Rho, Rac1, and Cdc42 activity assays

The small G protein affinity binding assays were performed using glutathione *S*-transferase (GST) fusion proteins with the Rho binding domain from rotekin (GST-RBD, for RhoA), with the Rac1 and Cdc42 interactive binding domain from PAK1 (GST-PBD, for Rac1/Cdc42), according to the manufacturer's protocol (Upstate Biotechnology, Charlottesville, VA). In brief, myocytes were lysed, and an equal volume of cell lysates was incubated with GST-RBD, GST-PBD, or GST beads (negative control). The samples were separated on SDS–PAGE. Activated RhoA, Rac1, and Cdc42 were detected by Western blotting, with antibodies against RhoA, Rac1, or Cdc42. Another set of equal amount of lysates was Western blotted with corresponding antibodies, to normalize the activated Rho GTPases in different samples. Scanning densitometry was used for semiquantitative analysis of the data.

In vitro kinase assay

ERK, JNK, and p38 MAPK activity was examined as previously described (Yamazaki et al., 1993). In brief, cardiomyocytes were lysed in buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 10 μg/ml leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The lysate was sedimented at 15,000*g* for 15 min at 4°C, and used for immunoprecipitation of p38, JNK1, or ERK 1/2. Immunoprecipitates were washed three times with the lysis buffer and two times with kinase buffer (25 mM HEPES, pH 7.4, 5 mM β-glycerophosphate, 2 mM dithiothreitol, and 10 mM $MgCl_2$) and resuspended in 40 μl of kinase buffer containing 4 μg of GST-ATF-2, GST-c-jun, or 20 μg of MBP, 50 μM ATP, and 10 μCi of [γ-^{32}P]ATP. The reaction mixture was incubated at 30°C for 20 min, terminated by the addition of 5× Laemmli's sample buffer. Samples were subjected to electrophoresis on SDS–PAGE, transferred to nitrocellulose membrane, and exposed to X-ray films. Loading differences were determined by blotting the membrane with anti-ERK, JNK1, p38, and MBP, c-jun, or ATF-2 antibodies.

Phosphorylation of Rho-GDI

Phosphorylation of Rho-GDI was performed using an in vivo labeling method (Mehta et al., 2001). In brief, serum-starved myocytes were labeled with 300 μCi/ml ^{32}P for 4 h in phosphate-free medium, and subjected to stretch for the indicated times. Cell lysates were immunoprecipitated with Rho-GDI or normal rabbit IgG (negative control) antibody. The phosphorylation of Rho-GDI was visualized by autoradiography. The blots were Western blotted with anti-Rho-GDI antibody to verify an equal amount of the protein in each sample. In vitro phosphorylation of GST-GDI was performed by incubating GST-GDI fusion proteins (Cytoskeleton, Inc., Denver, CO) with immunocomplexes of PKCα, δ, ζ, or ε obtained after immunoprecipitation of cell lysates with respective PKC antibodies or normal IgG (negative control), as previously described (Jain et al., 1999).

Subcellular fractionation and PKC translocation assay

Subcellular fractionation was performed as previously described (Strait and Samarel, 2000). Briefly, cardiomyocytes were sonicated in homogenization buffer (25 mM Tris-HCl, pH 7.5, 4 mM EGTA, 2 mM EDTA, 5 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin and leupeptin), and sedimented (100,000*g* for 60 min, 4°C), the supernatant fraction was saved as the cytosolic fraction. The pellet was resuspended in homogenization buffer with the addition of 1% Triton X-100. The sample was sonicated and sedimented again at 100,000*g* for 30 min at 4°C, the supernate was saved as the particulate sample.

Western blotting

Cardiomyocytes were lysed in lysis buffer. Equal amounts of extracted proteins were separated on 10% SDS–PAGE, transferred to nitrocellulose membrane, and the Western blots probed with antibodies specific for PKCα, δ, or the phosphorylated forms of MEK1, MEK3, MEK4, ERK1/2, JNK, or p38 MAPK. Membranes were reprobed with antibodies recognizing unphosphorylated forms of MAP kinases to confirm equal loading. Primary antibody binding was detected with horseradish-peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody and visualized by chemiluminescence (NEN Life Science, Boston, MA).

Leucine incorporation

Serum starved cardiomyocytes were subjected to cyclic stretch for 24 h. [^{3}H]-Leucine (1 μCi/ml) was added 4 h before harvest. The total radioactivity of incorporated [^{3}H]-Leucine into proteins was measured by liquid scintillation counting, as described previously (Yamazaki et al., 1995).

RNA isolation and Northern blot analysis

Total RNA was isolated using TRIzol reagent. Total RNA (20 μg) was separated on 1% MOPS-formaldehyde-agarose gel, and Northern blots were performed, as described previously (Manabe et al., 1999).

Immunocytochemistry

After 24 h of cyclic stretch, cardiomyocytes were fixed in 3.7% paraformaldehyde, and permeabilized with 0.3% Triton X-100. Cells were incubated with Texas Red-X phalloidin (1:40), or PKCα and δ antibodies (1:400). For characterization of PKC distribution, cells were further incubated with anti-rabbit FITC-conjugated secondary antibody (1:400, Sigma-Aldrich, St. Louis, MO). Immunostained cardiomyocytes were viewed by fluorescence microscopy. Quantitation of cell surface area was performed on actin-stained cardiomyocytes.

Statistical analysis

Data expressed as the mean ± SEM. Statistical differences were determined using a Student's *t*-test ($P < 0.05$).

RESULTS

Cyclic stretch induces activation of Rho GTPases in cardiomyocytes

By using a GST-RBD and GST-PBD pulldown assay, we observed that cyclic stretch induced a significant (sixfold) increase in RhoA-GTP, with maximal stimulation within 1–10 min. The binding of RhoA to GST-RBD was sustained at a higher level at 60 min (Fig. 1A,C). In contrast, stretch induced a rapid activation (about sevenfold) of Rac1 and Cdc42 in 1 min, which decreased to a basal level in 30 min (Fig. 1A,C). As a negative control, cell lysates prepared from stretched cells were incubated with GST beads, and activation of RhoA and Rac1 were examined. As shown in Figure 1B, we could not detect pulldown of GTP-RhoA and GTP-Rac1 (using GST beads), demonstrating the specificity of GST-RBD and GST-PBD assays.

Rho GTPases are involved in the cyclic stretch-induced hypertrophic responses

Cardiomyocytes were subjected to cyclic stretch for 24 h in the presence or absence of Toxin B or C3 exoenzyme (C3). Toxin B inactivates Rho family GTPases, but not Ras family proteins (Hofmann et al., 1997). C3 inactivates RhoA by ADP-ribosylating RhoA within the effector domain. It does not contain a binding or transfer component and therefore cannot efficiently permeate into the cell interior to access its enzymatic target. By using the liposome formulation lipofectamine, C3 can be efficiently introduced into cells (Borbiev et al., 2000). Cardiomyocytes were pretreated with lipofectamine (10 μg/ml) for 45 min, and exposed to C3 (5 μg/ml) overnight before subjecting to cyclic stretch for 24 h. Cyclic stretch induced a marked increase in cell size and an increase in both the staining intensity and the organization of the actin fibers in the cell cytoplasm (Fig. 2A). The increased cell size was inhibited by pretreatment with Toxin B and C3 (Fig. 2B). The myofibrillar reorganization was disrupted with a decreased intensity of actin immunostaining in Toxin B and C3 treated cells (Fig. 2A). Toxin B and C3 alone had no effect on the myocardial cell phenotype. Cyclic stretch promoted [^{3}H]-leucine incorporation, with an 80% increase observed, compared to control (Fig. 2C). Toxin B and C3 significantly inhibited the increase in leucine incorporation (70% and 65%, respectively). Both inhibitors had minimal effects on the basal level. Northern blot analysis demonstrated that stretch-induced expression of c-fos and ANP was blocked in Toxin B and C3 treated cells (Fig. 2D,E). These results suggest that Rho GTPases are required in cyclic stretch-induced cardiac hypertrophy.

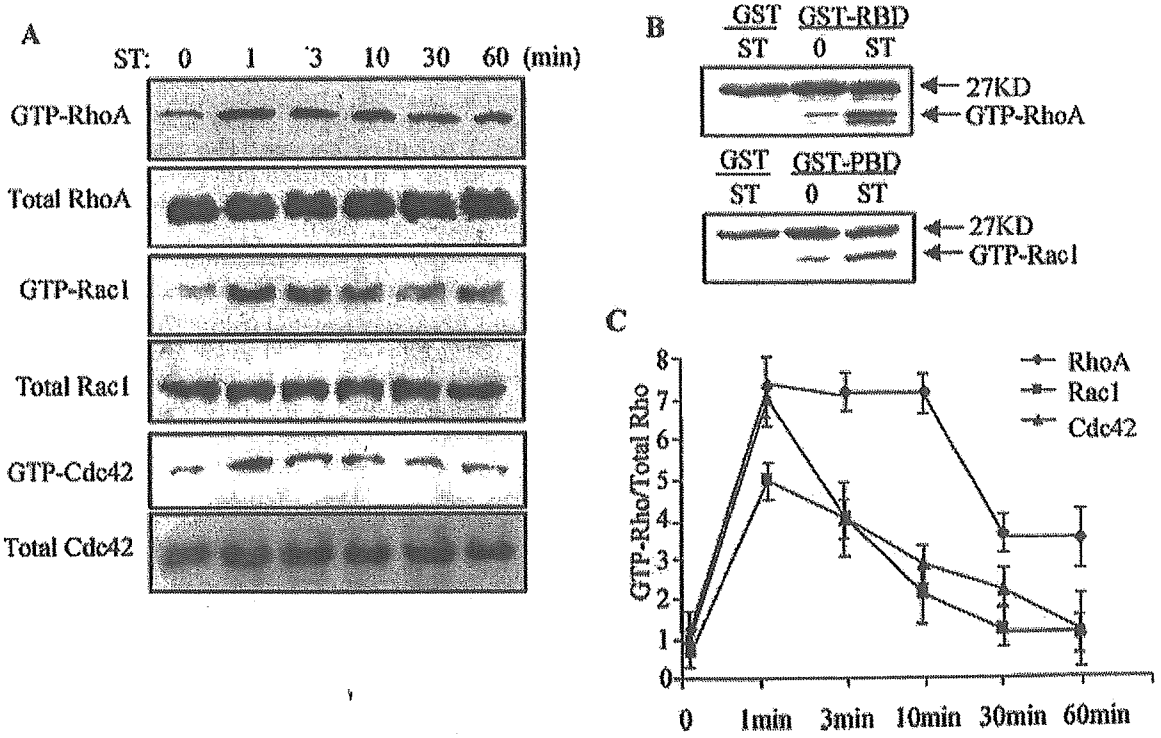

Fig. 1. Cyclic stretch induces activation of Rho GTPases. A: Cardiomyocytes were subjected to cyclic stretch (15% elongation, 60 cycle/min) for the times indicated. GTP-RhoA, GTP-Rac1, and GTP-Cdc42 were pulled down and detected by immunoblotting with anti-RhoA, -Rac1, and Cdc42 antibody, respectively. RhoA activity is indicated by the amount of RBD-bound RhoA normalized to the amount of RhoA in whole cell lysates; Rac1/Cdc42 activity is indicated by the amount of PBD-bound Rac1/or Cdc42 normalized to the amount of Rac1 or Cdc42 in whole cell lysates. B: Cardiomyocytes were subjected to cyclic stretch for 1 min, and the cell lysates were precipitated with GST-RBD, GST-PBD, or GST beads. The activation of RhoA and Rac1 was determined as in (A). GTP-RhoA, GTP-Rac1, and GTP-Cdc42 were quantified by scanning densitometry and were expressed relative to total proteins. Results are the mean ± SEM for three separate experiments (C).

Rho GTPases mediate cyclic stretch-induced activation of MAP Kinases

To further elucidate the downstream signaling pathways associated with the activated Rho GTPases, we determined the involvement of MAP Kinase pathways. Using an in vitro kinase assay, we demonstrated that ERK1/2, JNK, and p38MAPK were activated by cyclic stretch (Fig. 3A). ERK and JNK were activated at 5 min, and peaked at 15 min. The activation of p38 was observed at 2 min, with a maximum activation at 15 min. Stretch-induced activation of JNK and p38 was strongly inhibited, and that of ERK partially inhibited, by Toxin B (Fig. 3B, left part, and Fig. 3C). C3 significantly inhibited the activation of ERK and p38, and partially inhibited the activation of JNK. The membranes reprobed with antibody against ERK1/2, JNK1, or p38 and antibody against kinase substrates (MBP, c-jun, and ATF-2) showed an equal amount of protein in different samples. These results indicated that RhoA and Rac1/Cdc42 were differentially involved in mediating the activation of MAP kinases.

