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Protein Kinase C Isozyme-Specific Modulation of Cyclic AMP-Dependent Phosphodiesterase in Hypertrophic Cardiomyopathic Hamster Hearts

JOHN J. CAI and HON-CHI LEE

Cardiovascular Division, Department of Internal Medicine, The University of Iowa College of Medicine, Iowa City, Iowa 52242

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SUMMARY

We recently demonstrated that protein kinase C (PKC) activities were elevated in hypertrophic cardiomyopathic (HCM) hamster hearts and that activation of PKC resulted in stimulation of cAMP-dependent phosphodiesterase (PDE) activity. In this study, we determined the composition of PKC isozymes in control and HCM hearts and identified the PKC isozyme responsible for the modulation of PDE activity in HCM hearts. Using quantitative autoradiographic techniques with PKC isozyme-specific antibodies, we found that the PKCα, ϵ, and ζ isozymes were expressed in both control and HCM hearts. The immunoreactive amounts of cytosolic PKCα and PKCϵ and of membrane PKCζ were significantly increased in HCM hearts. The enzymatic activity of PKC in HCM hearts was significantly elevated in both membrane (148.0 ± 13.7 versus 78.9 ± 1.9 pmol/mg/min in controls, four experiments) and cytosol (117.3 ± 5.1 versus 75.7 ± 5.1 pmol/mg/min in controls, four experiments). Contribution of individual PKC isozyme activity was assessed by the immunoprecipitable PKC activity with isozyme-specific antibodies. The membrane PKCϵ (41.7 ± 4.9 versus 18.7 ± 0.3 pmol/mg/min in controls, four experiments, $p < 0.05$) and PKCζ (61.5 ± 14.0 versus 20.3 ± 2.7 pmol/mg/min in controls, four experiments, $p < 0.05$) but not PKCα (50.9 ± 6.8 versus 44.3 ± 1.5 pmol/mg/min, four experiments, p = N.S.) were increased in HCM hearts. On the other hand, the cytosolic PKCα (47.7 ± 4.1 versus 27.0 ± 1.4 pmol/mg/min, four experiments, $p < 0.05$) and PKCϵ (42.8 ± 3.1 versus 19.1 ± 3.9 pmol/mg/min, four experiments, $p < 0.05$) but not PKCζ (27.2 ± 3.0 versus 32.0 ± 2.1, four experiments, p = N.S.) were increased in HCM hearts. Furthermore, after immunoprecipitation of PKCα, activation of PKC could no longer potentiate the PDE activity in HCM hearts. Removal of PKCϵ or PKCζ, on the other hand, did not affect the PKC-mediated PDE stimulation in HCM hearts. These results suggest that there is an increase in the quantitative expression of PKC isozymes in HCM hearts and that the cross-talk between PKC and PDE in these hearts is mediated specifically via the PKCα isozyme.

PKC is a ubiquitous family of closely related serine/threonine protein kinases that mediate many cellular functions (1–5). Multiple PKC isozymes have been identified either by molecular cloning techniques or by immunochemical methods. Conventional PKCs, which include PKCα, PKCβI, PKCβII, and PKCγ, can be activated by Ca^{2+}, phospholipids, diacylglycerol, and phorbol ester. Novel PKCs, which include PKCδ, PKCϵ, PKCϵ', PKCλ, PKCζ, PKCθ, and PKCη, are structurally similar to the conventional PKCs but lack the Ca^{2+} binding domain and do not require Ca^{2+} for activation. However, little is known about the contribution of the activities of PKC isozymes to total PKC activity in the heart. Recently, the expression of the PKC isozymes in myocardium or myocytes has been reported in rats (6–8), cultured myocytes (9, 10), and bovine hearts (11), but their role in pathological states remains to be elicited. A condition that is known to be associated with abnormal PKC regulation is HCM. In most cardiac hypertrophy animal models, total PKC activities are found to be elevated (12, 13), but the expression of PKC isozymes and the distribution of isozyme activities in these animals have not been previously reported. The Syrian cardiomyopathic hamster (BIO 14.6) is a genetically inbred cardiac HCM animal model with a predictable clinical course (14). We previously reported that there is an increase in cardiac PKC activity due to an impairment in the developmental reduction in PKC activity (15). Whether this increased PKC activity was due to global increase in PKC isozymes, an increase in specific PKC isozyme(s), or expression of new PKC isozymes was unknown. More recently, we demonstrated that activation of PKC potentiates the PDE

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ABBREVIATIONS: PKC, protein kinase C; PDE, cAMP-dependent phosphodiesterase; HCM, hypertrophic cardiomyopathy; PMA, phorbol-12-myristate-13-acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; BSA, bovine serum albumin; TBS, Tris-buffered saline; TTBS, TBS containing 0.05% Tween 20.

activity in HCM hamster hearts but not in control hearts (16). Such an abnormal cross-talk was found to be associated with elevated PDE activities and reduced cAMP concentrations and may contribute to the derangements of excitation/contraction coupling in these animals. In the present study, we first identified the individual PKC isozymes expressed in hamster hearts by immunoblot analysis using anti-PKC isozyme-specific antibodies (anti-PKCα, PKCβ, PKCγ, PKCδ, PKCϵ, and PKCζ). Second, we determined the activity of each expressed PKC isozyme in control and HCM hamster hearts by immunoprecipitation techniques using the PKC isozyme-specific antibodies. Third, we sought to identify the PKC isozyme(s) in the BIO 14.6 hearts that was responsible for potentiation of PDE. We found that the PKCα, PKCϵ, and PKCζ isozymes are expressed in control and HCM hamster hearts, whereas the PKCβ, PKCγ, and PKCδ isozymes are not detectable. The patterns of increase in expression and activities of each PKC isozyme differ. Furthermore, cross-talk could be elicited only by PKCα in BIO 14.6 hearts. These results may have important pathophysiological implications.

