ SMCs treated with stretch stress.

Fig. 8

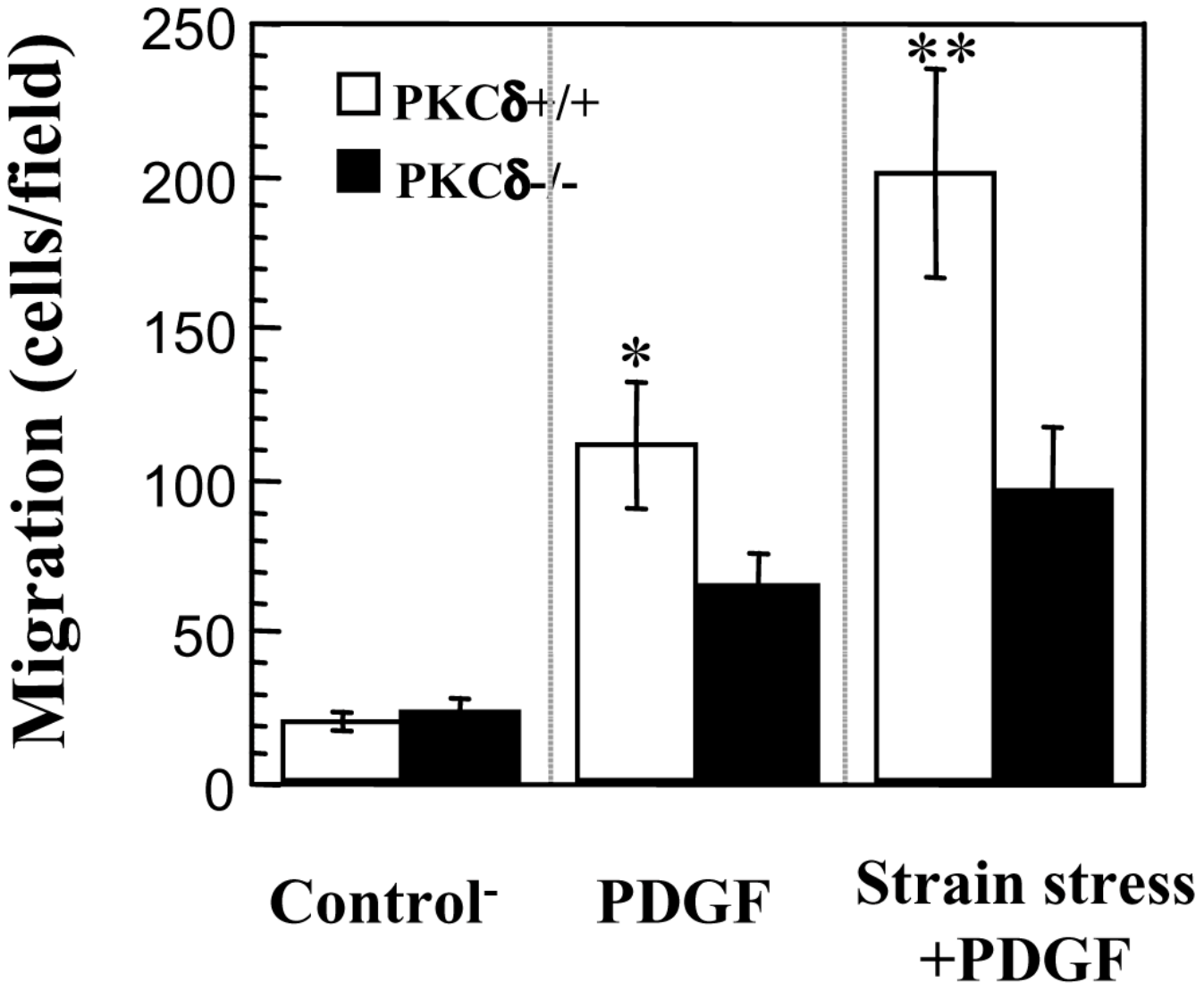

Figure 8. Migration in PKCδ+/+ and PKCδ–/– SMCs. A modified Boyden chemotaxis chamber was used for determining SMC migration. A polycarbonate filter with 8 μm pores was placed between the lower and upper chambers. SMCs with or without pre-treatment by strain stress in 50 μl of DMEM medium were added to the upper chambers, BSA (Control$^-$) or PDGF-BB in the lower chamber, and incubated at 37°C for 6 h. SMCs on the lower side of the filter were stained with a quick stain kit and counted under the microscope with a magnification ×200. Data represent means (±SD). *Significant difference from PKCδ–/– SMCs without strain stress treatment, **significant difference from PKCδ+/+ SMCs without strain stress treatment, $P<0.01$.