PKC regulates cyclic stretch-induced activation of Rho GTPases

To determine the role of PKC in cyclic stretch-mediated activation of Rho GTPases, chelerythrine

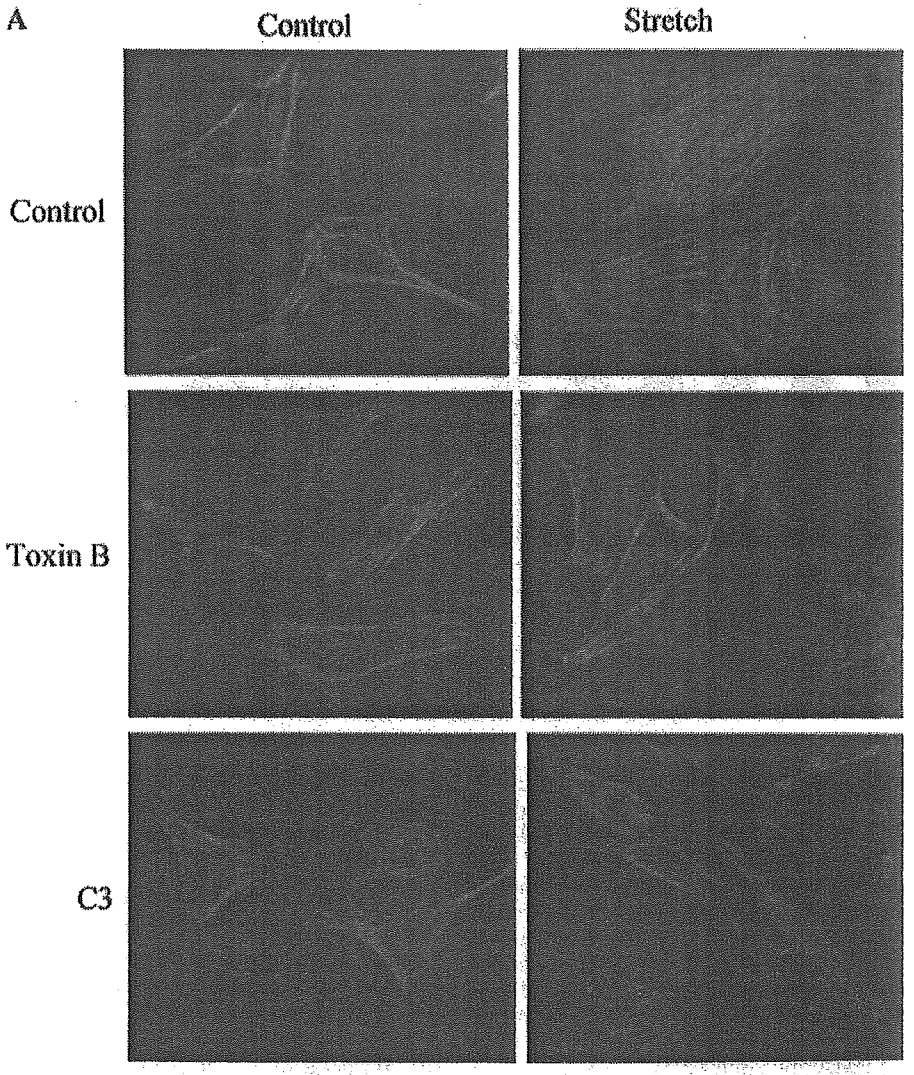

Fig. 2. Rho GTPases are involved in stretch-induced hypertrophic responses. **A:** Serum-starved cardiomyocytes were subjected to cyclic stretch for 24 h in the presence and absence of Toxin B (10 ng/ml) and C3 exoenzyme (5 μg/ml, overnight pretreatment, C3). Immunofluorescence was performed with Texas Red phalloidin antibody. **B:** Phalloidin-stained cells were imaged and cell surface areas calculated with the *Image J* Program (n = 100 cells each). $^{*}P < 0.001$ versus control; $^{\#}P < 0.001$ versus stretch. **C:** [^{3}H]-leucine (1 μCi/ml) was added 4 h before harvest. The total radioactivity of incorporated [^{3}H]-leucine into proteins was measured by liquid scintillation counting. Each bar represents the mean ± SEM of six separate experiments. $^{*}P < 0.05$ versus control and $^{\#}P < 0.05$ versus stretch. **D:** Cardiomyocytes were subjected to stretch for 30 min in the presence or absence of Toxin B and C3. Total RNA was extracted, and Northern blot was performed as described under Materials and Methods. **E:** Cardiomyocytes were subjected to stretch for 24 h in the presence or absence of Toxin B and C3. Total RNA was extracted, and Northern blot was performed.

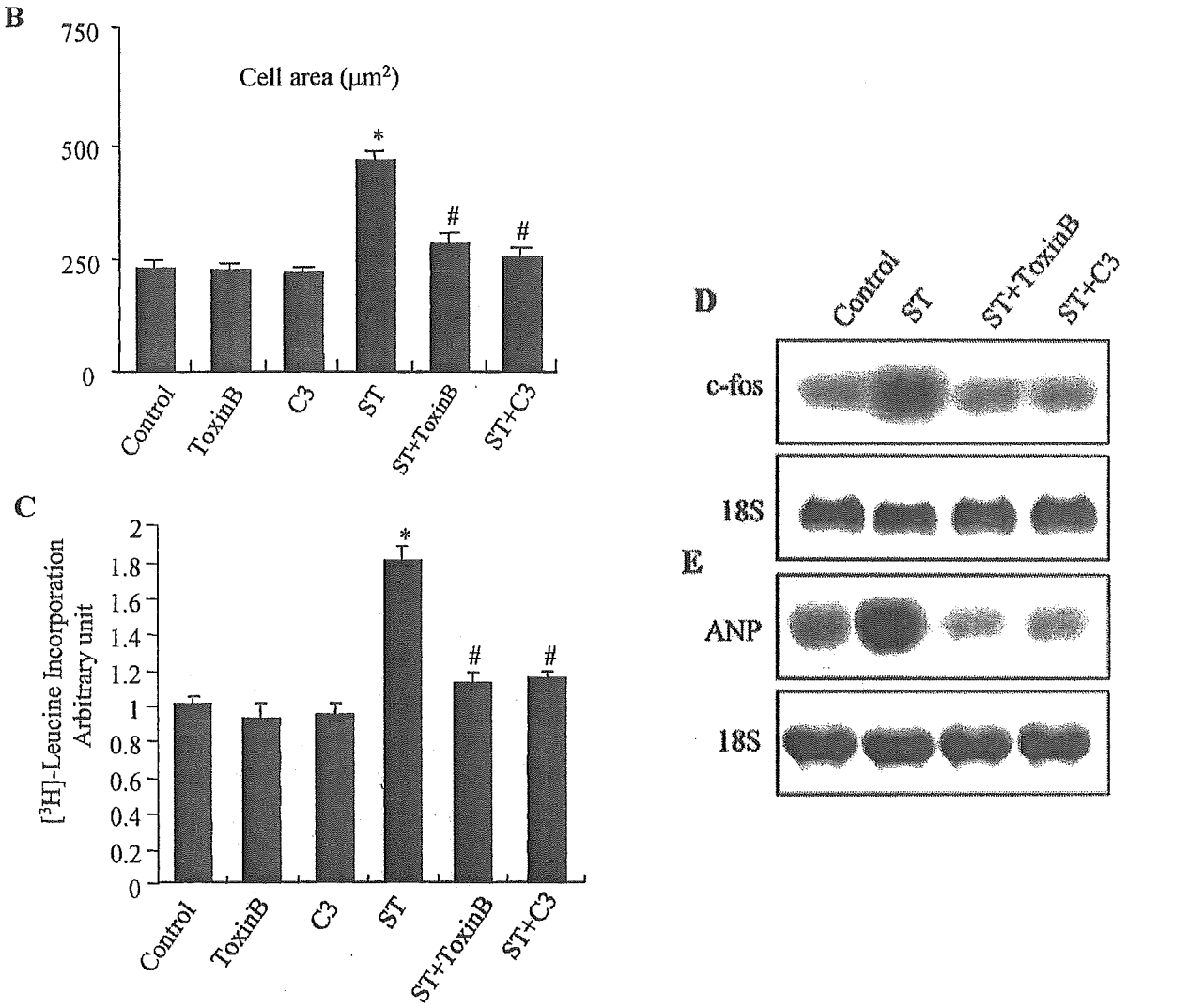

Fig. 2. (*Continued*)

chloride, a specific, but non-isozyme-selective PKC inhibitor was used. Cyclic stretch-induced activation of RhoA, and Rac1/Cdc42 was blocked by chelerythrine, indicating that PKC is an upstream regulator of Rho GTPases (Fig. 4A). We further determined whether the involved PKC isozymes were phorbol ester-sensitive, by depleting conventional and novel PKC isozymes using PMA (when applied chronically, it downregulates the expression of conventional and novel PKCs) (Szallasi et al., 1994). Overnight pretreatment of cardiomyocytes with PMA inhibited stretch-induced activation of Rho GTPases (Fig. 4A). PMA and chelerythrine alone had no effect on the activation of RhoA, Rac1, and Cdc42. To further understand the role of PKC activation in regulating Rho GTPases activation, we used PMA, as a direct activator of PKC. Cardiomyocytes were treated with PMA for 1–60 min, and the activation of Rho GTPases was determined. As shown in Figure 4B, PMA stimulation induced rapid activation of RhoA, Rac1, and Cdc42, which returned to basal level at 30 min. These data suggested that stretch-induced activation of Rho GTPases was regulated by phorbol ester-sensitive PKC isozymes.

PKC regulates cyclic stretch-induced phosphorylation of Rho-GDI

The dissociation of Rho-GDI is a prerequisite for membrane association and activation of Rho GTPases by GEFs (Bokoch et al., 1994). Rho-GDI phosphorylation/dephosphorylation has been implicated in regulation of the dissociation of the Rho GTPases/Rho-GDI complex (Bourmeyster and Vignais, 1996; Gorvel et al., 1998; Mehta et al., 2001). Using an in vivo labeling method, we observed that Rho-GDI was rapidly phosphorylated by cyclic stretch at 1 min, and returned to the basal level at 10 min, consistent with the activation of Rho GTPases (Fig. 5A). As a negative control, cell lysates from stretched cells were incubated with normal rabbit IgG, and the

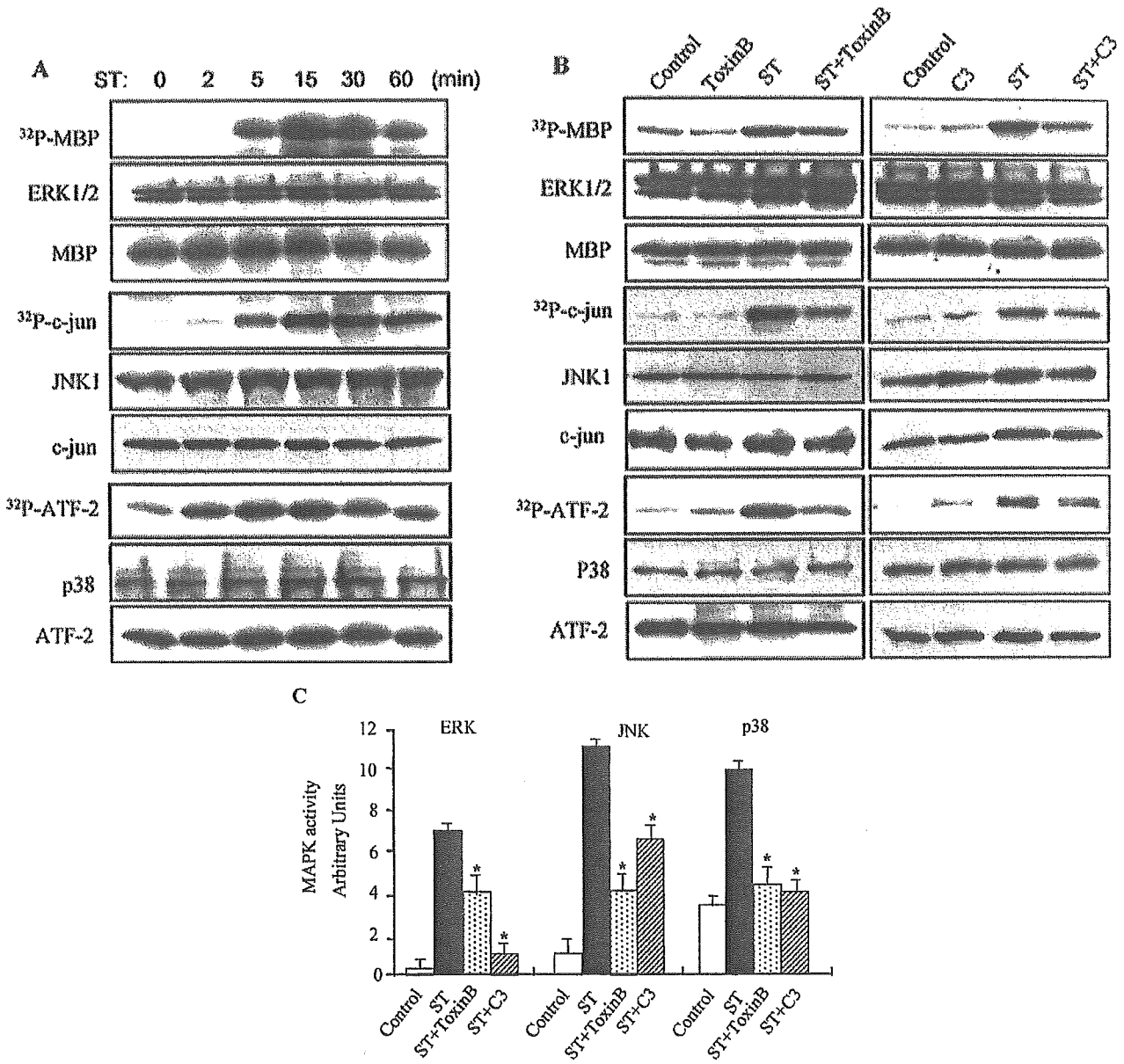

Fig. 3. Rho GTPase regulates cyclic stretch-induced activation of MAP kinases. **A:** Serum-starved cardiomyocytes were subjected to cyclic stretch for the times indicated. Cell lysates were used for immunoprecipitation of ERK, JNK1, and p38MAPK. A kinase assay was performed by adding 20 μg of myelin basic protein, 4μg of c-Jun or GST-ATF-2, respectively, and10 μCi of [γ-^{32}P] ATP. The samples were electrophoresed, transferred to nitrocellulose membrane, and exposed to X-ray films. The activity of ERK1/2, JNK, and p38MAPK was determined by autoradiography. The membranes were Western blotted with antibodies against ERK1/2, JNK p38, and corresponding kinase substrates, to verify an equal amount of protein in each sample. **B:** Cardiomyocytes were pretreated with or without Toxin B (10 ng/ml, 1 h) and C3 (5 μg/ml, overnight), then subjected to cyclic stretch for 15 min. The activity of ERK, JNK, and p38MAPK were determined as described above. **C:** Scanning densitometry was used for semiquantitative analysis of the data. Results are means ± SEM for three separate experiments. *$P < 0.05$ versus cyclic stretch.

