β-adrenergic stimulation activates protein kinase Cε and induces extracellular signal-regulated kinase phosphorylation and cardiomyocyte hypertrophy

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Abstract. The cardiac adrenergic signaling pathway is important in the induction of cardiac hypertrophy. The cardiac adrenergic pathway involves two main branches, phospholipase C (PLC)/protein kinase C (PKC) and the adenylate cyclase (cAMPase)/protein kinase A (PKA) signaling pathways. It is hypothesized that PLC/PKC and cAMPase/PKA are activated by the α-adrenergic receptor (αAR) and the β-adrenergic receptor (βAR), respectively. Previous studies have demonstrated that exchange protein directly activated by cAMP (Epac), a guanine exchange factor, activates phospholipase Cε. It is possible that there are βAR-activated PKC pathways mediated by Epac and PLC. In the present study, the role of Epac and PLC in βAR activated PKC pathways in cardiomyocytes was investigated. It was found that PKCε activation and translocation were induced by the βAR agonist, isoproterenol (Iso). Epac agonist 8-CPT-2'OMe-cAMP also enhanced PKCε activation. βAR stimulation activated PKCε in the cardiomyocytes and was regulated by Epac. Iso-induced change in PKCε was not affected in the cardiomyocytes, which were infected with adenovirus coding rabbit muscle cAMP-dependent protein kinase inhibitor. However, Iso-induced PKCε activation was inhibited by the PLC inhibitor, U73122. The results suggested that Iso-induced PKCε activation was independent of PKA, but was regulated by PLC. To further investigate the downstream signal target of PKCε activation, the expression of phosphorylated extracellular signal-regulated kinase (pERK)1/2 and the levels of ERK phosphorylation was analyzed. The results revealed that Iso-induced PKCε activation led to an increase in the expression of pERK1/2. ERK phosphorylation was inhibited by the PKCε inhibitor peptide. Taken together, these data demonstrated that the βAR is able to activate PKCε dependent on Epac and PLC, but independent of PKA.

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Introduction

Myocardial hypertrophy, which is a reaction of cardiomyocytes to a variety of pathological stimuli, is a common complication of hypertension, coronary heart disease, valvular heart disease, congenital heart disease, and other cardiovascular diseases. However, when stressors persist, the compensatory hypertrophy can evolve into a decompensated state, with profound changes in the gene expression program, contractile dysfunction and extracellular remodeling (1,2). Pathological hypertrophy increases myocardial oxygen consumption and reduces myocardial compliance, ultimately leading to heart failure, arrhythmia and sudden death (3-5). Cardiac hypertrophy is an independent risk factor of cardiovascular morbidity and mortality (3,4).

The β-adrenergic receptor (βAR) and its signal transduction pathway are important factors leading to cardiac hypertrophy (6,7). Although the acute stimulation of βAR may have a beneficial effect on cardiac function, evidence suggests that the long-term activation of human cardiac βAR may lead to heart dysfunction, apoptosis and cardiac remodeling (8,9). Studies have revealed that the chronic stimulation of βAR may lead to cardiomyocyte hypertrophy (10) and inappropriate cardiac hypertrophy may develop into heart failure (11). The clarification of the signal transduction mechanism of cardiomyocyte hypertrophy by βAR stimulation may contribute to improving the prevention and treatment of cardiac hypertrophy and heart failure.

Since the adrenergic receptor (AR) was categorized into αAR and βAR, it has been suggested that the protein kinase (PK)C signal transduction pathway is mediated by αAR, and that PKA is a βAR-exclusive downstream effector. A new family of proteins, designated exchange protein directly activated by cAMP (Epac), has been reported, which makes the cAMP-mediated signaling mechanism

more complex. These proteins have been identified as the nucleotide exchange factors for small GTPases from the Rap family (12,13). A previous study by Schmidt *et al* (14) demonstrated that the activated adenylate cyclase (AC)-coupled β2AR in HEK-293 cells can stimulate a novel phospholipase C (PLC)ε subtype of PLC independently of PKA, but is mediated by Epac, suggesting that Epac-mediated signal transduction pathways may exist between βAR and PKC (15). An investigation into the inflammatory pain mechanism for nociceptive neurons by Hucho *et al* (16) revealed that βAR stimulation in neurons may lead to PKCε activation, and that Epac mediates signal transduction between cAMP and PKCε. Sudies by Oestreich *et al* (17,18) demonstrated that Epac and PLCε can mediate the βAR regulation of calcium ion release in cardiomyocytes. These studies suggest that there is a possible association between PKC and βAR, with the exception of αAR signaling. It is possible that Epac and PLC mediate the interconnection between the β-adrenergic and PKC pathways.

PKCε, one of the major PKC isozymes expressed in the heart, is important in cardiac cell signal transduction and function, including involvement in ischemic preconditioning, cardiac hypertrophy, myocardial fibrosis, heart failure and other signal transduction pathways (19,20). Upon activation, PKCε undergoes translocation from the cytoplasm to the cell membrane (particulate fractions) to phosphorylate its targets, including extracellular signal-regulated kinases (ERK) (19-21). ERK1/2 are considered the downstream kinases of PKC and are implicated in a wide range of cellular processes, including cell growth, proliferation to apoptosis (22,23). The ERK pathway is involved in cardiac development and hypertrophy (24). The βAR may activate ERK and ERK may also be activated in a PKC-dependent way (7,22,24). It remains to be elucidated whether βAR activates PKCε, mediated by Epac and PLC, to induce cardiac hypertrophy and the phosphorylation of ERK1/2 in cardiomyocytes.