Materials and Methods

Preparation of cell fractions. Syrian cardiomyopathic hamsters (BIO 14.6, BioBreeders, Watertown, MA) and control hamsters (BIO RB) were studied at 6 months of age. At that age, the HCM in BIO 14.6 hamsters was fully developed (14–16). Handling of the animals and preparation of the cell fractions were performed as described previously (16). Briefly, the hamsters were anesthetized with ketamine (50–100 mg/kg intraperitoneal) and killed with a guillotine. A midline sternal dissection was made to expose the heart, which was rapidly excised and placed in an ice-cold buffer containing 0.25 M sucrose, 60 mM Tris·HCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM EGTA, 10 μM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitors, pH 7.4. The atria, great vessels, and adventitial tissues were quickly removed, and the ventricular myocardium was rinsed in buffer, minced, and then homogenized with a Tissuemizer (Mark II, model T25-S1, IKA Labortecknik, Germany) at 75% full speed using three pulses for 5 sec each. The homogenate was centrifuged at 1000 $\times$ *g* at 4° with a tabletop centrifuge (model TJ-6R, Beckman, Palo Alto, CA) for 15 min. The supernatant was further centrifuged at 100,000 $\times$ *g* at 4° for 60 min with an ultracentrifuge (model L7–55, Beckman). The supernatant was designated the cytosolic fraction, and the pellet was resuspended in a buffer containing 50 mM Tris·HCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol with 10 μM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitors, pH 7.4, and was designated the membrane fraction.

Partial purification of PKC and assay of PKC activities. Partial purification and assay of PKC activities were performed as previously described (16). Briefly, membrane and cytosolic fractions were extracted with 1% of Triton X-100 in a buffer containing 20 mM Tris·HCl, pH 7.5, 0.5 mM EDTA, and 0.5 mM EGTA on ice for 60 min. Then, partial purification of PKC was achieved by chromatography with DEAE cellulose (Whatman DE52) equilibrated with buffer containing 20 mM Tris·HCl, pH 7.5, 0.5 mM EDTA, and 0.5 mM EGTA and eluted with buffer containing 20 mM Tris·HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 0.1 M NaCl (17). The eluent was assayed for protein concentration according to the method of Bradford (18). Protein concentrations ranged between 0.1 and 0.2 mg/ml.

PKC activities were assayed based on the measurement of the phosphorylation of a synthetic peptide from myelin basic protein, Ac-MBP(4–14) (GIBCO-BRL Life Technologies, Gaithersburg, MD) (19). PKC specificity was confirmed by inhibition of PKC effects with the PKC pseudosubstrate inhibitor peptide PKC(19–31) (20). The PKC reaction mixture contained 20 mM Tris·HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM $CaCl_2$, 20 μM ATP, 50 μM Ac-MBP(4–14), 10 μM PMA, 0.28 mg/ml phosphatidyl serine, [γ-^{32}P]ATP (1–2 $\times$ 10^6 cpm), and 1–2 μg of a partially purified PKC fraction with or without 100 μM PKC peptide inhibitor, PKC(19–31), in a total reaction volume of 50 μl. The reaction was allowed to proceed at 30° for 5 min, and 25 μl of the reaction mixture was removed and spotted onto phosphocellulose discs. The discs were washed with two 500-ml aliquots of 75 mM phosphoric acid each for 5 min on a shaker. This was followed by two 500-ml aliquot washings of water. The amount of ^{32}P incorporated into the substrate Ac-MBP(4–14) was measured by scintillation counting of the phosphocellulose discs. PKC-specific phosphorylation was determined by the difference in substrate phosphorylation in the presence and absence of the PKC-specific pseudosubstrate peptide inhibitor. Samples were assayed in duplicates.

Immunoprecipitation of PKC isozymes. Immunoprecipitation of PKC isozymes was carried out by incubating 5–10 μg of a partially purified PKC fraction with 5 μg of anti-PKC isozyme IgG (GIBCO-BRL Life Technologies) in a total volume of 100 μl on a rotating shaker at 4° for 2 hr. The amount of antibodies used was ~5-fold in excess of the amount necessary to remove PKC isozymes based on dose-response curves. The incubation solution contained 50 mM Tris·HCl, pH 7.5, 0.15 M NaCl, 15 μg/ml leupeptin, 15 μg/ml aprotinin, 1 mM EDTA, 1 mM EGTA, 1% BSA, and 1% glycerol. The PKC/antibody complexes were then removed by the addition of 50 μl of 4% cross-linked protein A-agarose (GIBCO-BRL) in the incubation solution containing 1% BSA at 4° for 1 hr. The protein A-agarose used was >20-fold more than the amount required for binding the anti-PKC isozyme antibodies. After centrifugation at 15,000 $\times$ *g* for 5 min at 4°, the supernatant was removed for PKC assay. Individual PKC isozyme activity was calculated by subtracting the PKC activity after immunoprecipitation from the control sample that was incubated with IgG from nonimmunized rabbit serum IgG, followed by protein A-agarose incubation. Samples were performed in duplicates.

Quantitative immunoautoradiography of PKC isozymes. The amounts of PKC isozymes in hamster cardiac cell fractions were measured immunochemically using isozyme-specific antibodies to the α, β, γ, δ, ϵ, and ζ isozymes (GIBCO-BRL). Immunoblotting was carried out according to the method of Towbin *et al.* (21). Proteins from the cell fractions (20 μg) were resolved using sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis (22) and then transferred electrophoretically to a nitrocellulose membrane. The nitrocellulose membrane was incubated successively with 5% nonfat dry milk in TBS, which contained 20 mM Tris·HCl, pH 7.5, and 150 mM NaCl, for 30 min to block nonspecific binding sites. The nitrocellulose membrane was then exposed to PKC isozyme-specific IgG antibodies at a concentration of 4 μg/ml in TTBS and 5% nonfat dry milk for 2 hr at room temperature. The membrane was washed extensively with TTBS and, for quantitative analysis, was incubated with ^{125}I-labeled donkey anti-rabbit IgG antibodies (10^6 cpm/ml, Amersham Corp., Arlington Heights, IL) in TTBS with 5% dry milk for 1 hr at room temperature. After additional washings with TTBS, the nitrocellulose membrane was dried and exposed to Kodak X-OMAT AR film with intensifying screens in a cassette for 10–48 hr at −70°. The intensities of the autoradiographic bands were measured with a laser densitometer (Image Quant-Computing Densitometer 300/325, Molecular Dynamics, Sunnyvale, CA). Control (BIO RB) hamster brain homogenate (5 μg) was included in each polyacrylamide electrophoresis experiment and served as an internal standard. The levels of immunochemically detectable PKC isozymes in cardiac cell fractions from HCM and control hamsters were normalized according to the internal standard and then compared.