in vivo phosphorylation of Rho-GDI was determined. As shown in Figure 5B, no phosphorylation of Rho-GDI was detected (left 2 lanes). Inhibition of PKC by chelerythrine or depletion of PKC by PMA, attenuated stretch-induced phosphorylation of Rho-GDI (Fig. 5C), indicating that phorbol-sensitive PKC isozymes are involved. We further determined whether PKC directly phosphorylated Rho-GDI in vitro, using GST-GDI as a substrate. GST-GDI was modestly phosphorylated by PKCα and δ in unstretched myocytes; but the phosphorylation significantly increased following cyclic stretch. Under similar conditions, PKCζ and ε failed to induce phosphorylation of GST-GDI (Fig. 5D). These results indicated that PKCα and δ were the major regulators in

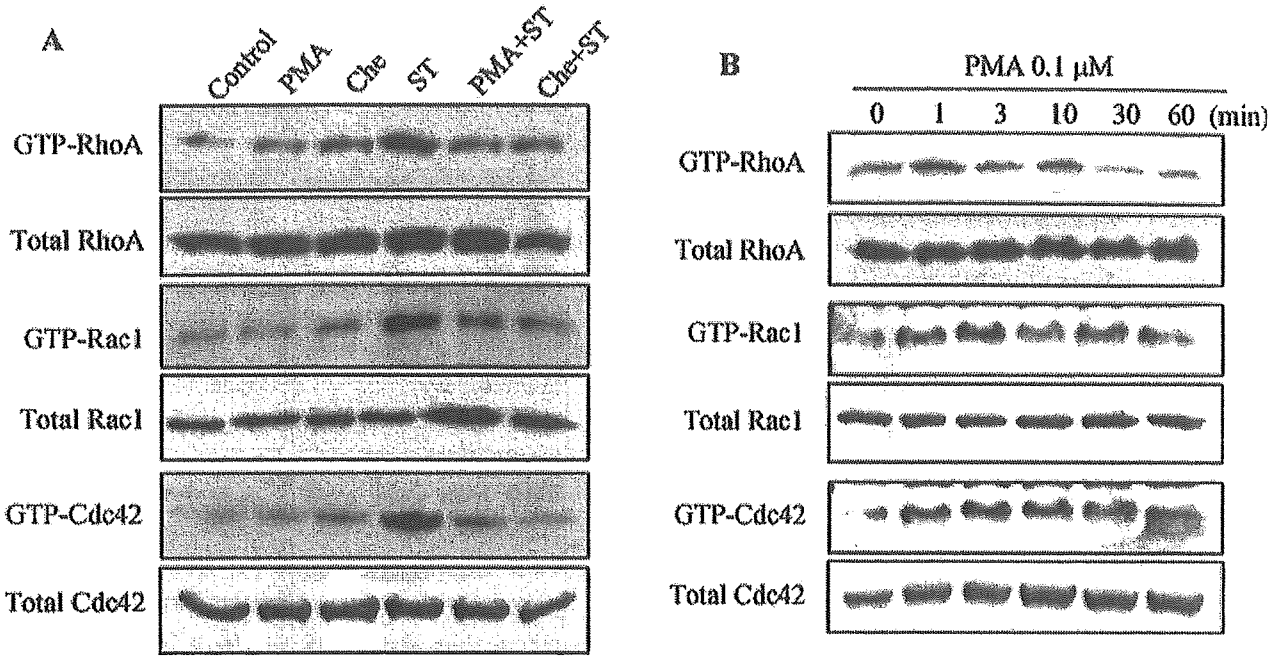

Fig. 4. PKC regulates stretch-induced activation of Rho GTPases. **A**: Cardiomyocytes were pretreated with or without chelerythrine (Che, 10 μM) for 30 min, or PMA (1 μM, overnight). After 1 min of stretching, Rho GTPases activity was assayed as described in Figure 1. **B**: Cardiomyocytes were exposed to PMA (0.1 μM) at the indicated times. Rho GTPase activity was assayed. Data are representative of three independent experiments.

mediating the phosphorylation of Rho-GDI. Another set of cell lysates were incubated with normal rabbit IgG, and in vitro kinase assay was performed to serve as a negative control (last part).

Subcellular localization of PKCα and δ

To determine the role of PKCα and δ in the regulation of cardiomyocyte growth and activation of Rho GTPases, the adenoviral-mediated gene transfer of WT and dominant-negative (DN) mutant of PKCα and δ was performed. Western blot analysis showed that the level of PKCα and δ overexpression was dose dependently increased after adenovirus infection (Fig. 6A). To verify the translocation of adenoviral-mediated overexpression of PKCα and δ in cardiomyocytes, cell membrane (particulate) and cytosolic (soluble) protein extracts were prepared and subjected to Western blotting. As shown in Figure 6B, endogenous PKCα and δ expression was predominantly located in the cytosolic fraction. AdPKCαWT and AdPKCδWT overexpression specifically increased the total PKC localization to the membrane fraction, while AdPKCαDN and AdPKCδDN overexpression increased total PKC localization to the cytosolic fraction. No crosstalk was observed between these two isozymes. To further characterize the subcellular localization of PKCα and δ, immunocytochemistry was performed. Overexpression of AdPKCαWT and AdPKCδWT demonstrated a diffuse distribution in cardiomyocytes; however, a significant concentration was observed surrounding the nucleus in AdPKCδWT infected cardiomyocytes. After 30 min of cyclic stretch stimulation, PKCα and δ demonstrated a distinct redistribution pattern (Fig. 6C). AdPKCαWT showed a localized perinuclear expression pattern, and AdPKCδWT redistributed into the nucleus. In contrast, overexpressing AdPKCαDN and AdPKCδDN blocked the redistribution of each PKC isozymes induced by cyclic stretch (Fig. 6C). In control virus AdLacZ infected myocytes, faint PKCα and δ antibody reactivity was observed in a diffuse pattern, which was consistent with the localization observed in WT overexpressing cells.

PKCα and δ differentially regulate the activation of Rho GTPases and phosphorylation of Rho-GDI

To determine the role of PKCα and δ in regulation of the activation of Rho GTPases and phosphorylation of Rho-GDI, cardiomyocytes were infected with adenoviral-mediated PKCα and δ mutants, and subjected to stretch for 1 min. Overexpression of AdPKCαDN blocked stretch-induced activation of RhoA and Rac1 (Fig. 7A), while AdPKCδDN overexpression inhibited stretch-induced activation of Rac1, and had no effect on stretch-induced activation of RhoA. However, the basal activation of RhoA was significantly increased in AdPKCδDN infected cardiomyocytes. Overexpression of AdPKCδWT inhibited the basal activation of RhoA, and mildly inhibited stretch-induced activation of RhoA. Stretch-induced phosphorylation of Rho-GDI was blocked by overexpression of AdPKCαDN, which is consistent with the regulation of Rho GTPases by PKCα (Fig. 7C). Overexpression of AdPKCδDN did not prevent the phosphorylation of Rho-GDI.

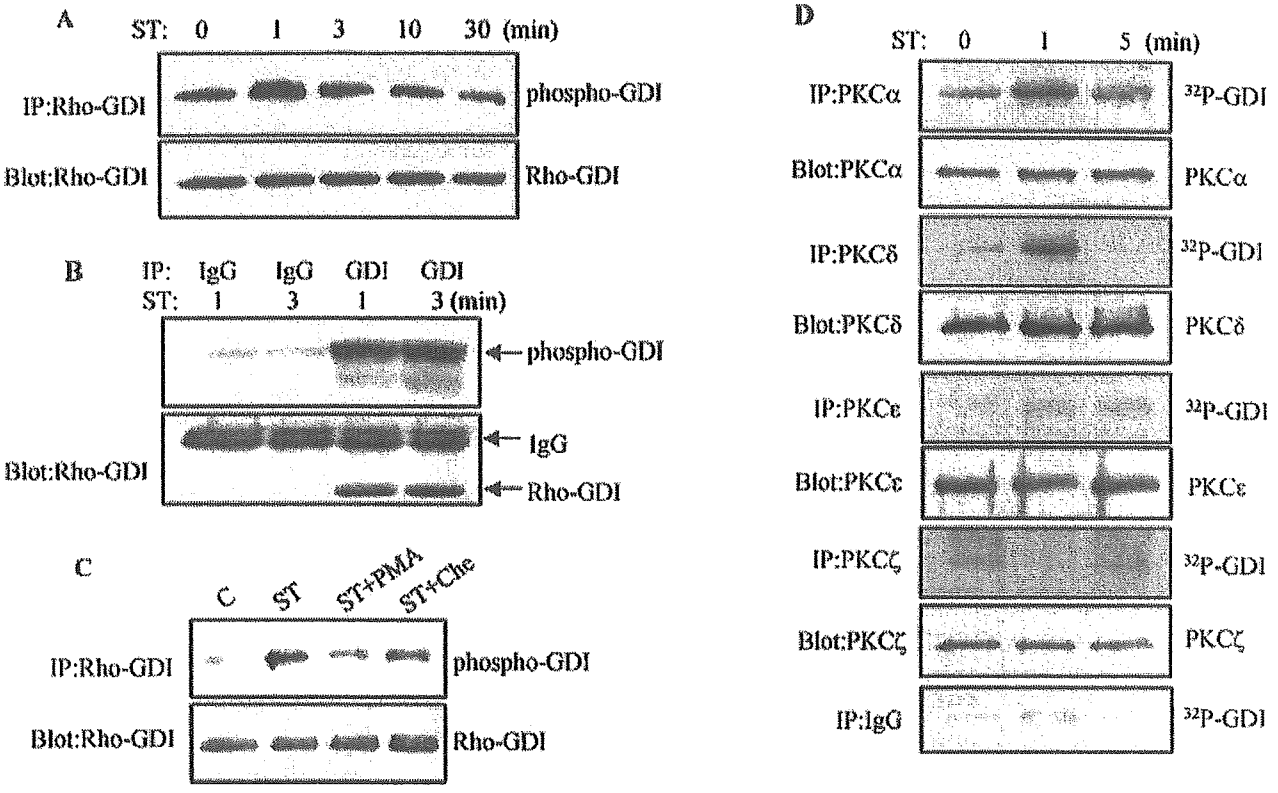

Fig. 5. PKC regulates stretch-induced Rho-GDI phosphorylation. A: Cardiomyocytes were subjected to stretch for the indicated times. Phosphorylation of Rho-GDI was determined as described under Materials and Methods. B: Cell lysates from stretched cells were immunoprecipitated with normal rabbit IgG (negative control) and Rho-GDI, phosphorylation of Rho-GDI was determined. C: Cardiomyocytes were pretreated with or without chelerythrine (Che, 10 μM) for 30 min, or PMA (1 μM, overnight). Phosphorylation of Rho-GDI was determined following 1 min of cyclic stretch. D: In vitro Rho-GDI phosphorylation. After stretching, cardiomyocytes were lysed to immunoprecipitate PKCα, δ, ζ, ε, or normal rabbit IgG (negative control, last part) as described under Materials and Methods. A kinase assay was performed using GST-GDI as a substrate. GDI phosphorylation was determined by autoradiography.

PKCα and δ are involved in cyclic stretch-induced cardiac hypertrophy

We have shown that stretch-activated Rho GTPases were required in the hypertrophic process (Fig. 2), and that the activation of Rho GTPases was differentially regulated by PKCα and δ (Fig. 7). Using adenoviral-mediated PKCα and δ mutants, we determined whether PKCα and δ were involved in stretch-induced cardiac hypertrophy. Overexpression of AdPKCαWT, but not AdPKCδWT, induced significant myofibrillar reorganization, increases in cell size and [^{3}H]-leucine incorporation (Fig. 8A–C). These data are consistent with previous studies (Braz et al., 2002), indicating that PKCα may be a unique inducer of cardiac hypertrophy. Cyclic stretch-induced increases in cell size and [^{3}H]-leucine incorporation were blocked by overexpression of AdPKCαDN and AdPKCδDN, and stretch-induced myofibrillar reorganization was also disrupted by AdPKCαDN and AdPKCδDN (Fig. 8A–C), suggesting that both PKCα and δ were required in cyclic stretch-induced cardiac hypertrophy.