The aim of the present study was to investigate PKCε translocation by stimulation with the βAR agonist, isoproterenol (Iso), in isolated cardiomyocytes, to clarify the role of Epac and PLC in the cross-talk between βAR and PKCε, and to investigate the effect of this signaling pathway on the phosphorylation of ERK and cardiomyocyte hypertrophy.

Materials and methods

Materials. Iso, phorbol 12-myristate 13-acetate (PMA), and PLC inhibitor (U73122) were obtained from Sigma-Aldrich (St. Louis, MI, USA). Rabbit polyclonal anti-PKCε (1:2,000; sc-214), rabbit polyclonal ERK2 antibody (1:1,000; sc-154), mouse monoclonal anti-pERK 1/2 (1:2,000; sc-81492), mouse monoclonal β-actin antibody (1:5,000; sc-47778), goat anti-rabbit horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) antibody [1:4,000(ERK2)-1:6,000(PKCε); sc-2004), goat anti-mouse IgG-HRP [1:5,000(p-ERK 1/2)- 1:7,500(β-actin); sc-2005] and the PKCε translocation inhibitor peptide (sc-3095) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The Epac agonist 8-CPT-2'OMe-cAMP (8-CPT) was obtained from Biolog Life Science Institute (Bremen, Germany). The green fluorescent protein (GFP) and human mutant Epac R279 K dominant negative (DN) in GFP structure were obtained from Genethon Center (Evry, France), and the adenovirus coding for rabbit muscle cAMP-dependent protein kinase inhibitor (Ad.PKI) was obtained from Rush University Medical Center (Chicago, IL, USA). The electrochemiluminescence (ECL) kit was obtained from GE Healthcare (Amersham, UK).

Primary culture of cardioyocytes. Cardiomyocyte culture was performed, as previously described, with a minor modification (25). Briefly, 20 pregnant female rats (Shanghai SLAC Laboratory Animal Co. Ltd., Shanghai, China) were maintained in a controlled environment (22-24˚C; 12 h light/12 h dark cycle) with *ad libitum* access to food and water. Subsequent to sacrifice by decapitation, the hearts were removed from the new-born Wistar rats (1-3 days old) and placed into pre-cooled 1X Ads buffer solution (NaCl, 17 g; Hepes, 11.9 g; NaH_2PO_4, 0.3 g; KCl, 1 g; glucose, 2.5 g; $MgSO_4$, 0.25 g; in 250 ml distilled water; Sigma-Aldrich), the ventricular muscle was cut into 1 mm^3 sections and a solution of pancreatin and collagenase (0.05%; Sigma-Aldrich) was added. Subsequently, 20 min digestion in pancreatin and collagenase at 37˚C and a centrifugation (500x g, 6 min) for supernatant removal was performed, 2 ml of newborn calf serum (HyClone Laboratories, Inc., Logan, UT, USA) was added and mixed evenly for subsequent use. The digested products were collected for all cells and subjected to a 6 min centrifugation at 500 x g. The ventricular myocytes were then purified with discontinuous Percoll density gradient centrifugation (2,000 x g, 30 min). The ventricular muscle cell layers were collected, washed with Ads two times (6 min centrifugation at 500 x g), the supernatant and culture medium [4:1 Dulbecco's modified Eagle's medium/M199 (Gibco Life Technologies, Carlsbad, CA, USA) containing 10% horse serum (HyClone Laboratories, Inc.), 5% newborn calf serum and 1% penicillin/streptomycin (Sigma-Aldrich)] was added. This was then mixed evenly, inoculated in a petri dish ($5x10^4$ cells/cm^2), and cultivated at 37˚C under conditions of 5% CO_2. After 48 h cultivation, the medium was replaced with serum-free medium and the subsequent experiments were carried out after 24 h. The study was approved by the Ethics Committee of Kunming Medical University (Kunming, China) and conformed to the standards set by the Yunnan Experimental Animal Management Board.

Cell treatment. The βAR agonist, Iso (1 μmol/l, 1 min), Epac agonist, 8-CPT (1 μmol/l, 10 min), PLC inhibitor, U73122 (2 μmol/l, 30 min), and PKC agonist, PMA (1 μmol/l, 5 min), were provided for cell treatment. Following cell infection ($5x10^2$/cm^2) by green fluorescent protein (GFP), Epac R279 K and Ad.PKI adenovirus, and the cell transfection by specific PKCε translocation inhibition peptide and scramble peptide, Iso (1 μmol/l, 1 or 10 min) was then provided for cell treatment and PKCε and pERK1/2 protein expression were detected.

Cardiomyocyte infection. The role of Epac was analyzed using Epac R279 K (dominant negative) to downregulate or inhibit Epac. The PKA-dependent activation of PKC was analyzed by overexpression of a specific PKA inhibitor peptide using Ad.PKI. Following infection with adenovirus coding for GFP, Epac R279 K with a multiplicity of infection (MOI) of 100 and Ad.PKI with an MOI of 100, the cells were treated with Iso (1 μmol/l) for 1 min.