PDE activity. PDE activities in cell fractions were assayed as previously described (16) by the two-step method of Thompson *et al.* (23). The enzymatic reaction was initiated by the addition of 25 μl of sample to an incubation mixture containing 40 mM Tris·HCl, pH 7.5, 4 mM mercaptoethanol, 0.5 mM EGTA, 5 mM $MgCl_2$, and 1 μM [8-^{3}H]cAMP (~1 $\times$ 10^5 cpm) with a total reaction volume of 150 μl.

Specificity was determined by the suppression of PDE activity with 0.5 mM 3-isobutyl-1-methylxanthine. The assay was terminated by heating for 45 sec in a 100° water bath, followed by incubation with snake venom 5′ nucleotidase (100 μg) at 30° for 10 min. Ten microliters of 10 mM adenosine were added to each sample as internal standards. The [8-^{3}H]adenosine formed was then isolated by chromatography with AG-1X8 resin (400 mesh) and eluted with methanol. The stock [8-^{3}H]cAMP was purified once every 2 weeks by high performance liquid chromatography using a SAX column (ISCO, Lincoln, NE). 3-Isobutyl-1-methylxanthine inhibited the [^{3}H]adenosine formation by >95%. The data were corrected for recovery of adenosine by monitoring absorbance at 260 nm. Samples were assayed in duplicates.

Statistical analysis. All experiments were performed in duplicates, and multiple determinations were obtained for each experimental condition described in the protocol. Group data are expressed as mean ± standard error. Statistical significance ($p < 0.05$) was determined by Student's t test.

Results

Cardiac PKC isozymes in control and HCM hamsters. The PKC isozymes in control and HCM BIO 14.6 hamster hearts were identified using anti-PKC specific antibodies, and quantitative analysis was performed with autoradiographic techniques. Specifically, antibodies against the α, β, γ, δ, ε, and ζ subtypes of PKC were incubated against Western blots of hamster cardiac cell fractions as described in Materials and Methods; results are shown in Fig. 1. The hamster brain internal standard was reactive against all antibodies tested except those against anti-PKCδ. However, control and HCM hamster hearts were only reactive against three anti-PKC isozyme antibodies. With hamster brain, protein bands at 81 and 67 kDa were reactive against anti-PKCβ and a single band at 81 kDa was reactive against anti-PKCγ, but no immunoreactivity against these antibodies was detected in any of the cardiac samples from control and HCM hamsters. Against anti-PKCα, a single band at 81 kDa was detected against the hamster brain and cardiac samples. Multiple bands were detected in hamster heart and brain samples with anti-PKCε and anti-PKCζ. To determine the specificity of these bands, we incubated the Western blot with these anti-PKC isozyme antibodies in the presence or absence of isozyme-specific peptides (1 μg of peptide/1 μg of IgG), against which the antibodies were raised (data not shown). Our results showed that only one protein band at 87 kDa was specific for PKCε and that two protein bands at 66 and 78 kDa were specific for PKCζ. In summary, PKCα, PKCε, and PKCζ were present in both membrane and cytosolic fractions in control and BIO 14.6 hamster hearts at 6 months of age, but the β, γ, and δ isozymes were not detectable.

Quantitative analysis of the three PKC isozymes expressed in the hamster hearts was determined by comparing each detectable PKC isozyme with an internal standard of 5 μg of hamster brain homogenate. These results are presented in Fig. 2. The amounts of the PKC isozymes were expressed as the percentage of immunoreactivity compared with the PKC isozyme-specific protein band in the hamster brain standard on each gel. The immunoreactive amounts of PKCα and PKCε were similar in the membrane fractions of control and HCM hearts (37.8 ± 2.7% in controls versus 34.3 ± 5.6% in HCM for PKCα and 23.5 ± 1.5% in controls versus 24.1 ± 1.9% in HCM for PKCε, five experiments, p = N.S.). In contrast, the cytosolic levels of both PKCα and PKCε were significantly elevated in HCM hamster hearts (34.0 ± 3.3% in controls versus 60.5 ± 3.2% in HCM for PKCα and 20.6 ± 2.9% in controls versus 29.3 ± 2.7% in HCM for PKCε, five experiments, $p < 0.05$ for both). Two different molecules of PKCζ (66 and 78 kDa) were detected in hamster hearts. The amounts of membrane and cytosolic PKCζ at 66 kDa did not differ between controls and HCM hearts (26.7 ± 4.9% in controls versus 28.4 ± 2.3% in HCM for membrane and 57.4 ± 10.6% in controls versus 49.8 ± 13.2% in HCM for cytosol, five experiments, p = N.S. for both). In comparison, the amounts of the cytosolic 78-kDa PKCζ was similar for control and HCM hearts (40.3 ± 6.2% and 32.0 ± 9.0%, respectively, five experiments, p = N.S.), but its levels were significantly increased in HCM cardiac membrane (8.6 ± 0.4% in controls versus 12.4 ± 1.3% in HCM, five experiments, $p < 0.05$). These results suggest that the increase in PKC in HCM hearts does not occur globally but rather occurs selectively for individual PKC isozymes in both membrane and cytosolic fractions.