PKCα and δ differentially regulate the activation of MEK/MAP kinase cascades

It has been reported that there is crosstalk between PKC and MAP kinase pathways in cardiomyocytes (Rohde et al., 2000; Heidkamp et al., 2001; Braz et al., 2002; Kerkela et al., 2002). To address the signaling mechanism, whereby PKC mediates stretch-induced hypertrophy, the activation of MAP kinases and their upstream regulator MAP kinase kinases (MKKs), in cardiomyocytes overexpressing PKCα and δ, was determined. Overexpression of AdPKCαWT induced phosphorylation of MEK1, MKK4, ERK1/2, and JNK, but not MKK3 and p38. In contrast, AdPKCδWT overexpression induced phosphorylation of MKK3 and p38, but had no effect on phosphorylation of MEK1, MKK4, ERK1/2, and JNK (Fig. 9A,B). Overexpression of AdPKCαWT and AdPKCδWT had no effect on the expression level of MEKs and MAPKs as demonstrated by Western blot using antibodies against normal MEKs and MAPKs. Another set of samples was Western blotted with actin antibody, to verify the equal amount of protein in each

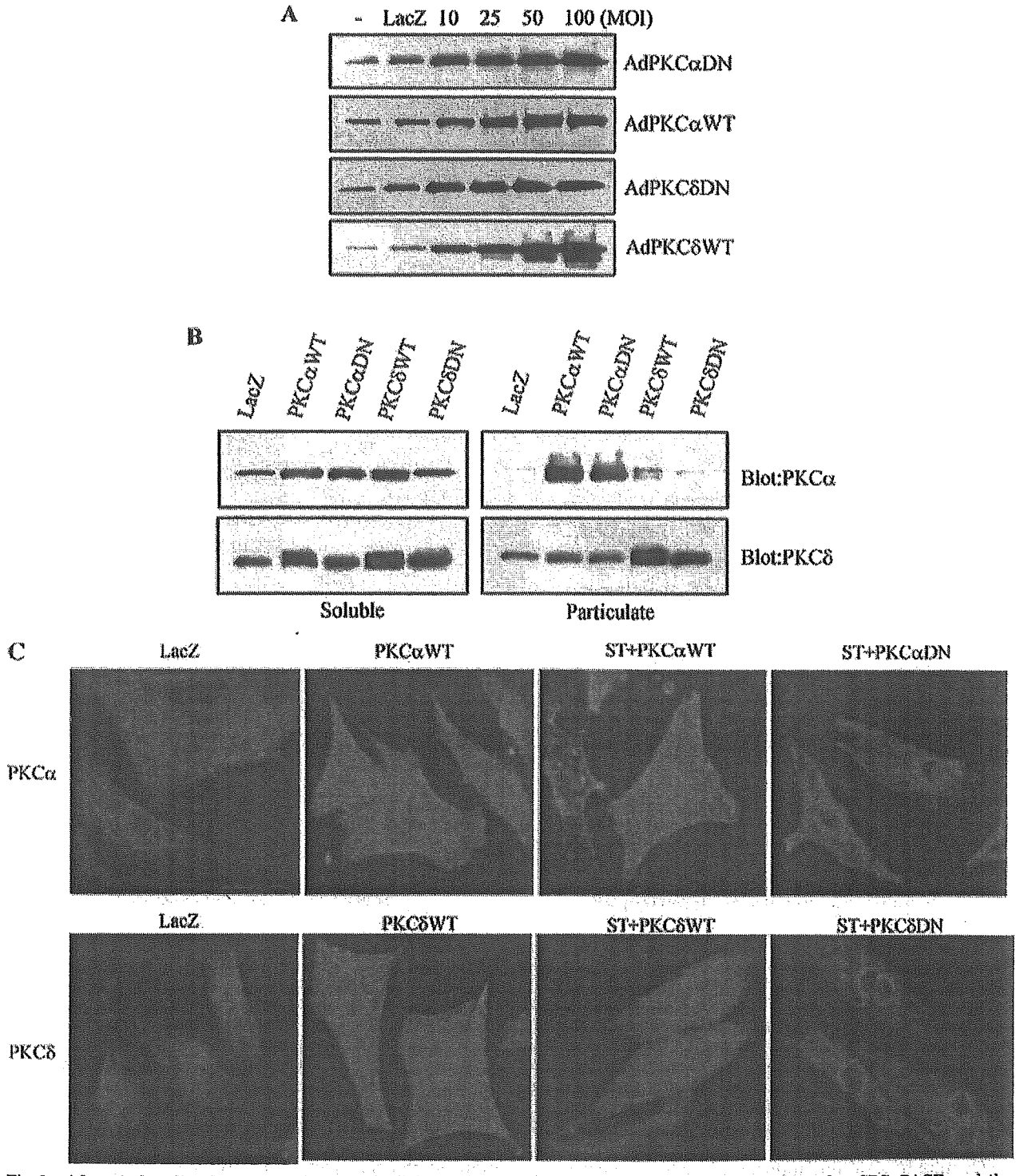

Fig. 6. Adenoviral-mediated overexpression and subcellular localization of PKCα and δ in cardiomyocytes. **A**: Cardiomyocytes were infected with AdLacZ, AdPKCαWT, AdPKCδWT, AdPKCαDN, and AdPKCδDN at different concentrations (multiplicity of infection, moi) for 6 h. Cells were cultured in serum-free medium for additional 24 h. Cell lysates (50 μg) were subjected to SDS–PAGE. The expression level was detected using anti-PKCα and δ antibodies. **B**: Cytosolic and particulate extracts (50 μg) were subjected to SDS–PAGE, and the subcellular protein distribution of PKCα and δ was determined by Western blotting. **C**: Cardiomyocytes infected with AdPKCα or AdPKCδ were subjected to cyclic stretch for 30 min. PKC isozyme distribution was characterized by immunocytochemical analysis using PKCα and δ antibody. The data are representative of three independent experiments.

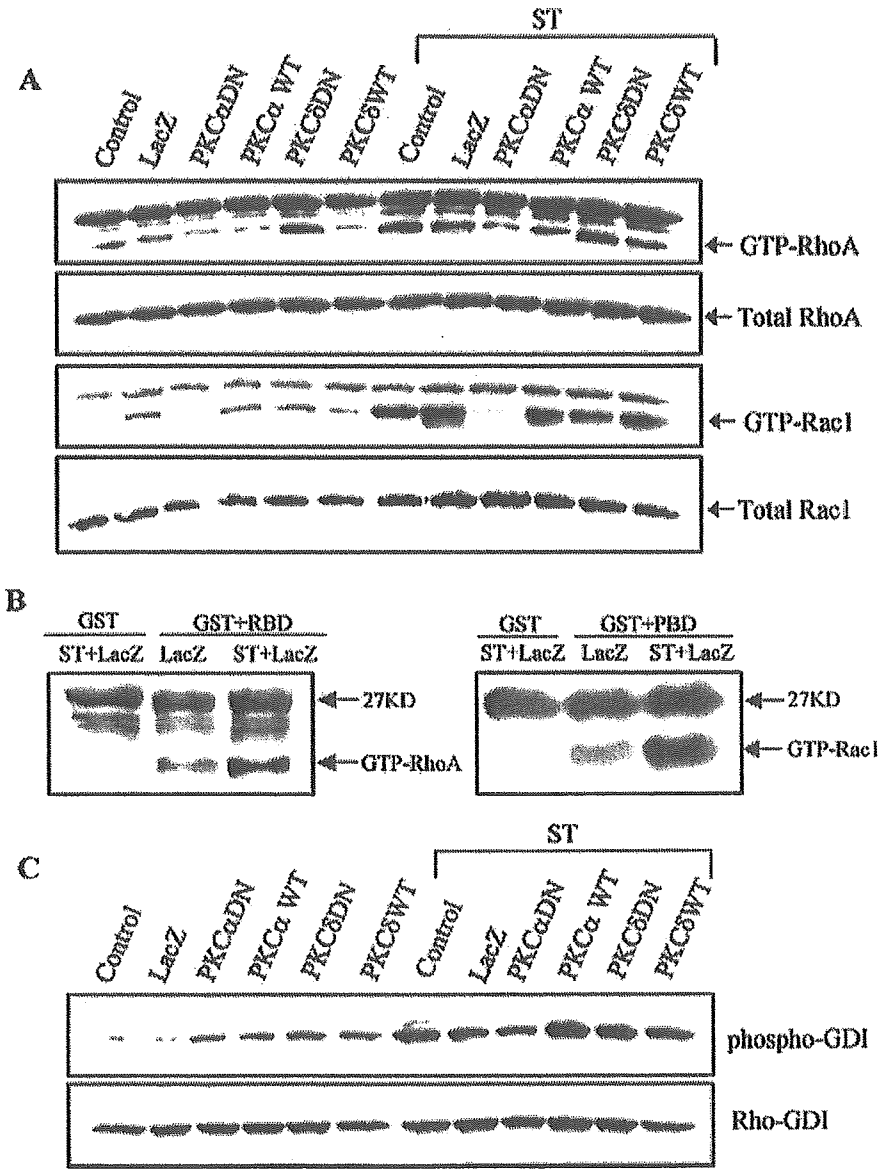

Fig. 7. PKCα and δ regulate cyclic stretch-induced activation of Rho GTPases and phosphorylation of Rho-GDI. A: Cardiomyocytes were infected with AdLacZ and AdPKC adenoviruses, after 24 h, cells were subjected to stretch for 1 min. Rho GTPase activity was determined as described in Figure 1. The data are representative of three independent experiments. Another set of samples was precipitated with GST beads to serve as a negative control (B). Following infection with PKCα and δ adenoviruses, cardiomyocytes were exposed to stretch for 1 min. The phosphorylation of Rho-GDI was observed by autoradiography, as described under Materials and Methods (C).

sample (last part). Since MAP kinases are differentially regulated by Rho GTPases in cardiac hypertrophy (Aikawa et al., 1999, 2001; Clerk et al., 2001), and stretch-induced activation of Rho GTPases was regulated by PKCα and δ (Fig. 7), we determined whether activation of MAP kinases induced by overexpression of PKCα and δ could be blocked by Toxin B and C3. Cardiomyocytes infected with AdPKCαWT or AdPKCδWT were treated with or without Toxin B or C3, and the phosphorylation of MAP kinases determined. As shown in Figure 9C, Toxin B and C3 inhibited PKCα-induced phosphorylation of ERK1/2, JNK, and PKCδ-induced phosphorylation of p38, indicating that PKCα and δ through Rho GTPases (at least partially) regulate the activation of MAP kinase cascades. We further determined the role of PKCα and δ in stretch-induced activation of MAP kinase cascades. Cardiomyocytes infected with or without AdPKCαDN and AdPKCδDN were subjected to cyclic stretch for 3 or 8 min, and the phosphorylation of MKKs and MAP kinases was examined. Overexpression of AdPKCαDN and AdPKCδDN inhibited stretch-induced phosphorylation of MEK1, MKK4, ERK1/2, and JNK; and phosphorylation of MKK3 and p38 was inhibited by overexpression of

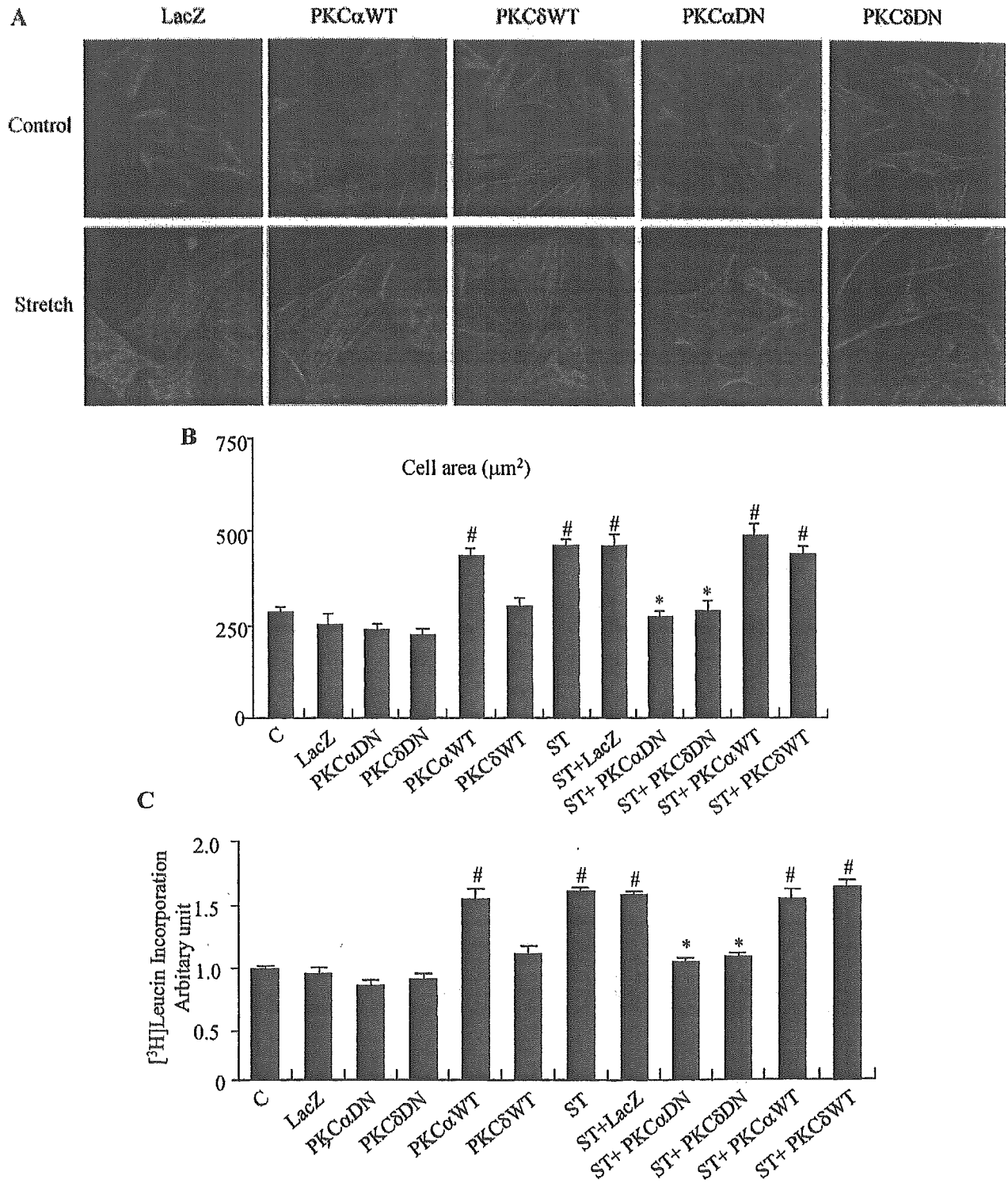

Fig. 8. PKCα and δ are involved in stretch-induced cardiac hypertrophy. **A**: Following infection with PKCα and δ adenoviruses, cardiomyocytes were subjected to stretch for 24 h. Immunofluorescence was performed with Texas Red phalloidin antibody. **B**: Phalloidin-stained cells were imaged and cell surface areas were calculated (n = 100 cells each). $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus stretch. **C**: Cardiomyocytes infected with or without PKCα and δ mutants were subjected to cyclic stretch for 24 h. [^{3}H]-leucine incorporation was measured, as described in Figure 2. Each bar represents the mean ± SEM of six separate experiments. $^{\#}P < 0.05$ versus control and $^{*}P < 0.05$ versus stretch.