Peptide transfection. It is hypothesized that the mechanism of activation of PKC involves translocation from a cytosolic fraction to a membrane-rich fraction. The translocation occurs when PKC in the cytoplasm binds to an isozyme-specific membrane-bound anchor protein or Receptor for Activated C Kinase (RACK). The RACK then transports the activated PKC isozyme to its target protein. Recently, small peptides of six to eight amino acids have been used to inhibit specific isozymes of PKC from binding to the specific RACK (26,27). Therefore, the specific PKCε inhibitor peptide was used in the present study to investigate the role of PKCε in ERK phosphorylation and cardiomyocyte hypertrophy induced by Iso stimulation (1 μmol/l for 10 min and 10 μmol/l for 48 h). The primary cardiomyocytes ($5x10^2/cm^2$) were exposed at room temperature to sterile phosphate buffered saline (PBS; HyClone Laboratories, Inc.) for 2 min and then exposed at room temperature to permeabilization buffer (20 mmol/l HEPES pH 7.4, 10 mmol/l EGTA, 140 mmol/l KCl, 50 μl/ml saponin, 5 mmol/l NaN_3 and 5 mmol/l oxalic acid dipotassium salt) containing either the PKCε translocation inhibitor peptide (22 μg/ml) or a control of scramble peptide (22 μg/ml) containing the same amino acids as the inhibitor peptide, but in a different sequence. This was removed after 2 min and the cells were washed twice with PBS at room temperature. The cells were then exposed to sterile PBS for 2 min at 37˚C prior to the addition of a fresh culture medium at 37˚C. The cells were allowed to recover for at least 1 h prior to the initiation of experiments, during which the cells began beating normally.

Immunocytochemical staining of cardiomyocytes. The cardiomyocytes ($5x10^2/cm^2$) were inoculated onto a Lab-Tec Chamber Slide system (Nunc®, Sigma-Aldrich) coated with 0.2% gelatine (Sigma-Aldrich) for culture and fixed with 4% paraformaldehyde (Sigma-Aldrich) (5 min, room temperature). They were then washed with NH_4Cl/PBS (Sigma-Aldrich) (pH 7.4, 0.5 mol/l) twice for 5 min each at room temperature and permeabilization treatment was performed using 1% Triton X-100/PBS (5 min, room temperature). Following washing with PBS and blocking of unspecific sites in 5% bovine serum albumin (BSA; Roche Diagnostics, Basel, Switzerland)/PBS, cells were incubated with a rabbit anti-PKCε (1:200) primary antibody in 1% BSA/PBS for 1 h at room temperature. After 1 h incubation with Alexa-488 conjugated goat anti-rabbit IgG antibody (Invitrogen Life Technologies, Carlsbad, CA, USA) at room temperature, the cells were washed with PBS, fixed with Vectashield mounting agent (Vector Laboratories, Burlingame, CA, USA), covered with a slide and sealed with nail polish. A confocal laser scanning microscope (Zeiss LSM510; Carl Zeiss Lase Optics GmbH, Oberkochen, Germany) was used to detect the translocation of PKCε.

Detection of the expression of PKCε and pERK1/2 by western blot analysis. Cell lysis buffer (Tris 12.5 mmol/l, pH 7.4, EDTA 1 mmol/l, EGTA 2.5 mmol/l, NaF 100 mmol/l) containing protease inhibitors was used to dissolve the cells and lysates were obtained following 15 min (4˚C) of centrifugation at 14,000 x g. The supernatant consisted of the cytosolic fractions. The centrifuged precipitate under a second suspension of lysis buffer containing 1% Triton X-100 consisted of the particulate fractions. The obtained cell particulate fractions were subject to protein quantification using Bradford's method and western blot analysis. Following cell lysis with radioimmunoprecipitation assay lysis buffer, containing Tris-HCl 25 mmol/l, pH 7.5, NaCl 150 mmol/l, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Roche Diagnostics), the lysates were collected, subject to a 10 min centrifugation (4˚C) at 10,000 x g, and the supernatant was collected for pERK1/2 protein expression detection by western blot analysis.

Western blot analysis. A total of 20 μg protein was used for 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane following electrophoresis at 35 V overnight. Non-specific sites were blocked (room temperature, 1 h) with Tween-tris-buffered saline (TTBS 0.1%) containing 5% nonfat dried milk, and PKCε antibody (1:2,000) and pERK1/2 (1:2,000) added to incubate at room temperature for 90 min. Following washing the membrane with TTBS, the membrane was incubated with horseradish peroxidase-conjugated IgG secondary antibody at room temperature for 1 h. All antibodies were visualized with chemiluminescence detection (ECL). β-actin (1:5,000) and ERK2 antibody (1:1,000) were used as the internal reference. The image signals were quantified by densitometric analysis of digitized films using the Scion Image (4.0.3) analysis system (National Institutes of Health, Bethesda, MD, USA).

Myocardial cell protein content and surface area determination after 48 h treatment with Iso. Following transfection with the PKCε inhibitor and scramble peptides, the cells ($5x10^2/cm^2$) were incubated with Iso (10 μmol/l) for 48 h. The protein content, DNA concentration and surface areas of the cells were then measured. Each dish was rinsed three times with PBS. The cell layer was scraped with standard sodium citrate containing 0.25% (w/v) SDS and frozen at -20˚C. Prior to use, the extracts were thawed and vortexed (Vortex-Genie 1; Scientific Industries, Inc., Bohemia, NY, USA). Total cell protein was assayed using Bradford's method. The results were normalized against the DNA content, which was measured fluorometrically using Hoechst staining, using calf thymus DNA as a standard. Hoechst 33258 and calf thymus DNA were obtained from Sigma-Aldrich. Hoechst 33258 dye was diluted to $1.5x10^{-4}$ mol/l in distilled water, and diluted five times with 20X saline-sodium citrate solution (SSC; 175.3 g NaCl and 88.2 g sodium citrate in 1 liter distilled water; Sigma-Aldrich) before testing. Calf thymus DNA was dissolved in SSC (1g/l). The cell extracts were mixed with Hoechst 33258 dye, and placed in the dark for 10 min. Absorbance was detected at 350 nm excitation wave and 450 nm emitting wave using a Fluorospectrophotometer 850 (Hitachi Ltd., Tokyo, Japan). The cell surface area measurements involved the use of microscope images (LV100POL; Nikon Corporation, Tokyo, Japan) (magnification, x10) captured with a digital camera (E5200; Nikon Corporation) and the cell perimeter was measured with an image analysis system (Scion Image). A total of five fields in each group were randomly selected, with 20 cells per field, to measure the cell surface area and to calculate the mean values (n=5).