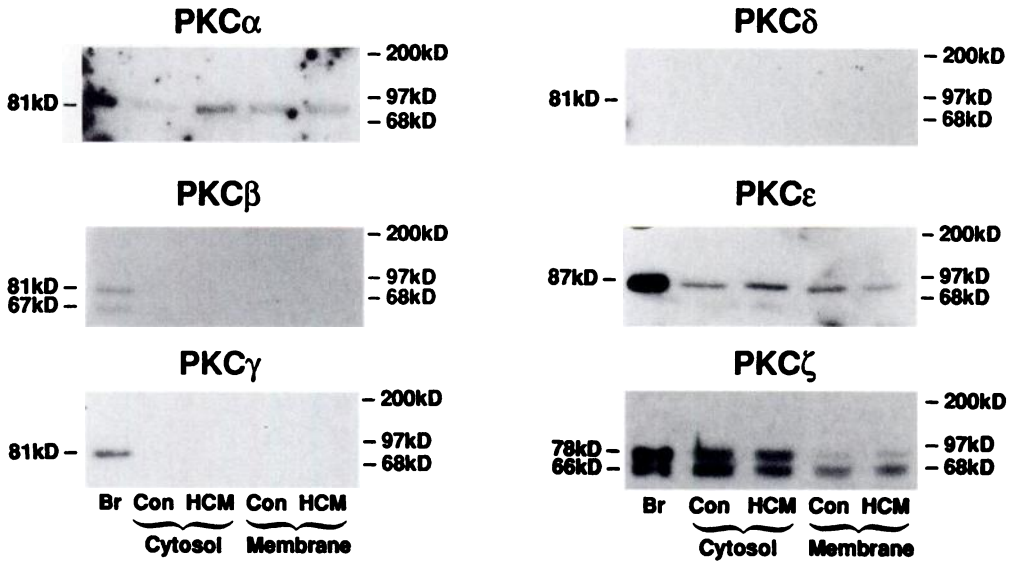

Fig. 1. Autoradiographs of PKC isozymes in control (*Con*) and HCM hamster hearts. Proteins (20 μg/lane) from cardiac membrane and cytosolic fractions were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%), transferred to nitrocellulose membranes, and subjected to immunoblotting procedures as described in Materials and Methods. Five micrograms of protein from hamster brain homogenate (*Br*) were included in each experiment and served as an internal standard for comparison between experiments. *Right*, positions for molecular mass standards. *Left*, calculated molecular masses for the identified immunoreactive bands.

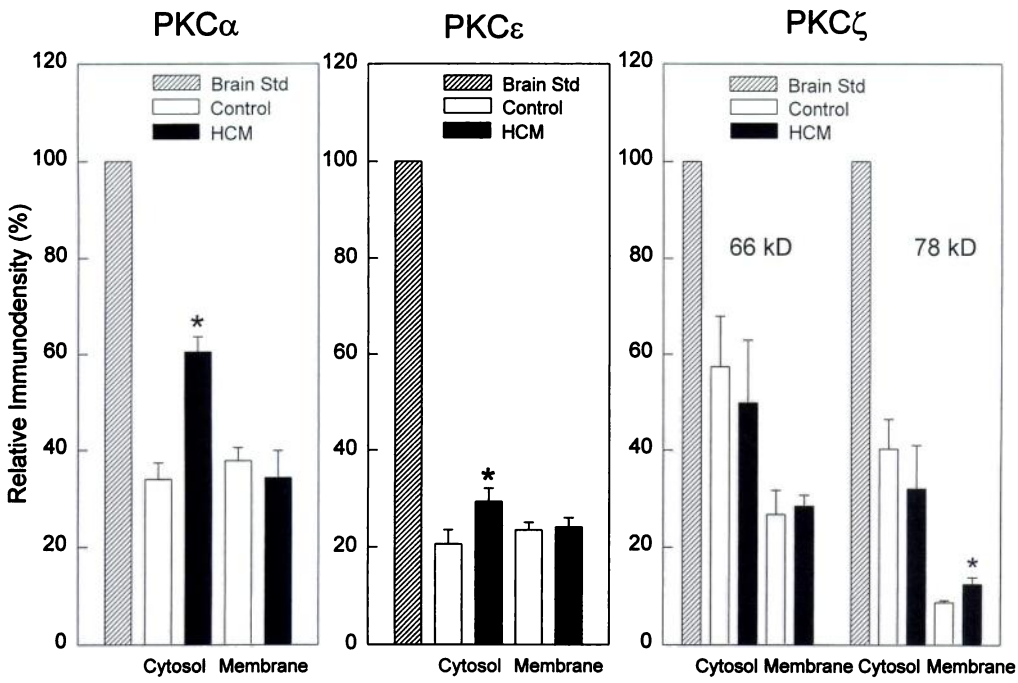

Fig. 2. Quantitative analysis of immunoreactive amounts of PKCα, PKCε, and PKCζ in cardiac cytosolic and membrane fractions from control and HCM hearts. PKCα was detected as a single 81-kDa band, PKCε as a single 87-kDa band, and PKCζ as two bands at 66 and 78 kDa. Results are expressed as percentage densitometric measurements on the autoradiographs relative to the hamster brain homogenate (5 μg) standard (100%) (five experiments). *, $p < 0.05$ compared with controls.

Activities of cardiac PKC isozyme in control and HCM hamsters. The activities of individual PKC isozymes were measured by the immunoprecipitable activity of PKC after incubation with anti-PKC isozyme-specific antibodies as described in Materials and Methods. To determine the amount of anti-PKC isozyme-specific IgG necessary to remove each PKC isozyme (PKCα, PKCε, and PKCζ) completely, we first established the dose-response curves, as shown in Fig. 3. Ten micrograms of partially purified PKC were incubated against 0, 0.1, 0.5, 1, 3, and 5 μg of anti-PKC isozyme IgGs, followed by the additional incubation with ~20-fold excess protein A-agarose in the presence of 1% BSA. After removal of the PKC isozymes with the isozyme-specific antibodies, the PKC activity in the supernatant was assayed. As indicated by the results, ~1 μg of the anti-PKC isozyme IgG was able to remove almost all of the PKC activity of that isozyme in 10 μg of partially purified PKC. The addition of 5 μg of anti-PKC antibodies allowed the PKC activities to reach a plateau without further reduction in PKC enzymatic activities. Subsequent immunoprecipitation experiments used <10 μg of partially purified PKC, and the 5 μg of anti-PKC IgGs, therefore, represented at ≥5-fold excess of the amount necessary to completely remove the specific PKC isozyme activities.