AdPKCδDN (Fig. 9D,E). These results indicate that PKCα and δ differentially regulate stretch-induced hypertrophy, at least partially via Rho GTPases and MAP kinases activation (Fig. 10).

DISCUSSION

Mechanical stretch plays an important role in the development of cardiac hypertrophy (Komuro et al., 1990; Sadoshima et al., 1992). However, the precise mechanism by which mechanical force is transduced into activation of downstream signaling cascades remains unknown. Rho GTPases have been implicated in cardiac hypertrophy in response to various hypertrophic stimuli (Aoki et al., 1998; Pracyk et al., 1998; Aikawa et al., 1999), including mechanical stretch (Aikawa et al., 1999, 2001). Kawamura et al. demonstrate that stretch activates and subsequently dissociates RhoA and Rac1 from a caveolar compartment in rat cardiomyocytes, suggesting that the compartmentalization of RhoA and Rac1 in caveolae, has a critical role in mechanotransduction in cardiomyocytes (Kawamura et al., 2003). Using affinity binding assays, selectively for GTP-bound small G proteins, we showed that stretch induced rapid and significant activation of RhoA, Rac1, and Cdc42 (Fig. 1), indicating that Rho GTPases may be directly activated by cyclic stretch. However, the involvement of autocrine/paracrine secreted growth factors, such as angiotensin II (Ang II) and endothelin-1 (ET-1), in stretch-induced activation of Rho GTPases, cannot be excluded. Previous studies demonstrated that RhoA and Rac1 were involved in Ang II and ET-1 induced cardiac hypertrophy (Aoki et al., 1998; Aikawa et al., 2000; Clerk et al., 2001). Cyclic stretch-induced early activation (1 min) of Rho GTPases was neither affected by the antagonists of Ang II AT1 and AT2 receptor, nor by the antagonist of ET-1 receptor (data not shown). These results suggest that cyclic stretch directly induces activation of Rho GTPases, at least during the early phase. Using Toxin B, which inactivates Rho family proteins, or C3 exoenzyme, which inactivates RhoA, our study further confirmed that the activation of Rho GTPases contributed to several

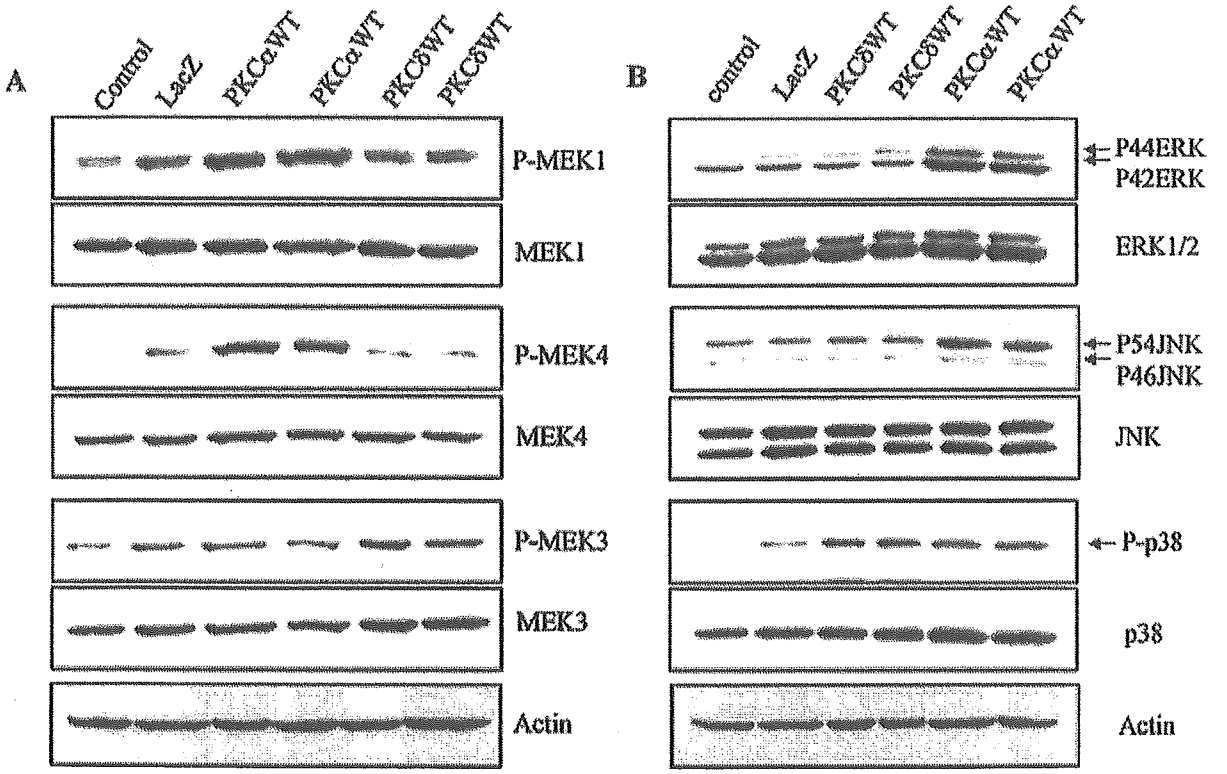

Fig. 9. PKCα and δ differentially regulate the activation of MAP kinase pathways. A: Cardiomyocytes were infected with 50 moi of AdLacZ, AdPKCαWT, or AdPKCδWT. After 24 h, cell extracts (50 μg) were subjected to SDS–PAGE and immunoblotted with specific phospho-MEK1, -MEK4, or -MEK3 antibodies. Membranes were reprobed for total MEK1, MEK4, and MEK3. Another set of samples was Western blotted with anti-actin antibody, to serve as a control for equal protein loading. B: Same cell extracts (50 μg) were subjected to SDS–PAGE and immunoblotted with specific phospho-ERK1/2, -JNK, or -p38 antibodies. Membranes were reprobed for total ERK1/2, JNK1, or p38 MAPK. Another set of samples was blotted with antibody against actin, to verify equal amount of protein in each sample. C: Cardiomyocytes infected with AdLacZ, AdPKCαWT, or AdPKCδWT were treated with or without Toxin B (10 ng/ml, 1 h) or C3 (5 μg/ml, overnight). The phosphorylation of ERK1/2, JNK, and p38 was observed, as described above. D: Cardiomyocytes were infected with AdLacZ (50 moi), AdPKCαDN (25 and 50 moi), or AdPKCδDN (25 and 50 moi). After 24 h, cells were subjected to stretch for 8 min. The phosphorylation of ERK1/2, JNK, and p38 was observed. E: Under similar condition with (D), after 24 h of infection, cells were subjected to stretch for 3 min. The phosphorylation of MEK1, MEK4, and MEK3 was observed, as described above. Data are representative of three independent experiments.

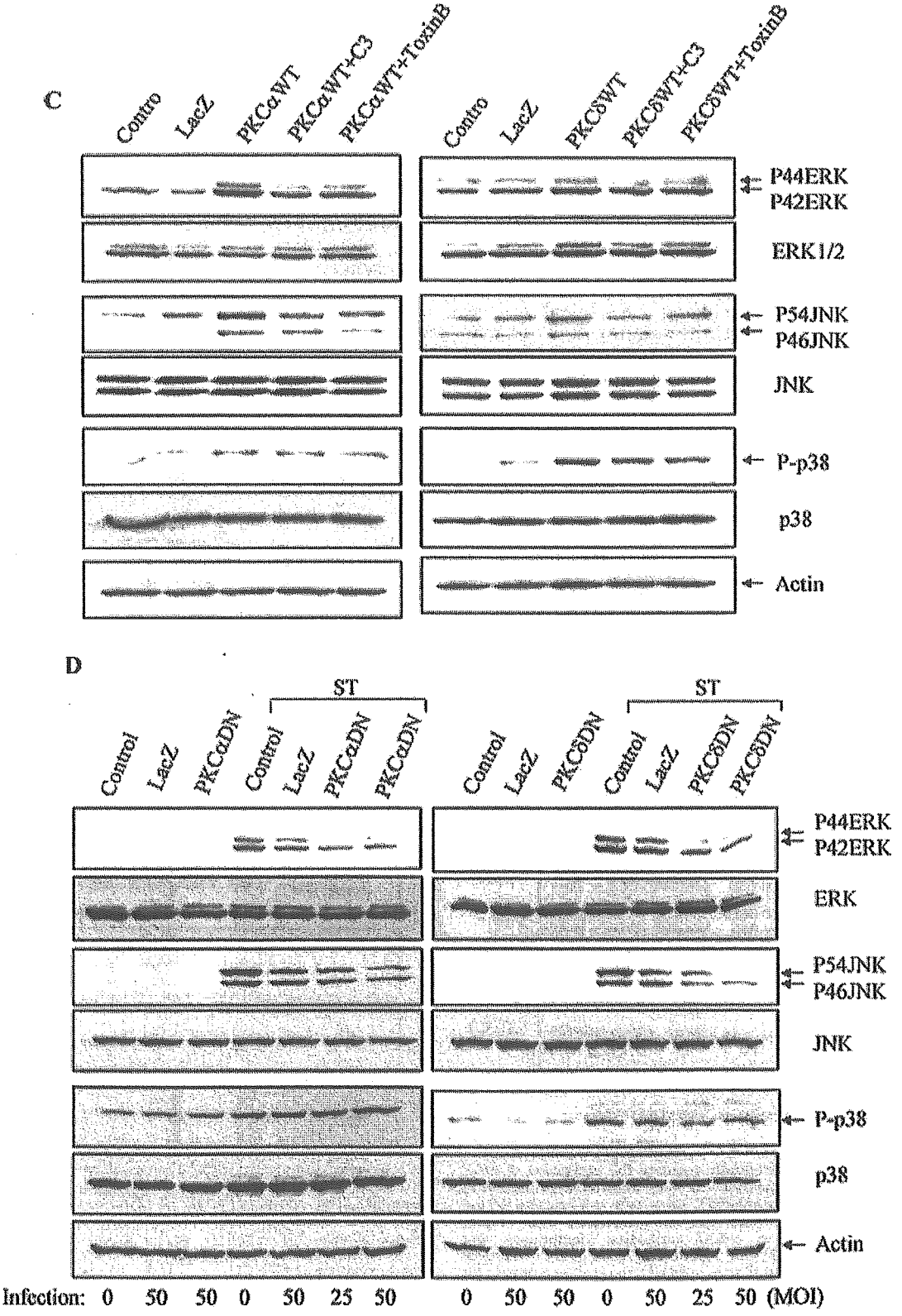

Fig. 9. (*Continued*)

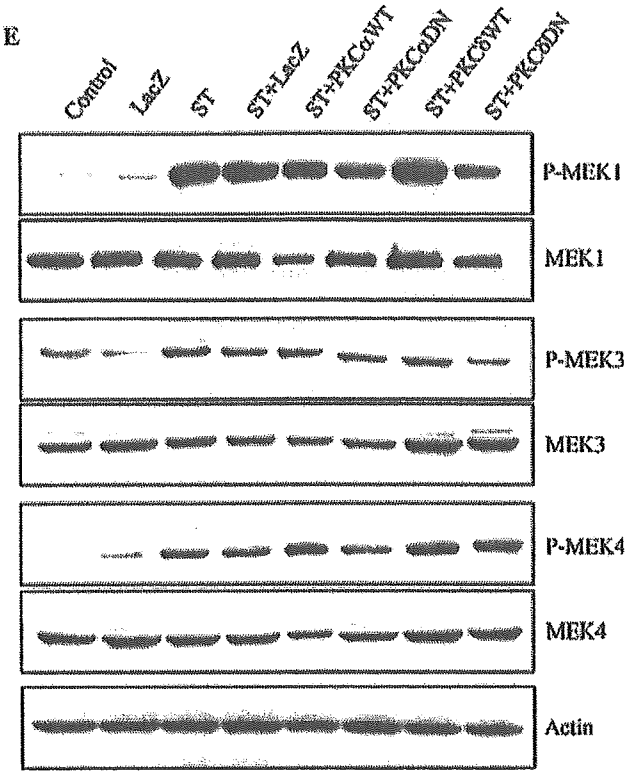

Fig. 9. (*Continued*)

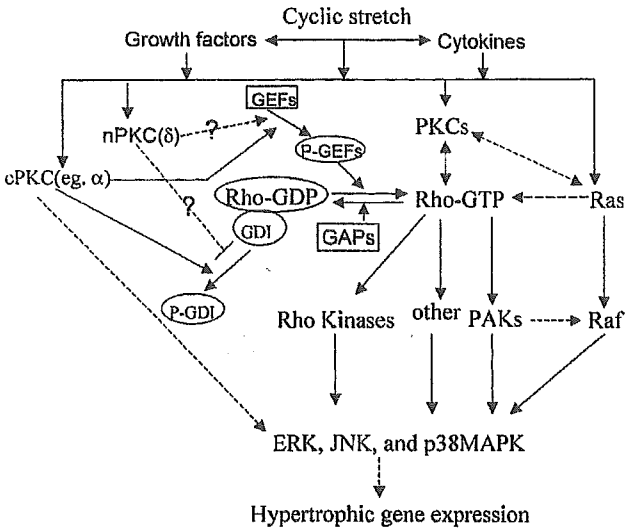

Fig. 10. Putative model depicting the role of PKC in regulating the activation of Rho GTPases and MAP kinases in cyclic stretch-induced hypertrophic process. Cyclic stretch directly or indirectly induced activation of RhoA, Rac1, and Cdc42, which is regulated by PKC (especially PKCα). PKCα, via phosphorylation of Rho-GDI, may promote dissociation of GDI from GDP form Rho GTPases, leads translocation and activation of Rho GTPases in cyclic stretched cardiomyocytes. PKCα may also via regulating the phosphorylation of Rho GEFs, promotes the activation of Rho GTPases. The mechanism of PKCδ in regulating the activation of Rho GTPases is not clear. The activated Rho GTPases may serve as a convergence point for various downstream signaling pathways that promote cardiac hypertrophy.

independent features of cardiac hypertrophy, such as increases in protein synthesis, cell size, and induction of hypertrophic gene expression.