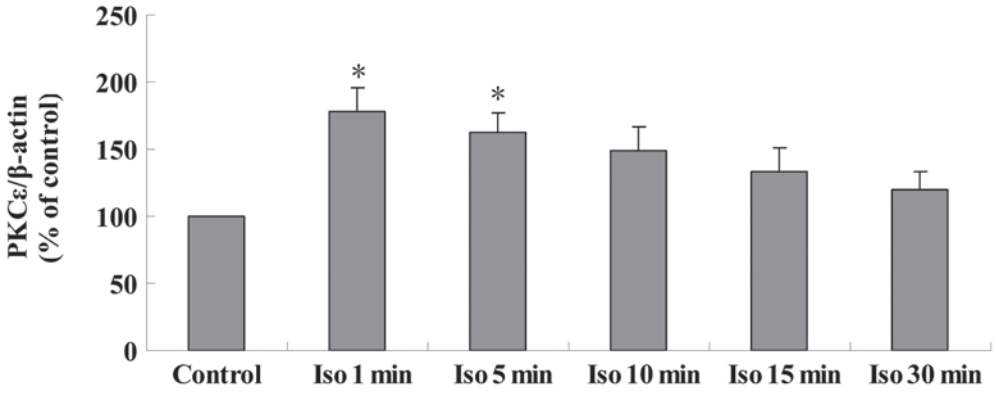

Figure 1. Time-dependent translocation of PKCε under Iso stimulation. The protein expression levels of PKCε of cellular particulate fractions in cardiomyocytes treated with Iso for different durations were detected using western blot analysis and β-actin was used as an internal reference (n=5). Data are presented as the means ± standard error of the mean of five independent experiments. *P<0.05, compared with the control. Iso, isoproterenol; PKC, protein kinase C.

Statistical analysis. The results are expressed as the mean ± standard error of the mean. Statistical analysis was performed using Student's t-test for single comparisons. Comparisons among three or more groups were made by one-way analysis of variance followed by the Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

βAR stimulation activates PKCε in cardiomyocytes. PKCε activation is associated with its translocation from the cytoplasm to the cellular particulate fractions (28). For evaluating PKCε translocation, cardiomyocytes were incubated with Iso (1 μmol/l) for different durations (1-30 min). Following cell scraping, differential centrifugation was performed in order to separate the particulate and cytosolic fractions. As shown in Fig. 1, the PKCε content in the particulate fractions was significantly increased following 1 and 5 min incubation with Iso (P<0.05). Compared with the control, this increase persisted for 15 min and decreased to the control level at 30 min. The precise sub-cellular PKCε localization was examined by immunostaining under confocal microscopy. As shown in Fig. 2, PKCε was translocated to the perinuclear area as early as 1 min after Iso incubation. Similarly, the western blot analysis revealed that this pattern persisted during the 15 min of Iso incubation, and homogeneous PKCε localization emerged after 30 min incubation. These results suggested that the Iso-induced translocation of PKCε was transient, with a translocation peak at 1 min. Therefore, this duration was used in the subsequent experiments to evaluate PKCε translocation.

PKCε activation by Epac agonist 8-CPT. Similar to that of Iso pre-incubation, following incubation of the cells with the Epac agonist (1 μmol/l 8-CPT for 10 min), the PKCε content in the cellular particulate fractions was significantly higher compared with those of the control (Fig. 3). The PKCε content of the particulate fractions increased by 168±23% compared with the control (P<0.05).

PKA-independent PKCε activation by Iso. Epac is activated by cAMP independently of PKA (29), therefore the present study subsequently examined whether the PKCε translocation produced by βAR stimulation was independent of PKA. Following infection with Ad.PKI, which specifically inactivates PKA activity, no inhibition of Iso-induced translocation of PKCε was observed (Fig. 4).

Inhibition of Iso-induced PKCε activation by Epac inhibitor Epac R279K. To further confirm whether Epac was involved in the Iso-induced PKCε activation, a dominant negative (DN) form of Epac (Epac R279K) was used to downregulate or inhibit the expression of Epac. The results revealed that DN Epac inhibited Iso-induced PKCε translocation (Fig. 5).

PLC-dependent PKCε activation by Iso. To assess the involvement of PLC, the cardiomyocytes were pre-incubated with the PLC inhibitor U73122 prior to stimulation with Iso. The translocation of PKCε was inhibited by this inhibitor (Fig. 6).

Iso-induced PKCε activation increases the expression of pERK1/2. Following introduction of the PKCε control scramble peptide into the cardiomyocytes and exposure of the cells to Iso (1 μmol/l, 10 min), there was a significant increase of pERK1/2 (P<0.05). However, when the PKCε inhibitor peptide was introduced into the cells prior to Iso exposure, no differences were identified in the expression of pERK1/2 compared with the PKCε inhibitor peptide alone (Fig. 7).