In the HCM hamster hearts, total PKC activities were significantly elevated in both cytosol (117.3 ± 5.1 versus 75.7 ± 5.1 pmol/mg/min in controls, four experiments, $p < 0.05$) and membrane (148.0 ± 13.7 pmol/mg/min versus 78.9 ± 1.9 pmol/mg/min in controls, four experiments, $p < 0.05$) (Fig. 4). As described in Materials and Methods, the activities of individual PKC isozymes were measured based on the amount of PKC activity inhibited by each of the PKC

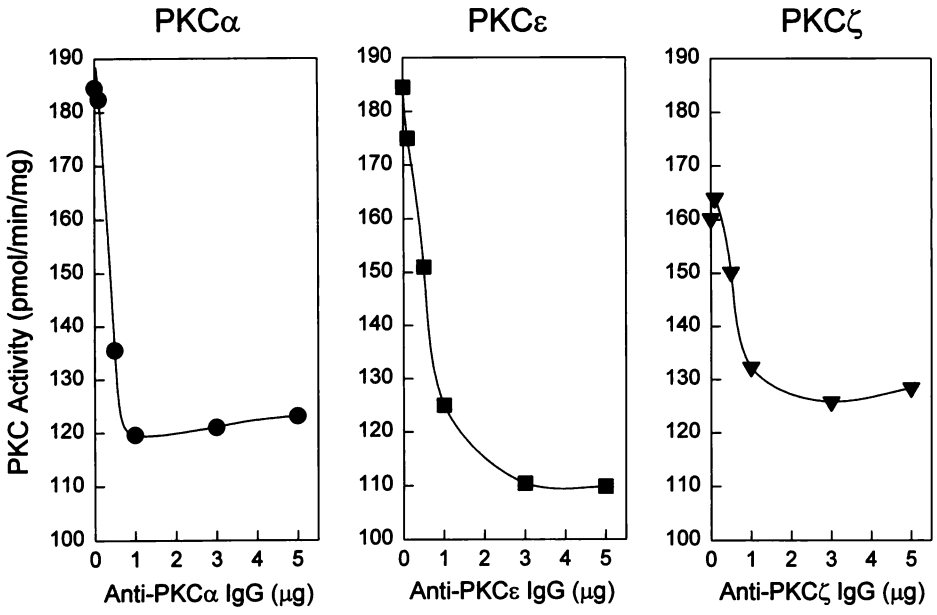

Fig. 3. Removal of PKC isozyme activities by immunoprecipitation with anti-PKC isozyme-specific antibodies. Ten micrograms of partially purified PKC from HCM membranes were incubated with 0, 0.1, 0.5, 1.0, 3.0, and 5.0 μg of anti-PKCα, anti-PKCε, or anti-PKCζ IgGs, followed by incubation with >20-fold excess of protein A-agarose in the presence of 1% BSA. Incubations with 5 μg of anti-PKC isozyme IgG allowed reduction of PKC activities to reach a plateau.

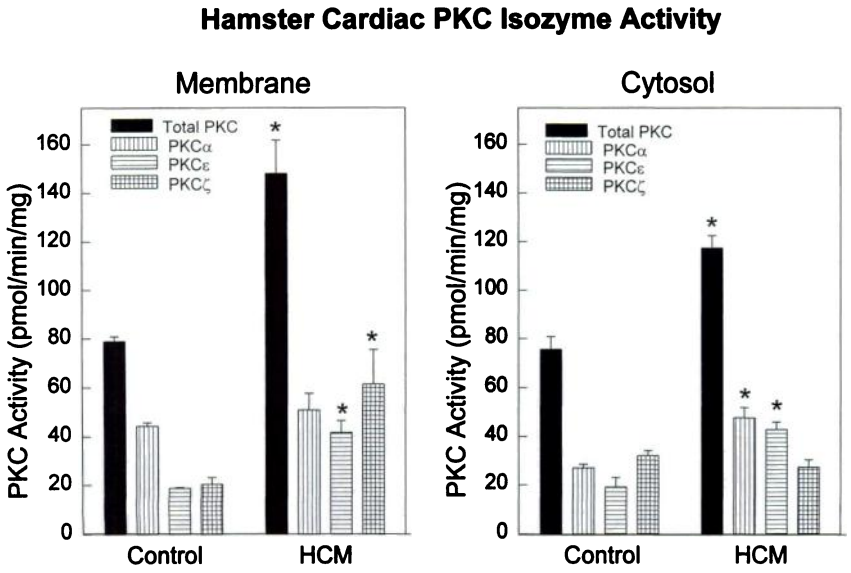

Fig. 4. Hamster cardiac PKC isozyme activities (four experiments). Compositions are given of PKC isozyme activities in membrane (*left*) and cytosol (*right*). *, $p < 0.05$ compared with the control counterpart. The activities of individual PKC isozymes were measured based on the amount of PKC activity inhibited by each of the PKC isozyme-specific antibodies as described in Materials and Methods.

isozyme-specific antibodies. The increase in cardiac membrane PKC activities was contributed to by increases in the activities of PKCε (41.7 ± 4.9 in HCM versus 18.7 ± 0.3 pmol/mg/min in controls, four experiments, $p < 0.05$) and PKCζ (61.5 ± 14.0 in HCM versus 20.3 ± 2.7 pmol/mg/min in controls, four experiments, $p < 0.05$). There was no significant difference in cardiac membrane PKCα activity between control and HCM hamsters (50.9 ± 6.8 versus 44.3 ± 1.5 pmol/mg/min, four experiments, p = N.S.). On the other hand, the increase in PKC activity in the cytosolic fraction was contributed to by increases in the activities of PKCα (47.7 ± 4.1 in HCM versus 27.0 ± 1.4 pmol/mg/min in controls, four experiments, $p < 0.05$) and of PKCε (42.8 ± 3.1 in HCM versus 19.1 ± 3.9 pmol/mg/min in controls, four experiments, $p < 0.05$) but not of PKCζ (27.2 ± 3.0 in HCM versus 32.0 ± 2.1 pmol/mg/min in controls, four experiments, p = N.S.). In summary, our results indicate that the increase in cardiac membrane PKC activity was due to increases in PKCε and PKCζ activities and the increase in cardiac cytosolic PKC activity was contributed to by increases in PKCα and PKCε activities.