Rho GTPases exert effects by a GTP-dependent activation of effector proteins, after dissociation from Rho-GDI. Rho-GDI phosphorylation/dephosphorylation has been implicated in regulation of the dissociation of the Rho GTPases/Rho-GDI complex, and in mediating activation of Rho GTPases (Bourmeyster and Vignais, 1996; Gorvel et al., 1998; Mehta et al., 2001). The phosphorylation of Rho-GDI may also be involved in regulating stretch-induced activation of Rho GTPases. However, the associated activation mechanism is not clear. Previous studies have shown that PKC interacts with Rho GTPases, leading to crosstalk between respective pathways (Tominaga et al., 1993). Studies in C2C12 myoblasts and endothelial cells have implicated PKCα in regulating sphingosin 1-phosphate- and thrombin-induced activation of RhoA (Meacci et al., 2000; Mehta et al., 2001; Holinstat et al., 2003), via promoting the phosphorylation of Rho-GDI and/or p115RhoGEF. Rho-GDI was rapidly phosphorylated by cyclic stretch, and the phosphorylation was also regulated by phorbol sensitive PKC isozymes, analogous to the regulation of Rho GTPases by PKC. These data suggest the possibility that the phosphorylation of Rho-GDI promotes the dissociation of Rho-GDI from Rho GTPases, leading to membrane translocation and activation of Rho GTPases in cyclic stretched cardiomyocytes. Although there is no direct evidence, the activation pattern of Rho GTPases and phosphorylation of Rho-GDI induced by stretch, and the regulation pattern by PKC, provided indirect evidence that cyclic stretch-induced activation of PKC, via phosphorylating Rho-GDI, was involved in regulating stretch-induced activation of Rho-GTPases.

PKC isozymes α, $\beta_{1/2}$, δ, ε, and ζ have been identified in neonatal rat cardiomyocytes (Disatnik et al., 1994). We found that phorbol-sensitive PKC isozymes were involved in stretch-induced activation of Rho GTPases and phosphorylation of Rho-GDI, and stretch-induced in vitro phosphorylation of Rho-GDI was mainly regulated by PKCα and δ, indicating that PKCα and δ may have a major role in regulating stretch-induced activation of Rho GTPases. To further confirm the role of PKCα and δ in regulating the activation of Rho GTPases, we used adenoviral-mediated gene transfer of a PKCα and δ mutant. Our result demonstrated that stretch-induced Rho-GDI phosphorylation and Rho GTPases activation were mainly regulated by PKCα, which via phosphorylation of Rho-GDI, may promote the dissociation of GDI from Rho GTPase, and result in activation of Rho GTPases. PKCδ was involved in the regulation of stretch-induced activation of Rac1; however, we did not observe the inhibitory effect of AdPKCδDN on stretch-induced activation of RhoA and phosphorylation of Rho-GDI. Since overexpression of AdPKCδDN alone induced a significant increase of basal activation of RhoA, it may interfere with the effect of PKCδ. We have also used Rottlerin, a specific inhibitor for PKCδ isozyme (Gschwendt et al., 1994), which inhibited stretch-induced activation of RhoA, Rac1, and the phosphorylation of Rho-GDI (data not shown). The role of PKCδ in regulating the activation of RhoA needs further study.

We have observed that overexpression of AdPKCδWT inhibited the basal activation of RhoA, and that stretch-induced activation of RhoA was partially inhibited, indicating that PKCδ might have a negative role in regulating the activation of RhoA.

Previous studies suggest that PKC isozyme specific localization may determine phosphorylation of different protein substrates at respective translocation sites and the resulting PKC-mediated cellular responses (Disatnik et al., 1994; Braz et al., 2002). PKCα prominently redistributed to the perinuclear region in stretched cardiomyocytes, where a number of signaling molecules have been localized (such as Cdc42 and Pyk2) (Klingbeil et al., 2001; Murphy et al., 2001), whereas PKCδ redistributed into the nucleus. The different localization of PKCα and δ may be associated with different cellular functions. We demonstrated that overexpression of AdPKCαWT alone induced increases in cell size, myofibrillar reorganization, and protein synthesis, suggesting that PKCα may be a unique hypertrophic inducer, consistent with a previous study (Braz et al., 2002). PKCδ has been implicated in regulating agonist-induced cardiac hypertrophy and ischemia-related apoptosis (Strait and Samarel, 2000; Chen et al., 2001; Braun et al., 2002, 2003; Simonis et al., 2002; Bayer et al., 2003). Overexpression of AdPKCδWT in cardiomyocytes did not stimulate hypertrophic growth after 48 h. However, overexpression of AdPKCαDN and AdPKCδDN in cyclic stretch-stimulated cardiomyocytes, suggested that both PKCα and δ were required in stretch-induced cardiac hypertrophy. After 48 h of infection with AdPKCαDN or AdPKCδDN, a number of beating myocytes were observed, and no significant cell number loss was found (data not shown), indicating that both PKCα and δ had no effect on the cell viability. These latter data suggest that the inhibitory effect of PKCα and δ on stretch-induced hypertrophic growth is not due to cell viability. PKC and Rho GTPases both have effects on the actin cytoskeleton, and our results further confirmed that cyclic stretch-induced increases in actin fibers and myofibrillar reorganization were regulated by Rho GTPases and PKC; though PKCα and δ may through RhoA and Rac1 differentially affect actin reorganization in the stretch-induced hypertrophic process.

The signaling mechanism, whereby PKCα and δ regulate cardiac hypertrophy, has been associated with MAP kinases activation (Rohde et al., 2000; Heidkamp et al., 2001; Braz et al., 2002; Kerkela et al., 2002). The phenylephrine-induced increase in ERK activity is inhibited by antisense PKCα treatment, and PMA-induced phosphorylation of ERK is inhibited by AdPKCαDN, implicating ERK as a downstream mediator of PKCα signaling (Braz et al., 2002; Kerkela et al., 2002). Infection of cells with adenoviruses encoding DN PKCδ or ε, inhibited the activation of ERK by phenylephrine (Wang et al., 2003). Heidkamp et al. have shown that overexpression of a constitutively active mutant of PKCδ induced significant phosphorylation of JNK and p38 in myocytes (Heidkamp et al., 2001). In this study, we showed that the overexpression of AdPKCαWT induced significant phosphorylation of MEK1, MKK4, and the downstream targets ERK1/2 and JNK; and that overexpression of AdPKCδWT induced phosphorylation of MKK3 and the downstream target p38. We did not observe any activation of JNK by PKCδ, which may be due to the difference between the WT and constitutively active mutant. Stretch-induced phosphorylation of MEK1, MEK4, ERK1/2, and JNK was inhibited by overexpression of AdPKCαDN and AdPKCδDN, and that of MEK3 and p38 was only inhibited by AdPKCδDN. These results suggested that MEK1/ERK1/2 and MEK4/JNK were downstream regulators of PKCα, and that MEK3/p38 was the major downstream regulator of PKCδ in cyclic stretch-mediated hypertrophic signaling. We have shown that Rho GTPases-mediated activation of MAP kinases are involved in stretch-induced hypertrophy. PKCα and δ had different roles in regulating the activation of RhoA and Rac1. Therefore, we determined whether Rho GTPases were involved in PKCα- and δ-mediated activation of MAP kinases. PKCα-induced phosphorylation of ERK1/2 and JNK, and PKCδ-induced phosphorylation of p38 were inhibited by pretreating the cells with Toxin B or C3, suggesting that PKCα regulates the activation of MAP kinases, through RhoA and Rac1, and that PKCδ may through Rac1, or other signaling pathways, mediate the activation of MAP kinases.

This study represents the first evidence that cyclic stretch mediated signaling of Rho GTPases specifically involves PKCα and δ, which via phosphorylation of Rho-GDI, regulate stretch-induced activation of Rho GTPases and MAP kinase pathways (Fig. 10). Our results suggest that the interconnectivity of PKC, Rho GTPases and MAPK signaling pathways has an important role in regulating stretch-induced cardiac hypertrophy.

LITERATURE CITED

Aikawa R, Komuro I, Yamazaki T, Zou Y, Kudoh S, Zhu W, Kadowaki T, Yazaki Y. 1999. Rho family small G proteins play critical roles in mechanical stress-induced hypertrophic responses in cardiac myocytes. Circ Res 84(4):458–466.

Aikawa R, Komuro I, Nagai R, Yazaki Y. 2000. Rho plays an important role in angiotensin II-induced hypertrophic responses in cardiac myocytes. Mol Cell Biochem 212(1–2):177–182.

Aikawa R, Nagai T, Tanaka M, Zou Y, Ishihara T, Takano H, Hasegawa H, Akazawa H, Mizukami M, Nagai R, Komuro I. 2001. Reactive oxygen species in mechanical stress-induced cardiac hypertrophy. Biochem Biophys Res Commun 289(4):901–907.

Aoki H, Izumo S, Sadoshima J. 1998. Angiotensin II activates RhoA in cardiac myocytes: A critical role of RhoA in angiotensin II-induced premyofibril formation. Circ Res 82(6):666–676.

Bayer AL, Heidkamp MC, Patel N, Porter M, Engman S, Samarel AM. 2003. Alterations in protein kinase C isoenzyme expression and autophosphorylation during the progression of pressure overload-induced left ventricular hypertrophy. Mol Cell Biochem 242(1–2): 145–152.

Bokoch GM, Bohl BP, Chuang TH. 1994. Guanine nucleotide exchange regulates membrane translocation of Rac/Rho GTP-binding proteins. J Biol Chem 269(50):31674–31679.

Borbiev T, Nurmukhambetova S, Liu F, Verin AD, Garcia JG. 2000. Introduction of C3 exoenzyme into cultured endothelium by lipofectamine. Anal Biochem 285(2):260–264.

Bourmeyster N, Vignais PV. 1996. Phosphorylation of Rho GDI stabilizes the Rho A-Rho GDI complex in neutrophil cytosol. Biochem Biophys Res Commun 218(1):54–60.

Braun MU, LaRosee P, Schon S, Borst MM, Strasser RH. 2002. Differential regulation of cardiac protein kinase C isozyme expression after aortic banding in rat. Cardiovasc Res 56(1):52–63.

Braun M, Simonis G, Birkner K, Pauke B, Strasser RH. 2003. Regulation of protein kinase C isozyme and calcineurin expression in isoproterenol induced cardiac hypertrophy. J Cardiovasc Pharmacol 41(6):946–954.

Braz JC, Bueno OF, De Windt LJ, Molkentin JD. 2002. PKC alpha regulates the hypertrophic growth of cardiomyocytes through extracellular signal-regulated kinase1/2 (ERK1/2). J Cell Biol 156(5):905–919.

Cerione RA, Zheng Y. 1996. The Dbl family of oncogenes. Curr Opin Cell Biol 8(2):216–222.

Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D, Cavallaro G, Banci L, Guo Y, Bolli R, Dorn GW 2nd, Mochly-Rosen D. 2001. Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. Proc Natl Acad Sci USA 98(20): 11114–11119.

Clerk A, Pham FH, Fuller SJ, Sahai E, Aktories K, Marais R, Marshall C, Sugden PH. 2001. Regulation of mitogen-activated protein kinases in cardiac myocytes through the small G protein Rac1. Mol Cell Biol 21(4):1173–1184.

Coghlan MP, Chou MM, Carpenter CL. 2000. Atypical protein kinases Clambda and -zeta associate with the GTP-binding protein Cdc42 and mediate stress fiber loss. Mol Cell Biol 20(8):2880–2889.

Cooper Gt, Kent RL, Uboh CE, Thompson EW, Marino TA. 1985. Hemodynamic versus adrenergic control of cat right ventricular hypertrophy. J Clin Invest 75(5):1403–1414.

Cox D, Chang P, Zhang Q, Reddy PG, Bokoch GM, Greenberg S. 1997. Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. J Exp Med 186(9):1487–1494.

Disatnik MH, Buraggi G, Mochly-Rosen D. 1994. Localization of protein kinase C isozymes in cardiac myocytes. Exp Cell Res 210(2): 287–297.

Faure J, Vignais PV, Dagher MC. 1999. Phosphoinositide-dependent activation of Rho A involves partial opening of the RhoA/Rho-GDI complex. Eur J Biochem 262(3):879–889.

Fukumoto Y, Kaibuchi K, Hori Y, Fujioka H, Araki S, Ueda T, Kikuchi A, Takai Y. 1990. Molecular cloning and characterization of a novel type of regulatory protein (GDI) for the rho proteins, ras p21-like small GTP-binding proteins. Oncogene 5(9):1321–1328.

Gorvel JP, Chang TC, Boretto J, Azuma T, Chavrier P. 1998. Differential properties of D4/LyGDI versus RhoGDI: Phosphorylation and rho GTPase selectivity. FEBS Lett 422(2):269–273.

Gschwendt M, Muller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, Marks F. 1994. Rottlerin, a novel protein kinase inhibitor. Biochem Biophys Res Commun 199(1):93–98.

Hall A. 1998. Rho GTPases and the actin cytoskeleton. Science 279(5350):509–514.

Heidkamp MC, Bayer AL, Martin JL, Samarel AM. 2001. Differential activation of mitogen-activated protein kinase cascades and apoptosis by protein kinase C epsilon and delta in neonatal rat ventricular myocytes. Circ Res 89(10):882–890.

Hofmann F, Busch C, Prepens U, Just I, Aktories K. 1997. Localization of the glucosyltransferase activity of Clostridium difficile toxin B to the N-terminal part of the holotoxin. J Biol Chem 272(17):11074–11078.