Iso-induced PKCε activation leads to hypertrophy in cardiomyocytes. The cells, which were exposed to the control scramble peptide and followed by Iso (10 μmol/l, 48 h) revealed significant increases in the cell protein/DNA ratio and in the cell surface area compared with the cells, which were exposed to the scramble peptide alone. However, following transfection of the cells with the PKCε inhibitor peptide and exposure to Iso, no significant differences were identified in the cell protein/DNA ratio or in the cell surface area compared with the PKCε inhibitor peptide alone (Figs. 8 and 9). These findings suggested that Iso-induced cardiomyocyte hypertrophy was mediated by PKCε and, following inhibition of PKCε activation by the PKCε-specific

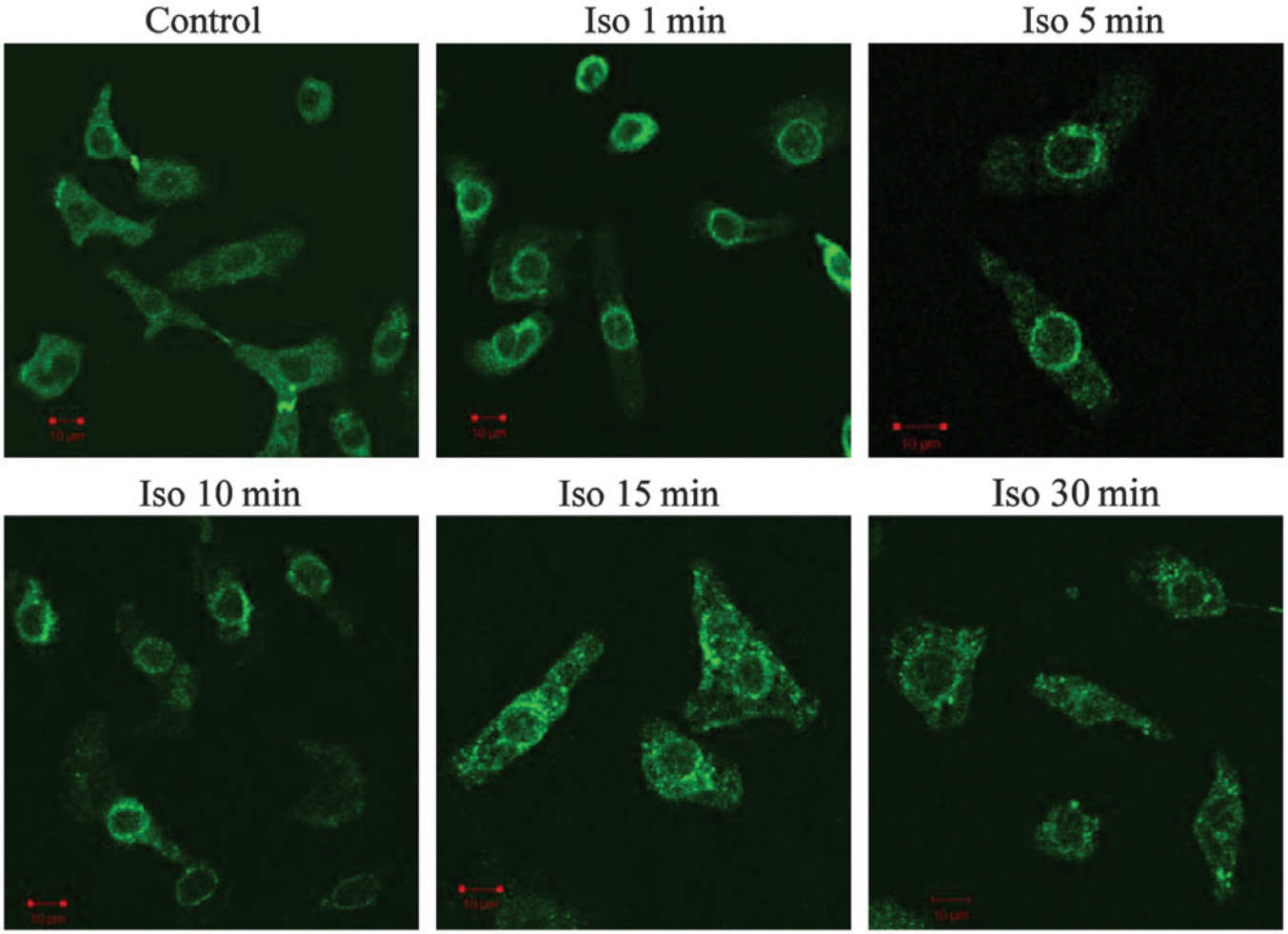

Figure 2. Confocal microscope images of Iso stimulation-induced time-dependent PKCε activation (magnification, x1,000; confocal immunofluorescence staining). PKCε was distributed evenly in the cytoplasm without Iso treatment. PKCε underwent translocation following incubation with Iso, with translocation around the nucleus. Translocation was most marked between 1 and 15 min, and was distributed in the cytoplasm at 30 min (n=3). Iso, isoproterenol; PKC, protein kinase C.

translocation inhibitor peptide, this hypertrophic effect was eliminated.