Modulation of PDE activity by PKC isozymes in HCM hearts. We recently reported that activation of PKC by the phorbol ester PMA produced significant stimulation of PDE activity in HCM hamster hearts but not in control hamster hearts (16). This PMA-induced increase in PDE activity was blocked by the PKC-specific pseudosubstrate peptide inhibitor PKC (19–31), suggesting the phorbol-induced effects were PKC specific. In addition, after removal of PKC with an anti-PKC antibody (monoclonal antibody 1.9, GIBCO-BRL) that recognizes an epitope in the PKC catalytic domain and cross-reacts with all the PKC isozymes (24), PMA could no longer elicit stimulatory effects on PDE activity (16). In this study, we used anti-PKC isozyme-specific antibodies to determine and identify the specific PKC isozyme(s) responsible for interacting with PDE in HCM hamster hearts. Results given in Fig. 5 were expressed as percentage of baseline PDE activity. In the presence of phosphatidylserine (0.28 mg/ml) and 100 nM free Ca^{2+}, PMA significantly stimulated PDE activity (240.8 ± 37.6 pmol/min/mg versus 197.5 ± 23.5 pmol/min/mg at baseline, four experiments, $p < 0.05$), and this effect was completely blocked by the PKC peptide inhibitor PKC(19–31) (198.9 ± 30.6 pmol/min/mg, four experiments, $p < 0.05$ compared with PMA alone). After removal of PKCα with anti-PKCα antibodies, PMA could no longer potentiate the activity of PDE (203.0 ± 50.4 pmol/min/mg at baseline versus 201.3 ± 51.9 pmol/min/mg with PMA stimulation, four experiments, p = N.S.). In contrast, removal of PKCε and PKCζ did not abolish the potentiation of PDE activity by activation of PKC. In comparison, PMA had no effect on PDE activity in control hamster hearts with or without incubation with anti-PKC isozyme antibodies (Fig. 5B). These results again confirmed that cross-talk between PKC and PDE occurs in HCM but not normal hamster hearts. PKCα appears to be the only isozyme that is responsible for such interaction between the two signal transduction pathways.

Discussion

In the present study, we report several new findings. First, the isozyme compositions of cardiac PKC in control and HCM hamsters were determined. We found that only the α, ε, and ζ PKC isozymes are detectable in hamster hearts. The elevated PKC activities in HCM hamster hearts result from neither a global increase in all PKC isozymes present nor the expression of new isozymes. Instead, different PKC isozymes are increased in different cell fractions. Second, we determined the enzymatic activity of the individual PKC isozymes in control and HCM hamster hearts. To our knowledge, this has not been previously reported in mammalian hearts. Third, we identified that the cross-talk between PKC and PDE in HCM hamster hearts is solely due to the effects of PKCα activation. PKCε and PKCζ as well as the PKCα in control hamster hearts are incapable of modulating PDE activity.

PKC isozymes in control and HCM hamster hearts. Our results demonstrate that only PKCα, PKCε, and PKCζ are present and PKCβ, PKCγ, and PKCδ are not detectable in control and HCM hamster hearts. Based on previous reports, there is considerable variability in the expression of PKC isozymes in mammalian hearts. Using column chro-

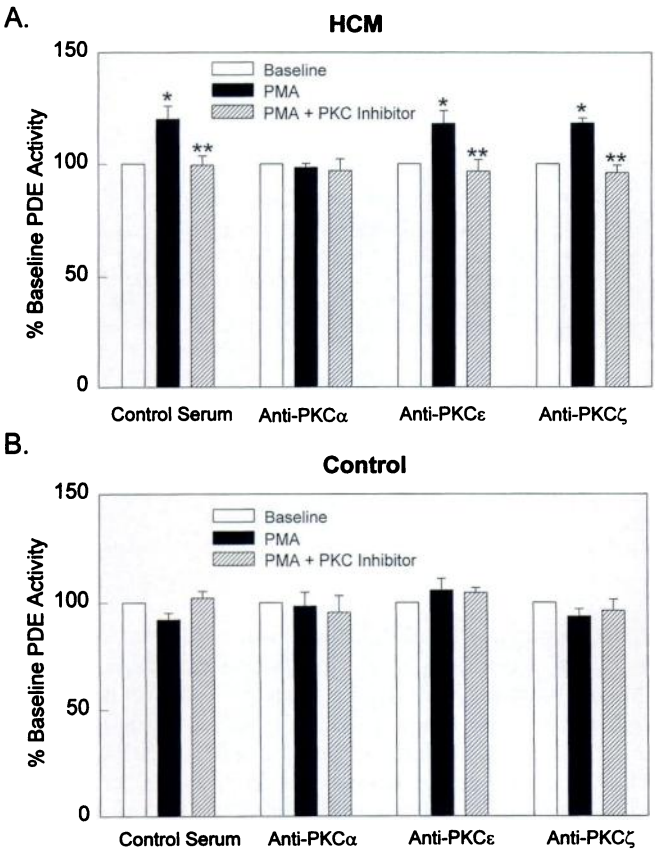

Fig. 5. Effect of individual PKC isozyme removal by immunoprecipitation with anti-PKC isozyme antibodies on phorbol ester-induced PDE stimulation. Partially purified PKC (5–10 μg) was incubated with 5 μg of anti-PKC isozyme IgG (~5-fold excess) for 2 hr, followed by incubation with protein A-agarose (in amounts >20-fold the amount required to bind the anti-PKC isozyme IgG) for 1 hr. The bound PKC isozymes were removed by centrifugation. The supernatant was recovered and assayed for PDE activity in the presence of PMA (10 μM) or PMA plus the PKC inhibitor peptide PKC(19–31) (20 μM). Results are expressed as percentage of baseline PDE activity (four experiments). *, $p < 0.05$ compared with baseline; **, $p < 0.05$ compared with PMA.