Holinstat M, Mehta D, Kozasa T, Minshall RD, Malik AB. 2003. PKCa-induced p115RhoGEF phosphorylation signals endothelial cytoskeletal rearrangement. J Biol Chem 278(31):28793–28798.

Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, Morii N, Narumiya S. 1996. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J 15(8):1885–1893.

Jain N, Zhang T, Kee WH, Li W, Cao X. 1999. Protein kinase C delta associates with and phosphorylates Stat3 in an interleukin-6-dependent manner. J Biol Chem 274(34):24392–24400.

Kashiwagi Y, Haneda T, Osaki J, Miyata S, Kikuchi K. 1998. Mechanical stretch activates a pathway linked to mevalonate metabolism in cultured neonatal rat heart cells. Hypertens Res 21(2):109–119.

Kawamura S, Miyamoto S, Brown JH. 2003. Initiation and transduction of stretch-induced RhoA and Rac1 activation through caveolae: Cytoskeletal regulation of ERK translocation. J Biol Chem 278(33): 31111–31117.

Keenan C, Kelleher D. 1998. Protein kinase C and the cytoskeleton. Cell Signal 10(4):225–232.

Kerkela R, Ilves M, Pikkarainen S, Tokola H, Ronkainen J, Vuolteenaho O, Leppaluoto J, Ruskoaho H. 2002. Identification of PKCalpha isoform-specific effects in cardiac myocytes using antisense phosphorothioate oligonucleotides. Mol Pharmacol 62(6): 1482–1491.

Klingbeil CK, Hauck CR, Hsia DA, Jones KC, Reider SR, Schlaepfer DD. 2001. Targeting Pyk2 to beta 1-integrin-containing focal contacts rescues fibronectin-stimulated signaling and haptotactic motility defects of focal adhesion kinase-null cells. J Cell Biol 152(1):97–110.

Kodama H, Fukuda K, Pan J, Makino S, Baba A, Hori S, Ogawa S. 1997. Leukemia inhibitory factor, a potent cardiac hypertrophic cytokine, activates the JAK/STAT pathway in rat cardiomyocytes. Circ Res 81(5):656–663.

Komuro I, Kaida T, Shibazaki Y, Kurabayashi M, Katoh Y, Hoh E, Takaku F, Yazaki Y. 1990. Stretching cardiac myocytes stimulates protooncogene expression. J Biol Chem 265(7):3595–3598.

Lamarche N, Hall A. 1994. GAPs for rho-related GTPases. Trends Genet 10(12):436–440.

Mammoto A, Takahashi K, Sasaki T, Takai Y. 2000. Stimulation of Rho GDI release by ERM proteins. Methods Enzymol 325:91–101.

Manabe T, Fukuda K, Pan J, Nagasaki K, Yamaguchi K, Ogawa S. 1999. Hypertrophic stimuli augment expression of cMG1/ERF-1, a putative zinc-finger motif transcription factor, in rat cardiomyocytes. FEBS Lett 463(1–2):39–42.

Meacci E, Donati C, Cencetti F, Romiti E, Bruni P. 2000. Permissive role of protein kinase C alpha but not protein kinase C delta in sphingosine 1-phosphate-induced Rho A activation in C2C12 myoblasts. FEBS Lett 482(1–2):97–101.

Mehta D, Rahman A, Malik AB. 2001. Protein kinase C-alpha signals rho-guanine nucleotide dissociation inhibitor phosphorylation and rho activation and regulates the endothelial cell barrier function. J Biol Chem 276(25):22614–22620.

Mende U, Kagen A, Meister M, Neer EJ. 1999. Signal transduction in atria and ventricles of mice with transient cardiac expression of activated G protein alpha(q). Circ Res 85(11):1085–1091.

Morissette MR, Sah VP, Glembotski CC, Brown JH. 2000. The Rho effector, PKN, regulates ANF gene transcription in cardiomyocytes through a serum response element. Am J Physiol Heart Circ Physiol 278(6):H1769–1774.

Murphy GA, Jillian SA, Michaelson D, Philips MR, D'Eustachio P, Rush MG. 2001. Signaling mediated by the closely related mammalian Rho family GTPases TC10 and Cdc42 suggests distinct functional pathways. Cell Growth Differ 12(3):157–167.

Nagai T, Tanaka-Ishikawa M, Aikawa R, Ishihara H, Zhu W, Yazaki Y, Nagai R, Komuro I. 2003. Cdc42 plays a critical role in assembly of sarcomere units in series of cardiac myocytes. Biochem Biophys Res Commun 305(4):806–810.

Nishizuka Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258(5082):607–614.

Nobes CD, Hall A. 1999. Rho GTPases control polarity, protrusion, and adhesion during cell movement. J Cell Biol 144(6):1235–1244.

Nosaka Y, Arai A, Kanda E, Akasaki T, Sumimoto H, Miyasaka N, Miura O. 2001. Rac is activated by tumor necrosis factor alpha and is involved in activation of Erk. Biochem Biophys Res Commun 285(3):675–679.

Nozu F, Tsunoda Y, Ibitayo AI, Bitar KN, Owyang C. 1999. Involvement of RhoA and its interaction with protein kinase C and Src in CCK-stimulated pancreatic acini. Am J Physiol 276(4 Pt 1): G915–923.

Olofsson B. 1999. Rho guanine dissociation inhibitors: Pivotal molecules in cellular signalling. Cell Signal 11(8):545–554.

Pan J, Fukuda K, Saito M, Matsuzaki J, Kodama H, Sano M, Takahashi T, Kato T, Ogawa S. 1999. Mechanical stretch activates the JAK/STAT pathway in rat cardiomyocytes. Circ Res 84(10): 1127–1136.

Pracyk JB, Tanaka K, Hegland DD, Kim KS, Sethi R, Rovira II, Blazina DR, Lee L, Bruder JT, Kovesdi I, Goldshmidt-Clermont PJ, Irani K, Finkel T. 1998. A requirement for the rac1 GTPase in the signal transduction pathway leading to cardiac myocyte hypertrophy. J Clin Invest 102(5):929–937.

Ridley AJ, Hall A. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70(3):389–399.

Rohde S, Sabri A, Kamasamudran R, Steinberg SF. 2000. The alpha(1)-adrenoceptor subtype- and protein kinase C isoform-dependence of Norepinephrine's actions in cardiomyocytes. J Mol Cell Cardiol 32(7):1193–1209.

Sadoshima J, Izumo S. 1993. Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: Potential involvement of an autocrine/paracrine mechanism. EMBO J 12(4):1681–1692.

Sadoshima J, Jahn L, Takahashi T, Kulik TJ, Izumo S. 1992. Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. An in vitro model of load-induced cardiac hypertrophy. J Biol Chem 267(15):10551–10560.

Schmitz U, Thommes K, Beier I, Wagner W, Sachinidis A, Dusing R, Vetter H. 2001. Angiotensin II-induced stimulation of p21-activated kinase and c-Jun NH2-terminal kinase is mediated by Rac1 and Nck. J Biol Chem 276(25):22003–22010.

Seko Y, Takahashi N, Tobe K, Kadowaki T, Yazaki Y. 1999. Pulsatile stretch activates mitogen-activated protein kinase (MAPK) family members and focal adhesion kinase (p125(FAK)) in cultured rat cardiac myocytes. Biochem Biophys Res Commun 259(1):8–14.

Simonis G, Honold J, Schwarz K, Braun MU, Strasser RH. 2002. Regulation of the isozymes of protein kinase C in the surviving rat myocardium after myocardial infarction: Distinct modulation for PKC-alpha and for PKC-delta. Basic Res Cardiol 97(3):223–231.

Slater SJ, Seiz JL, Stagliano BA, Stubbs CD. 2001. Interaction of protein kinase C isozymes with Rho GTPases. Biochemistry 40(14): 4437–4445.

Strait JB, Samarel AM. 2000. Isoenzyme-specific protein kinase C and c-Jun N-terminal kinase activation by electrically stimulated contraction of neonatal rat ventricular myocytes. J Mol Cell Cardiol 32(8):1553–1566.

Strait JB 3rd, Martin JL, Bayer A, Mestril R, Eble DM, Samarel AM. 2001. Role of protein kinase C-epsilon in hypertrophy of cultured neonatal rat ventricular myocytes. Am J Physiol Heart Circ Physiol 280(2):H756–H766.

Szallasi Z, Smith CB, Pettit GR, Blumberg PM. 1994. Differential regulation of protein kinase C isozymes by bryostatin 1 and phorbol 12-myristate 13-acetate in NIH 3T3 fibroblasts. J Biol Chem 269(3): 2118–2124.

Takeishi Y, Ping P, Bolli R, Kirkpatrick DL, Hoit BD, Walsh RA. 2000. Transgenic overexpression of constitutively active protein kinase C epsilon causes concentric cardiac hypertrophy. Circ Res 86(12): 1218–1223.

Toker A. 1998. Signaling through protein kinase C. Front Biosci 3: D1134–1147.

Tominaga T, Sugie K, Hirata M, Morii N, Fukata J, Uchida A, Imura H, Narumiya S. 1993. Inhibition of PMA-induced, LFA-1-dependent lymphocyte aggregation by ADP ribosylation of the small molecular weight GTP binding protein, rho. J Cell Biol 120(6):1529–1537.

Wang L, Rolfe M, Proud CG. 2003. Ca(2+)-independent protein kinase C activity is required for alpha1-adrenergic-receptor-mediated regulation of ribosomal protein S6 kinases in adult cardiomyocytes. Biochem J 373(Pt 2):603–611.

Watanabe G, Saito Y, Madaule P, Ishizaki T, Fujisawa K, Morii N, Mukai H, Ono Y, Kakizuka A, Narumiya S. 1996. Protein kinase N (PKN) and PKN-related protein rhophilin as targets of small GTPase Rho. Science 271(5249):645–648.

Yamazaki T, Tobe K, Hoh E, Maemura K, Kaida T, Komuro I, Tamemoto H, Kadowaki T, Nagai R, Yazaki Y. 1993. Mechanical loading activates mitogen-activated protein kinase and S6 peptide kinase in cultured rat cardiac myocytes. J Biol Chem 268(16): 12069–12076.

Yamazaki T, Komuro I, Kudoh S, Zou Y, Shiojima I, Mizuno T, Takano H, Hiroi Y, Ueki K, Tobe K, Kadowaki T, Nagai R, Yazaki Y. 1995. Mechanical stress activates protein kinase cascade of phosphorylation in neonatal rat cardiac myocytes. J Clin Invest 96(1):438–446.

Yanazume T, Hasegawa K, Wada H, Morimoto T, Abe M, Kawamura T, Sasayama S. 2002. Rho/ROCK pathway contributes to the activation of extracellular signal-regulated kinase/GATA-4 during myocardial cell hypertrophy. J Biol Chem 277(10):8618–8625.

Zhang S, Han J, Sells MA, Chernoff J, Knaus UG, Ulevitch RJ, Bokoch GM. 1995. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J Biol Chem 270(41):23934–23936.

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ORIGINAL ARTICLE

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Genetic variants in the calpain-10 gene and the development of type 2 diabetes in the Japanese population

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Abstract Variation in the gene encoding the cysteine protease calpain-10 has been linked and associated with risk of type 2 diabetes. We have examined the effect of three polymorphisms in the calpain-10 gene (SNP-43, Indel-19, and SNP-63) on the development of type 2 diabetes in the Japanese population in a pooled analysis of 927 patients and 929 controls. We observed that SNP-43, Indel-19, and SNP-63 either individually or as a haplotype were not associated with altered risk of type 2 diabetes with the exception of the rare 111/221 haplogenotype (odds ratio (OR) $=3.53$, $P=0.02$). However, stratification based on the median age-at-diagnosis in the pooled study population (<50 and ≥ 50 years) revealed that allele 2 of Indel-19 and the 121 haplotype were associated with reduced risk in patients with later age-at-diagnosis (age-at-diagnosis ≥ 50 years OR $=0.82$ and 0.80, respectively; $P=0.04$ and 0.02). Thus, variation in the calpain-10 gene may affect risk of type 2 diabetes in Japanese, especially in older individuals.

Keywords Association study · Age-at-diagnosis · Calpain-10 · Genetics · Polymorphism · Type 2 diabetes

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Introduction

The results of association and linkage studies indicate that multiple genes are involved in determining susceptibility to type 2 diabetes in Japanese with each gene making a modest contribution to overall risk (Seino et al. 2001; Mori et al. 2001, 2002; Iwasaki et al. 2003). The gene encoding the cysteine protease calpain-10 (CAPN10) was first found to be associated with risk of type 2 diabetes in studies carried out in Mexican Americans (Horikawa et al. 2000). Two recent meta-analyses and a large association study have confirmed that single nucleotide polymorphisms (SNP)-43 and SNP-44 are associated with a 1.19- and 1.17-fold increased risk, respectively, of type 2 diabetes (Weedon et al. 2003; Song et al. 2004). SNP-43 may be a functional polymorphism affecting transcriptional regulation of the calpain-10 gene (Horikawa et al. 2000; Baier et al. 2000). However, Indel-19 and SNP-63 are just tagging SNPs, and their effect on transcriptional regulation or other functions of calpain-10 are unknown. The effect of the core CAPN10 polymorphisms SNP-43, Indel-19, and SNP-63 on risk of type 2 diabetes in Japanese has been examined in three small studies (Daimon et al. 2002; Horikawa et al. 2003; Shima et al. 2003). The results suggest that variation in CAPN10 is not a major risk factor. However, these studies were not able to quantify the effect of CAPN10 on risk because of the small number of cases and controls in the individual studies. Here, we reexamine the role of the CAPN10 in the development of type 2 diabetes in the Japanese population.