Discussion

At least 11 different isozymes have been identified as members of the serine/threonine PKC family, which can be divided into three categories based on their molecular structures and activation modes, comprising the classical or conventional PKC, the novel PKC and the atypical PKC (30). The G-proteins Gαq and Gβγ, rather than Gαs, have been hypothesized to activate phospholipase for PKC activation (31). However, previous studies on nociceptors have suggested that Gαs may activate PKC (32,33). In the Gαs signaling pathway, Gαs/AC/cAMP may induce PKCε activation, and this pathway is important in βAR signal transduction. Therefore, it was hypothesized that the βAR agonist Iso activates PKCε in cardiomyocytes, resulting in a series of pathological and physiological reactions.

In the present study, western blot analysis and confocal laser scanning microscopy were used to detect PKCε translocation following Iso stimulation in cardiomyocytes. The results revealed that Iso enhanced the activation of PKCε. The Iso-stimulated PKCε activation was relatively short and time-dependent, peaking following 1 min of Iso stimulation and returning to the baseline level after 30 min. Iso also induced PKCε to translocate to the perinuclear area. Since the subcellular localization of PKC was key in determining its function and specificity regulation, the localization of PKCε around the nucleus may lead to the phosphorylation of target proteins, thus regulating intranuclear transcription and protein synthesis. The results demonstrated that βAR stimulation activated PKCε in cardiomyocytes.

It has been reported that the PKA inhibitor CMIQ does not inhibit β2-AR-induced translocation of PKCε in neurons (16,18,34) and the signaling pathway from AC/cAMP to PKC does not involve PKA, indicating other upstream branches of the Gs/cAMP second-messenger signaling pathway at PKA prior to the activation of PKCε (35). To verify whether the Iso-induced PKCε activation passed through PKA in cardiomyocytes, Ad.PKI was used to infect cardiomyocytes. Following specific inhibition of PKA activity, Iso remained able to activate PKCε, suggesting that PKA did not mediate Iso-induced PKCε activation and that PKCε activation was PKA-independent, possibly mediated by the novel PKA-independent signaling pathway factor, Epac.

Epac, a protein identified by Kawasaki *et al* (12) and de Rooij *et al* (13), may be directly activated by cAMP and

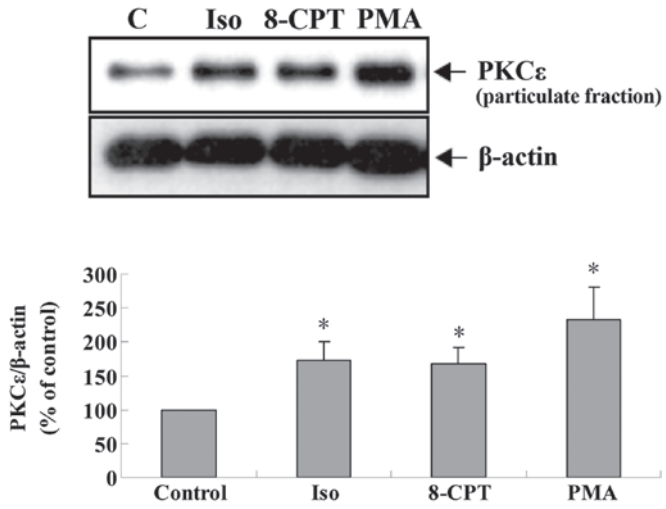

Figure 3. Effects of Iso and 8-CPT on PKCε translocation in cardiomyocytes. Western blotting was used to determine the PKCε translocation in cellular particulate fractions and β-actin was used as an internal reference (n=6). PMA was used as a positive control. Data are presented as the means ± standard error of the mean of six independent experiments. *P<0.05, compared with the control. Iso, isoproterenol; PKC, protein kinase C; 8-CPT, 8-CPT-2'OMe-cAMP; PMA, phorbol 12-myristate 13-acetate; C, control.

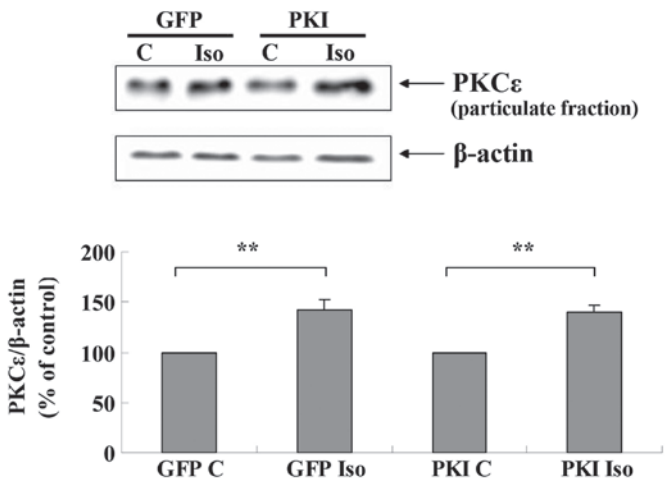

Figure 4. Effect of the PKA inhibitor, Ad.PKI, on Iso-induced PKCε activation. Following infection of the cardiomyocytes by Ad.PKI and GFP, Iso treatment (1 μmol/l for 1 min) was performed. Western blot analysis was used to determine the PKCε content in the cellular particulate fractions, with β-actin as an internal reference (n=4). Data are presented as the means ± standard error of the mean of four independent experiments. **P<0.01, compared with the GFP and PKI control group. Iso, isoproterenol; PK, protein kinase; Ad.PKI, adenovirus-coded rabbit muscle cAMP-dependent protein kinase inhibitor; GFP, green fluorescent protein; C, control.