matographic techniques, we found that bovine hearts consist of PKCα and PKCβ (11). Experiments using immunofluorescence techniques showed that the α, βI, βII, δ, ε, and ζ PKC isozymes were all detectable in 1-day-old rat cardiac myocytes and in neonatal rat ventricular myocytes in culture (10). Using a reverse transcriptase-polymerase chain reaction technique, the α, δ, ε, η, and ζ PKC isozymes mRNA were found to be expressed in adult rat cardiac myocytes and in neonatal rat ventricular myocytes in culture (9). However, results from immunoblots with anti-PKC isozyme-specific antibodies were variable from different laboratories examining the PKC isozyme expression in rat hearts. Gu and Bishop (8) showed that the α, β, δ, ε, and ζ PKC isozymes were all present in control and HCM rat hearts induced by pressure overload. In an examination of the tissue and cellular distribution of the PKC isozymes, Wetsel *et al.* found that the α, βI, βII, δ, and ζ isozymes were present, whereas the γ and ε isozymes were absent in adult rat hearts (25). On the other hand, Rybin and Steinberg (7) showed that PKCδ and PKCε were present in cardiac myocytes, whereas PKCα and PKCζ were present in nonmyocytes only, and PKCβ was totally absent in the rat heart. However, Bogoyevitch *et al.* (6) reported that only PKCε and PKCζ were present in cardiac myocytes and that PKCη was found only in nonmyocytes in the heart, whereas the α, βI, γ, and δ PKC isozymes were not detected. The discrepancy in results from different studies in the cardiac content of PKC isozymes is not immediately clear and may be due to the methods of isolation and detection; assay conditions, including substrate specificity of PKC isozymes; specificity of isozyme-specific antibodies; cell types being examined; and potential species differences. In the Syrian hamster, the α, ε, and ζ isozymes are present in control and HCM hearts. Why the expression of cardiac PKC isozymes in hamster is different from that in other species and the functional significance of such a difference are not clear. The hamster cardiac PKC isozymes represent three different subtypes with different activation requirements. PKCα requires Ca^{2+} and phospholipids for activation. PKCε and PKCζ do not contain the C2 domain where Ca^{2+} binds and are independent of Ca^{2+} in their activation. In addition, PKCζ contains only one zinc finger motif instead of two and does not bind diacylglycerol or phorbol ester (26). PKCζ has been shown to exhibit a constitutively active protein kinase activity *in vitro* (27) and could be stimulated by phosphatidylinositol-3,4,5-triphosphate (28). Our results showed that PKCα is slightly more prevalent in the membrane than in the cytosol fraction in control hamster hearts. Compared with controls, the HCM hearts showed a significant increase in PKCα in the cytosolic but not in the membrane fraction, so most of the PKCα is not membrane bound. PKCε showed a similar pattern of increase in the cytosolic fraction in HCM hearts. In contrast, PKCζ showed a selective increase in the 78-kDa membrane-associated band in HCM hearts. Therefore, the different PKC isozymes appear to be differently and independently regulated during the developmental reduction of PKC activities in HCM hearts (15).

Activities of cardiac PKC isozymes in control and HCM hamsters. The enzyme activities of individual PKC isozymes has not been previously reported in mammalian hearts. We used the removal of enzyme activity by anti-PKC isozyme-specific antibodies to measure the activity of individual isozymes. Such an approach has been previously used to completely eliminate the PKC/PDE cross-talk in the HCM hearts, suggesting quantitative removal of PKC by anti-PKC antibodies (16). In the present study, the results showed that PKCα and PKCε are both responsible for the increase in cytosolic PKC activities, whereas PKCε and PKCζ account for the increase in enzyme activity in the membranes of HCM hearts. In control hamster hearts, both PKCα and PKCζ constitute the major isozymes in the cytosolic fraction with a PKCα/PKCε/PKCζ ratio of 34.6:24.4:41.0%. This ratio is changed to 40.5:36.4:23.1% in HCM hearts due to increases in both PKCα and PKCε activities. The 1.8-fold increase in cytosolic activity of PKCα is consistent with the finding based on immunoblot analysis of a 1.8-fold increase in PKCα amounts in HCM hearts. In contrast, the 1.4-fold increase in cytosolic amounts of PKCε is associated with a 2.3-fold increase in isozyme activity. Examination of the membrane fractions suggests that PKCα constitutes the major isozyme in control hamster hearts, with a PKCα/PKCε/PKCζ ratio of 53.2:22.4:24.4%. In HCM hearts, the membrane PKC is predominantly PKCζ, with a PKCα/PKCε/PKCζ ratio of 33.0:27.1:39.9%. Interestingly, the 2.2-fold increase in the enzyme activity of membrane PKCε is not accompanied by a signifi-

cant increase in immunoreactive amounts of the isozyme in HCM hearts, and the 1.4-fold increase in immunoreactive amounts of the membrane-associated 78-kDa PKCζ is accompanied by a 3.1-fold increase in enzyme activity. These results suggest that the increase in PKC activity in HCM hearts is isozyme and cell fraction specific. In addition, there appears to be significant increases in the specific activities of PKCε and PKCζ in HCM hearts. The underlying mechanism of such changes is not immediate clear. The changes in enzyme activities are not associated with any detectable changes in the molecular masses of the isozymes. However, the HCM hamster hearts are known to have elevated activities of phosphatidylinositide metabolism with markedly enhanced release of arachidonic acid and diacylglycerol (29). Arachidonic acid is known to be a potent activator of both PKCε (27) and PKCζ (30) and may be a potential cause of enhanced PKC isozyme activities. Our results also suggest that assessment of isozyme activities using an immunoprecipitation approach is feasible because the sum of the individual isozyme activities correlates very well with the total PKC activity in each cell fraction. This finding also suggests that the total PKC activities can be accounted for entirely by the activities of PKCα, PKCε, and PKCζ without a contribution from other PKC isozymes. In addition, we added the antibodies against PKCα, PKCε, and PKCζ, and they produced 93.1 ± 4.1% (four experiments) inhibition of the total PKC activities. The addition of the isozyme-specific peptides prevents the immunoprecipitations by 91.3 ± 5.2% (four experiments). These results again support our contention that quantitative assessment of isozyme activities with an immunoprecipitation approach is feasible.