Fig. 9

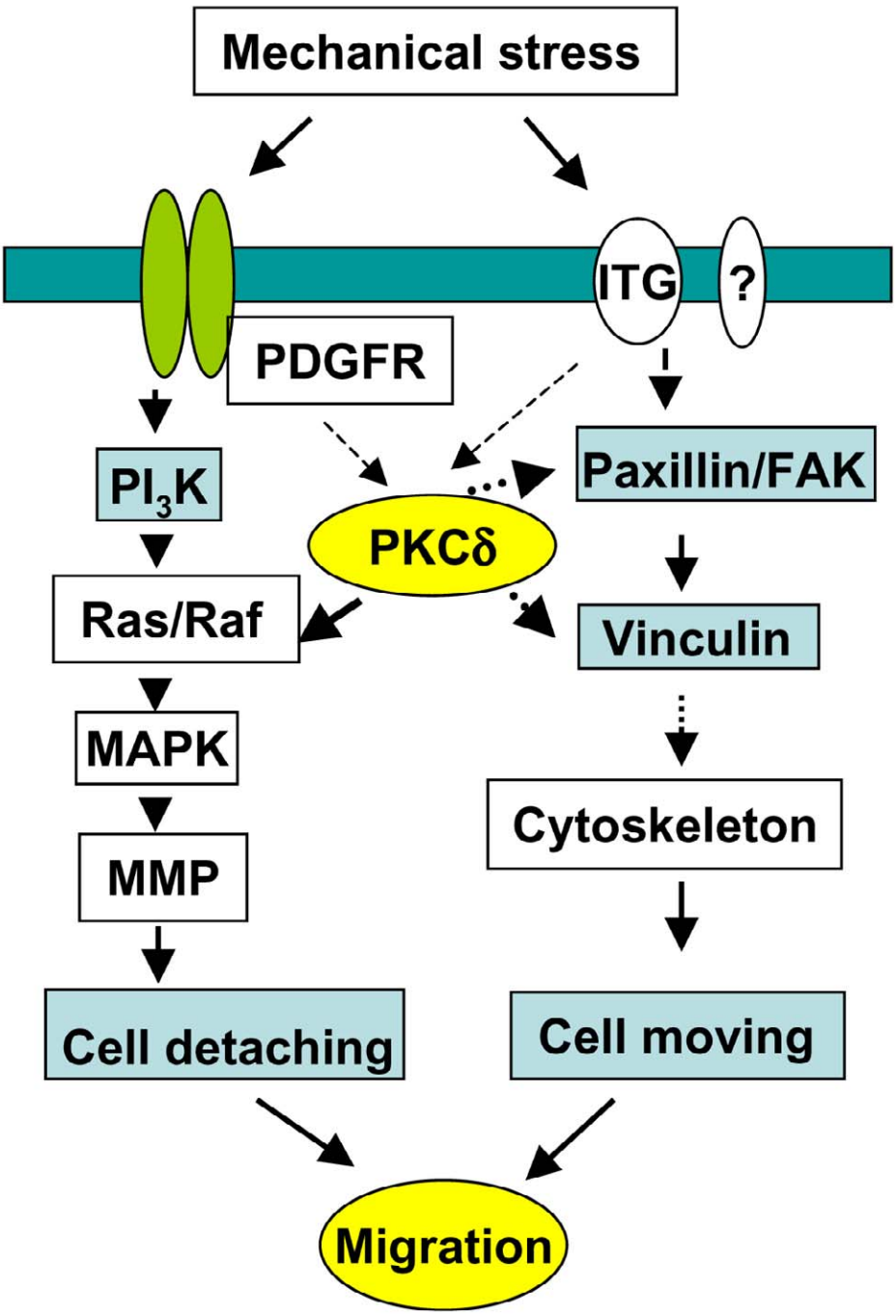

Figure 9. Schematic representation of PKCδ-dependent migration stimulated by mechanical stress. Mechanical stress can directly activate PDGF receptor (PDGFR)-MAPK pathways leading to matrix metalloproteinase (MMP) production, which is responsible for cell detachment from the matrix proteins. In parallel, mechanical stress activates integrins (ITG) resulting in cytoskeleton rearrangement, which is essential for cell movement. Both pathways are co-ordinately regulated by PKCδ leading to SMC migration.