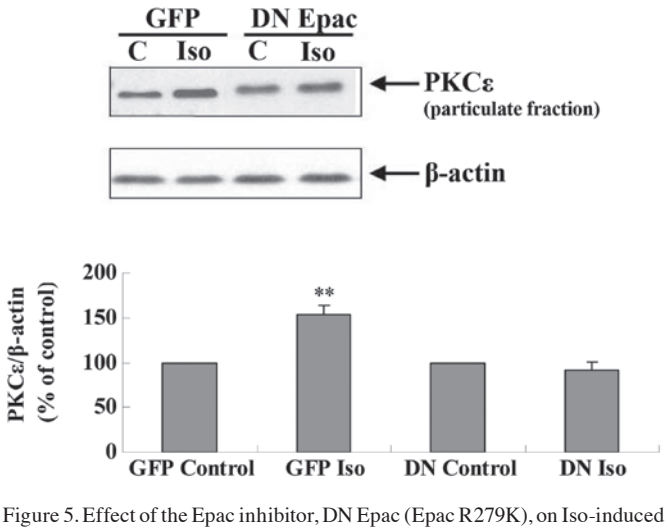

Figure 5. Effect of the Epac inhibitor, DN Epac (Epac R279K), on Iso-induced PKCε activation and translocation. Following infection of the cardiomyocytes by adenoviruses carrying EpacR279K (DN) and GFP, Western blot analysis was used to determine the PKCε content in the particulate fractions of the cardiomyocytes, with β-actin as an internal reference (n=5). Data are presented as the means ± standard error of the mean of five independent experiments. **P<0.01, compared with the GFP control group. Iso, isoproterenol; PKC, protein kinase C; GFP, green fluorescent protein; Epac, exchange protein directly activated by cAMP; C, control.

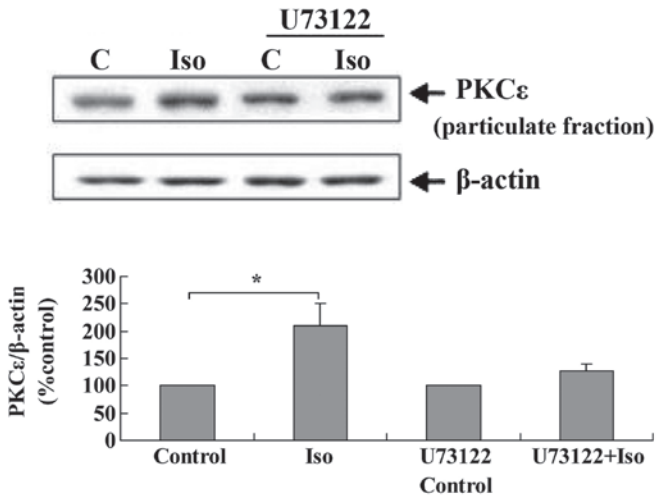

Figure 6. Effect of the phospholipase C Inhibitor, U73122, on Iso-induced PKCε activation. Following preincubation with U73122, western blot was used to determine the PKCε content in the particulate fractions of the cardiomyocytes, with β-actin as an internal reference (n=6). Data are presented as the means ± standard error of the mean of six independent experiments. *P<0.05, compared with the control. Iso, isoproterenol; PKC, protein kinase C; C, control.

mediate the cAMP signal transduction process. Epac is involved in numerous physiological processes, including cell division and differentiation, exocytosis, insulin secretion, cell adhesion and amyloid protein secretion (36,37). Studies have revealed that Epac may be a positive regulatory protein for cardiomyocyte hypertrophy (38). It has been demonstrated that Epac1 is involved in the hypertrophic effects of βAR in a PKA-independent manner in adult rat ventricular cardiomyocytes (39). In the present study, following incubation of cardiomyocytes with the Epac activator, 8-CPT, PKCε translocation to the cellular particulate fractions was observed, suggesting that Epac activated PKCε. The pattern of 8-CPT-induced PKCε translocation was similar to that of Iso and it was hypothesized that there may be causal association between Epac and Iso. To further clarify the role of Epac in the Iso-activated PKCε signaling pathway, the mutant Epac R279K (DN) was constructed to inhibit the action of the wild-type Epac protein. The results revealed that Iso-induced PKCε activation was inhibited in the mutant, suggesting an Epac-mediated βAR/PKCε signaling pathway in cardiomyocytes.

Epac in cardiomyocytes is considered to mediate the activation of PLCε and the βAR-dependent Ca^{2+} release (17) and a signal transduction pathway, beginning with β2AR

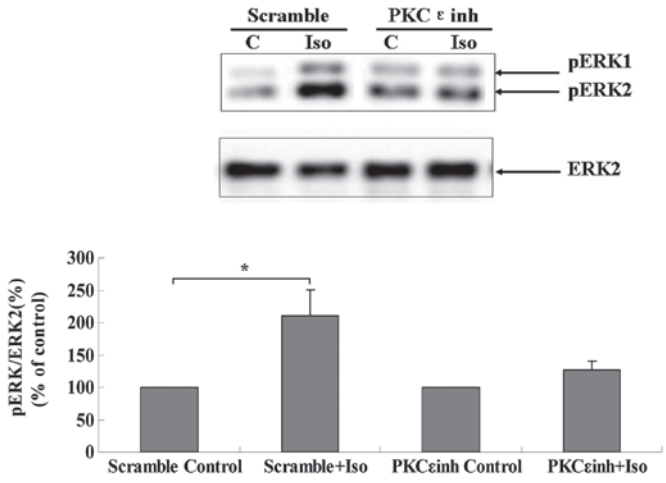

Figure 7. Effect of Iso-induced PKCε activation on the expression of pERK1/2 in cardiomyocytes. Following transfection of cardiomyocytes with the PKCε-specific inhibitor and scramble peptides, western blot analysis was used to determine the expression of pERK1/2 in the cardiomyocytes, with ERK2 as an internal reference (n=4). Data are presented as the means ± standard error of the mean of six independent experiments. *P<0.05, compared with the scramble peptide group. Iso, isoproterenol; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; Scramble, scramble peptide; PKCε inh, PKCε inhibitor peptide; C, control.