PKC isozyme-specific modulation of PDE activity. Signal transduction across the cell membrane involves a complex network of signaling systems that include various membrane receptors, G proteins, second messenger-generating enzymes, protein kinases, target functional proteins, and regulatory proteins. It is now known there is diverse interaction among signaling systems, and knowledge of such interactions is vital to an understanding of the regulation of physiological and pathological cellular responses (31). PKC is a prominent player in signal transduction pathway cross-talk and is known to modulate various receptors, second messenger pathways, and kinases (32). In mammalian hearts, PKC is known to have important effects on the regulation of excitation/contraction coupling by modulation of ion channel activities (33), cytosolic Ca^{2+} activities (34), Na^+/H^+ exchange activities (35), and sarcoplasmic reticulum function (36). Activation of PKC has also been shown to cause the induction of immediate-early genes such as c-*fos* and *Egr*-1 and activate cardiac gene transcription (37). Nevertheless, the effect of PKC in the regulation of other signal transduction pathways in mammalian hearts has not been extensively studied. Recently, we reported that activation of PKC results in the potentiation of PDE activity resulting in acceleration of cAMP degradation and cAMP deficiency in HCM hearts (16). It is intriguing that the PKC/PDE interaction is only observed in HCM hearts and not in normal control hearts. In the present study, we further substantiated this observation by demonstrating convincingly that the α PKC isozyme in HCM hearts is solely responsible for such cross-talk. Activation of PKCε and PKCζ does not lead to potentiation of PDE activities, and PKCα in control hamster hearts also lacks such capability. Different PKC isozymes have been shown to have different patterns of translocation on activation (10), but they are not known to regulate different functions in the heart. Our findings represent a first demonstration of PKC isozyme-specific regulation of cellular processes in HCM hearts. The fact that PKCα exhibits an aberrant function is interesting. Of the PKC isozymes expressed in Syrian hamster hearts, PKCα is the only isozyme that is Ca^{2+} dependent, and intracellular Ca^{2+} is known to be elevated in the HCM hearts (38), lending further support to the pathophysiological relevance in our finding. Our results, however, cannot rule out that the primary abnormality for the PKC/PDE cross-talk resides in the PDEs of HCM hearts. The difference in PDE regulation between control and HCM hearts may not be due to the elevated PKCα levels in the HCM hearts because substantial levels of this isozyme exist even in the control hearts. However, PDE activities in the control hearts is not altered by PKC activation. The primary abnormality could be due to altered PKCα function or altered PDE structure or function in one or more of its isozymes. Whether the elevation in PKCε and PKCζ activities is associated with abnormal regulation of other cellular metabolic pathways or functions in HCM hearts is not known. The novel PKC isozymes have different substrate requirements and specificities compared with the conventional PKC isozymes (2, 39), and this may account for their lack of effect on PDE. PKCε is a major isozyme in the rat heart (6) and is greatly activated by arachidonic acid (40). PKCε is believed to be localized in the cell nucleus as well as with myofibrils, and activation of PKCε appears to be associated with translocation from nuclear locations to contractile elements (10). In contrast to other isozymes, PKCζ does not require diacylglycerol for activation and does not down-regulate in response to phorbol ester (26). On exposure to norepinephrine, PKCζ appears to translocate from cytosolic sites to the perinuclear region in cardiac myocytes (10). Similar to PKCε, PKCζ activity is stimulated by arachidonic acid (27); in addition, phosphoinositol-3,4,5-triphosphate produces substantial stimulation of PKCζ (28). Both phosphoinositide metabolism and arachidonic acid release are significantly increased in HCM hamster hearts (29), suggesting the presence of enhanced stimuli for the activation of these PKC isozymes that may be pathophysiologically important in HCM hearts, even though their exact roles are still unknown. Nevertheless, our results suggest that PKCα is capable of accelerating the degradation of cAMP through potentiation of PDE activities in HCM hearts. Such cross-talk may result in cAMP deficiency and lead to the associated abnormalities in excitation/contraction coupling in HCM hearts.

In summary, we demonstrated the presence of the α, ε, and ζ PKC isozymes and determined the enzymatic composition of the PKC isozymes in control and HCM hearts. We did not evaluate the role of PKCη and PKCθ in the hamster hearts. However, these PKC isozymes have not been previously identified in mammalian hearts. PKCη is found in lung and skin tissues (41), whereas PKCθ is expressed in skin and skeletal muscle (42). We cannot rule out the presence of these isozymes in the HCM hamster hearts; however, the activities of PKCα, PKCε, and PKCζ can account for the total PKC activities in the cardiac cytosolic and membrane fractions. Therefore, even if PKCη and PKCθ isozymes were present, they would not contribute significantly to the cellular PKC

activities. It would therefore be reasonable to conclude that PKCα, PKCϵ, and PKCζ are the major cardiac PKC isozymes in hamster hearts. HCM hearts are associated with elevation of enzyme activities of specific isozymes in specific cell fractions. However, PKCα appears to be solely responsible for the aberrant interaction with PDE in HCM hearts and may lead to the deficiency in second messengers in HCM hearts.

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Send reprint requests to: Hon-Chi Lee, M.D., Ph.D., Cardiovascular Division, Department of Internal Medicine, University of Iowa Hospitals and Clinics, Iowa City, IA 52242.