Material and methods

Subjects

All subjects were Japanese. We studied three groups of cases and controls. The first group (study 1) included 205 unrelated subjects with type 2 diabetes recruited from the outpatient clinic in the Diabetes Center, Tokyo Women's Medical University and 208 unrelated normoglycemic subjects recruited from the Seijin Igaku Medical Clinic of Tokyo Women's Medical University using the following inclusion criteria: age >60 years, HbA1c $<5.6\%$, and no family history of diabetes. The second group (study 2) consisted of 281 unrelated normal glucose-tolerant (by oral glucose tolerance testing) subjects who were recruited at four outpatient clinics: Diabetes Center, Tokyo Women's Medical University ($n=50$); Third Department of Internal Medicine, Yamaguchi University ($n=121$); Department of Internal Medicine, University of Tokyo ($n=30$); and Shiseikai Daini Hospital, Tokyo ($n=80$). The third group of subjects (study 3) comprised 454 patients with type 2 diabetes and 192 nondiabetic controls who were recruited from Gunma University Hospital and affiliated hospitals and Fujita Health University School of Medicine. The genetic studies were approved by the institutional review board of each participating institution. Informed consent was obtained from all participants.

The pooled analyses included the datasets above as well as the data from three published studies: Daimon et al. 2002, SNP-43; Horikawa et al. 2003, SNP-43, Indel-19 and SNP-63; and Shima et al. 2003, SNP-43, Indel-19, and SNP-63.

Linkage disequilibrium (LD)

We examined the structure of the linkage disequilibrium (LD) in the CAPN10 region using the software package GOLD [graphical overview of linkage disequilibrium (Abecasis and Cookson 2000)] and a common set of 14 SNPs having a minor allele frequency ≥ 0.10 in diabetic ($n=30$) and nondiabetic subjects ($n=30$).

Genotyping

Genomic DNA was prepared from peripheral blood lymphocytes by standard procedures. We typed three polymorphisms in CAPN10: SNP-43, CAPN10-g.4852G>A (rs3792267); insertion/deletion (Indel)-19, CAPN10-g.7920 (32 bp-repeats) (rs3842570); and CAPN10-g.16378C>T (rs5030952) as described previously (Horikawa et al. 2003) or using TaqMan-based assays with custom probes/primers (Applied Biosystems, Foster City, CA, USA). Additional SNPs used for studies of LD in the CAPN10 region were genotyped using TaqMan technology. Previous studies have shown that the three core polymorphisms lead to four common haplotypes described as 111, 112, 121, and 221 (allele 1 or 2 at SNP-43, Indel-19, and SNP-63, respectively). The haplogenotypes were assigned by inspection of the genotypes at SNP-43, Indel-19, and SNP-63.

Statistical analyses

Polymorphisms were tested for deviation from Hardy-Weinberg equilibrium, heterogeneity in allele and genotype among studies and differences in allele, genotype, haplotype, and haplogenotype between groups using a chi-squared test. All P values are two sided.

Results

Haplotype structure across the CAPN10 region

The analysis of LD in the region of CAPN10 revealed a single region of strong LD (Fig. 1).

CAPN10 and SNP-43, and Indel-19 and SNP-63 are contained within this single LD block, which does not

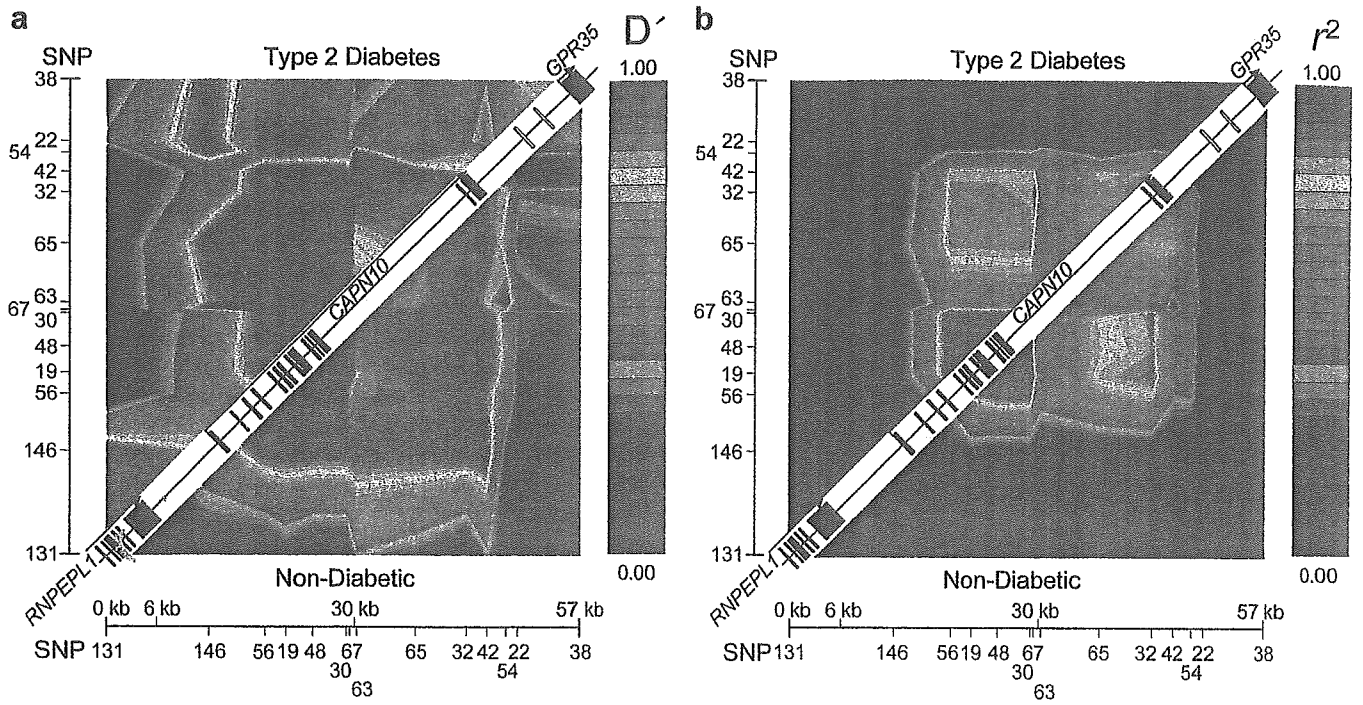

Fig. 1 Linkage disequilibrium (LD) in the CAPN10 region visualized using GOLD. The red and orange regions denote strong LD as defined using D' and r^2. The exons of CAPN10 and the adjacent genes RNPEPL1 and GPR35 are shown as *filled boxes* along the diagonal. The two variable number of tandem repeats (*VNTRs*) between CAPN10 and GPR35 are shown as *open boxes*. The SNPs used in this analysis are described in Horikawa et al. (2000). SNP-43 was not included in this analysis because the minor allele frequency was < 0.10 in the Japanese population

include the flanking RNPEPL1 and GPR35 genes. Thus, association of polymorphisms in this block with type 2 diabetes or a type-2-diabetes-related trait in the Japanese population implies that it is a variation in CAPN10 itself and not an adjacent locus that is responsible for the effect. The associated variant may or may not be causal depending on the LD with other variants in the block.

Genetic variation in CAPN10 and type 2 diabetes

We typed SNP-43, Indel-19, and SNP-63 in the three study groups described above (Table 1). There was no significant difference in the frequency of SNP-43 or Indel-19 between cases and controls (Table 2), which is in agreement with previous studies in Japanese (Daimon et al. 2002; Horikawa et al. 2003). However, we observed a significant difference in SNP-63 allele frequency between cases and controls in the subjects from study 1 (0.66 and 0.73, respectively, $P = 0.04$) but not in the subjects from study 3 (Table 2). In order to gain a better understanding of what role SNP-63 may play in the progression of type 2 diabetes, we examined the effect of SNP-63 genotype on various clinical and metabolic characteristics assessed by a standard 75-g oral glucose tolerance test in a group of 281 normal glucose-tolerant subjects from study 2 (Table 3). No significant effects of SNP-63 genotype on phenotype were observed except for area-under-the-curve plasma glucose level from 0 min to 120 min ($P = 0.03$).

We then carried out a pooled analysis using data from all known studies carried out in the Japanese population (Daimon et al. 2002; Horikawa et al. 2003; Shima et al. 2003). The 281 nondiabetic subjects from study 2 were excluded from the primary analyses because their mean age-at-study in this group was significantly younger than other the control groups. The pooled study population included 927 patients and 929 controls although Indel-19 and SNP-63 were not typed in all subjects. There was no significant difference in SNP-43, Indel-19, or SNP-63 genotype or allele frequencies between the type 2 diabetic and control groups in the overall analysis (Table 4). There was also no significant difference in SNP-43/Indel-19/SNP-63 haplotype (Table 5) or haplogenotype frequency (Table 6) except for the rare 111/221 combination, which was associated with significantly increased risk of type 2 diabetes (OR = 3.53, $P = 0.02$).

Genetic variation in CAPN10 may modify risk of type 2 diabetes in older patients

Since age is a risk factor for type 2 diabetes, we split the type 2 diabetic group based on median age-at-diagnosis, which was 50 years in the pooled sample, and repeated the comparisons but using only those cases for whom age-at-diagnosis was available: Patients with age-at-diagnosis < 50 years included 118 patients from study 1, 103 from study 3, and 68 from Horikawa et al. (2003); and patients with age-at-diag-

Table 1 Clinical characteristics of study populations. Data are mean ± SD. *NA* data not available, *Ctrl* control, *T2D* type 2 diabetes

Trait	Study population											
	Study 1		Study 2			Study 3		Horikawa et al. (2003)		Shima et al. (2003)		Daimon et al. (2002)	
Subjects	Ctrl[a]	T2D	Ctrl	T2D		Ctrl[a]	T2D	Ctrl[a]	T2D	Ctrl	T2D	Ctrl	T2D
n	208	205	281	–		192	454	172	177	276	10	81	81
Gender (F/M)	68/140	84/121	135/146	–		70/122	206/248	90/82	63/114	NA	NA	46/35	45/36
Age-at-study (years)	67.9 ± 5.5	59.1 ± 13.0	44.4 ± 15.9	–		67.8 ± 5.6	59.9 ± 11.7	68.0 ± 5.7	62.0 ± 11.0	NA	NA	62.3 ± 8.2	65.1 ± 10.7
Age-at-diagnosis (years)	–	45.9 ± 12.7	–	–		–	51.0 ± 12.0[b]	–	49.8 ± 11.4	–	NA	–	NA
BMI	23.1 ± 2.4	23.5 ± 3.6	22.3 ± 3.0	–		23.1 ± 2.8	24.2 ± 4.2	22.8 ± 3.3	23.9 ± 3.3	NA	NA	23.8 ± 3.6	25.6 ± 3.9
HbA_{1c} (%)	5.0 ± 0.3	8.2 ± 2.0	4.9 ± 0.4	–		4.9 ± 0.3	7.9 ± 1.9	5.0 ± 0.4	6.7 ± 1.0	NA	NA	5.3 ± 0.3	6.7 ± 1.1
Fasting glucose (mg/dl)	95.0 ± 8.7	160.5 ± 48.5	92.8 ± 9.4	–		–	NA	NA	NA	NA	NA	NA	NA
Treatment (diet/oral agents/insulin)	–	42/81/82	–	–		–	NA	–	46/70/61	–	NA	–	NA

[a] All subjects were > 60 years old
[b] Data are available for only 246 subjects

nosis ≥50 years included 87 patients from study 1, 143 from study 3, and 85 from Horikawa et al. (2003). There was no significant difference in SNP-43, Indel-19, and SNP-63 allele or genotype frequencies between the type 2 diabetic group with age-at-diagnosis < 50 years and the controls (Table 4). The SNP-43 and SNP-63 frequencies were also not different between the type 2 diabetic group with age-at-diagnosis ≥50 years and the controls. However, there was a small but significant difference in Indel-19 allele frequency (Table 4). The 3R allele at Indel-19 (allele 2 in the haplotype) was associated with lower risk of type 2 diabetes (OR = 0.82, $P = 0.04$).

The 121 haplotype was associated with significantly decreased risk (OR = 0.80, $P = 0.02$) of type 2 diabetes in the group of patients with age-at-diagnosis ≥50 years (Table 5). The 111 haplotype had the highest risk (OR = 1.33, $P = 0.046$) in the older group of patients. This effect of the 111 haplotype likely reflects the contribution of SNP-44 to type 2 diabetes risk (Weedon et al. 2003) since 88% of the 111 haplotypes in Japanese carry the at-risk C-allele at SNP-44. The rare 111/221 haplogenotype was associated with increased risk of type 2 diabetes irrespective of age-at-diagnosis (Table 6). The 121/121 haplogenotype had a protective effect against type 2 diabetes that approached significance in patients with age-at-diagnosis ≥50 years (OR = 0.76, $P = 0.06$). Individuals with haplotypes 111/111, 111/112, and 111/221 had the highest risk of type 2 diabetes (OR = 1.83, 1.25, and 4.12, respectively) although the increase in risk was not significant because of the small numbers of individuals studied. If the nondiabetic subjects in study 2 (Table 1) are included in the pooled control group, the results are similar, including the effects of the 121/121 haplogenotype on risk in patients with age-at-diagnosis ≥50 years (OR = 0.75, $P = 0.04$).

Discussion

The results suggest that genetic variation in CAPN10 may affect risk of type 2 diabetes in the Japanese population, especially in older individuals. Interestingly, the common 121 haplotype appears to be protective in Japanese, suggesting the overall effect of CAPN10 in this population is to reduce the risk of diabetes rather than increase it. It is important to note, though, that the statistical significance of the comparison is marginal ($P = 0.01$–0.04), and none of the comparisons would be significant if corrected for multiple testing. Thus, the results presented here need to be confirmed through studies of a much larger dataset. However, if our results are correct, they suggest an interaction between genetic (CAPN10) and nongenetic (age) factors to modify risk of type 2 diabetes. In this regard, recent studies have shown that calpain-10 is part of a novel apoptotic pathway in insulin-secreting pancreatic beta cells and thus may