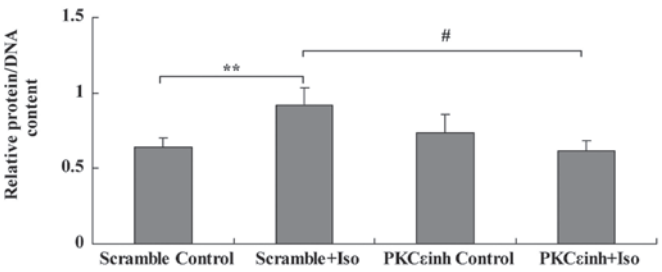

Figure 8. Effect of Iso-induced PKCε activation on cardiomyocyte protein content. The relative ratio of cardiomyocyte protein/DNA following transfection of cells with the PKCε inhibitor and scramble peptides followed by 48 h Iso treatment (n=6). Data are presented as the means ± standard error of the mean of six independent experiments. **P<0.01, compared with the scramble peptide group; #P<0.05, compared with the scramble peptide+Iso group. Scramble, scramble peptide; PKCε inh, PKCε inhibitor peptide; Iso, isoproterenol; PKC, protein kinase C.

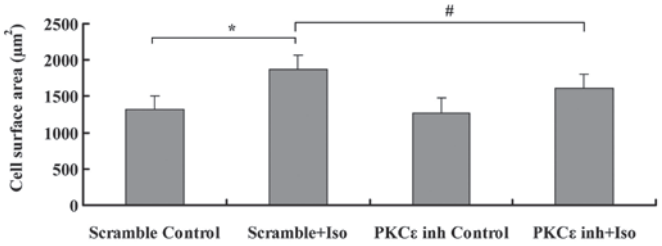

Figure 9. Effect of Iso-induced PKCε activation on cardiomyocyte surface area. The cell surface area of cardiomyocytes following transfection with the PKCε inhibitor and scramble peptided followed by 48 h Iso treatment (n=6). Data are presented as the means ± standard error of the mean of six independent experiments. *P<0.05, compared with the scramble peptide group; #P<0.05, compared with the scramble peptide+Iso group. Scramble: scramble peptide; PKCε inh: PKCε inhibitor peptide.

and followed by cAMP, Epac and Rap2B, leading to PLCε activation and calcium ion release, has been suggested (15). The results of the present study demonstrated that βAR stimulation activated PKCε in cardiomyocytes and that Epac mediated this signal transduction pathway. Therefore, it was hypothesized that Epac may activate PLC and subsequently activate PKCε. Following the preincubation of cardiomyocytes with the PLC inhibitor, U73122, the effect of PLC on Iso-induced PKCε activation suggested that PLC mediated Iso-induced PKCε activation.

ERK signaling is closely associated with the pathological processes of cardiomyocyte hypertrophy and apoptosis (40-42). Iso-induced cardiac hypertrophy is associated with ERK by increasing ERK1/2 mRNA transcription in cardiomyocytes (43). Iso may also activate MAPK in cardiomyocytes and phosphorylate the Raf/MEK/ERK pathways (44,45). PKCε may phosphorylate Ras/Raf and lead to activation of ERK1/2, inducing myocardial protein synthesis and cardiomyocyte hypertrophy. Although βAR activates ERK, it remains to be elucidated whether βAR acts on ERK by activating PKC. In the present study, the PKCε-specific translocation inhibitor peptide was used to inhibit the activation of PKCε to observe the effect of PKCε on pERK1/2. The results revealed that the PKCε-specific inhibitor peptide inhibited the Iso-induced pERK1/2 increase, suggesting that PKCε mediated the Iso-induced phosphorylation of ERK1/2. The increase in pERK1/2 was one of the downstream effectors of Iso-induced PKCε activation and Iso may induce ERK1/2 phosphorylation through the activation of PKCε, resulting in cardiomyocyte hypertrophy.

There is considerable evidence demonstrating that chronic βAR activation may result in myocardial hypertrophy and heart failure, accompanied by cardiomyocyte injury, apoptosis, necrosis and cardiac remodeling (46). The results presented in the present study demonstrated an interaction between βAR and PKCε in cardiomyocytes. The possible pathway of PKCε activation by βAR suggested that βAR stimulation may activate the Epac guanine exchange protein, which in turn activated PLC, resulting in PKCε translocation to the cellular particulate fractions. ERK phosphorylation and cardiomyocyte hypertrophy are effects of Iso-induced PKCε activation. The clarification of signal transduction of cardiac hypertrophy resulting from βAR activation in cardiomyocytes assists in further understanding the molecular mechanisms underlying cardiac hypertrophy, providing a reference for examining new preventative methods and developing effective drugs against cardiac hypertrophy.

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