Protein Kinase C Isoform Expression and Regulation in the Developing Rat Heart

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Abstract To determine whether age-dependent differences in cardiac responses to autonomic agonists could result from developmental changes in protein kinase C (PKC) isoform expression, we probed extracts from the fetal, neonatal, and adult heart as well as cultured neonatal and isolated adult ventricular myocytes with specific antisera to calcium-dependent (α and β) and calcium-independent (δ, ε, and ζ) isoforms of the enzyme. Although PKC-β immunoreactivity could not be detected in cultured neonatal or isolated adult ventricular myocytes, adult and neonatal myocytes expressed multiple other isoforms of PKC. Our studies revealed an age-dependent decline in the immunoreactivity for three PKC isoforms. PKC-α was detected in extracts from the fetal and 2-day-old neonatal heart as well as cultured neonatal rat ventricular myocytes. Only faint PKC-α immunoreactivity was detected in extracts from the adult heart, and PKC-α was not detected in extracts from isolated adult ventricular myocytes, suggesting that PKC-α resides in nonmyocyte elements in the adult heart. PKC-δ also was detected in greater abundance in fetal and neonatal than in adult myocardial extracts. The decline in PKC-α and PKC-δ expression occurred during the first 2 postnatal weeks. PKC-ζ was detected in greatest abundance in extracts from the fetal heart. PKC-ζ expression declined markedly by the second postnatal day, and only faint PKC-ζ immunoreactivity was detected in extracts from adult myocardium. Failure to detect PKC-ζ in extracts from isolated adult ventricular myocytes suggests that PKC-ζ resides primarily in nonmyocyte elements in the adult heart. PKC-ε was detected in all preparations, but it was detected in greatest abundance in extracts from neonatal hearts. In vitro sympathetic innervation of previously noninnervated neonatal ventricular myocytes or in vivo chemical sympathectomy of the neonatal heart did not modulate PKC isoform expression, suggesting that sympathetic innervation does not significantly regulate PKC isoform expression. PKC-α partitioned to the soluble fraction of unstimulated myocytes and was selectively translocated to the particulate fraction by Ca²⁺. In contrast, a major portion of the novel PKC isoforms partitioned to the particulate fraction of unstimulated myocytes. The subcellular distribution of novel PKC isoforms was not influenced by Ca²⁺, 12-O-tetradecanoylphorbol 13-acetate (TPA, 300 nmol/L) induced translocation of soluble PKC-α, PKC-δ, and PKC-ε to the particulate fraction at 30 minutes and complete (PKC-α and PKC-δ) or 80% (PKC-ε) downregulation at 24 hours. PKC-ζ was not affected by TPA. We conclude that multiple PKC isoforms, which differ in their subcellular distribution and regulation by Ca²⁺ and phorbol esters, are expressed in the heart in an age-dependent fashion. The observation that the developmental decline in PKC-ζ precedes the fall in PKC-α and PKC-δ suggests that PKC isoform expression is controlled by distinct mechanisms that are regulated differently during development. (Circ Res. 1994;74:399-309.)

Key Words: protein kinase C • development • cardiac myocytes • phorbol esters

The cardiac response to a variety of autonomic agonists is dependent on the developmental stage of the heart. It is now well established that at least some of these differences result from age-dependent changes in components of the membrane receptor complex and/or intracellular signaling cascade.¹ Previous studies in our laboratory have focused on developmental changes in the cardiac response to α₁-adrenergic receptor agonists.² ³ Activation of this receptor stimulates the phospholipase C-dependent hydrolysis of membrane phosphoinositides, resulting in the generation of at least two intracellular second-messenger molecules: inositol triphosphate (IP₃), which mobilizes intracellular calcium, and diacylglycerol (DAG), the endogenous activator of protein kinase C (PKC). PKC is key in the regulation of signal transduction and cell growth and differentiation, as well as in tumor promotion in many cell types.⁴ In cardiac myocytes, it has been implicated as a candidate-signaling molecule in receptor-dependent induction of myocyte hypertrophy.⁵ ⁶ Although phorbol esters also have been reported to modulate contractile function, the results are inconsistent.⁷ ¹⁰ As a result, the precise role of PKC in mediating receptor-dependent regulation of myocardial cell contractility remains controversial.

PKC is a family of closely related serine-threonine protein kinases that can be classified into two major categories. The conventional PKC isoforms (α, βI, βII, and γ) are characterized enzymatically by their requirement for calcium, phospholipids, and DAG or phorbol ester for activation. This reflects the presence of four conserved domains. The C1 region is the putative phospholipid– and DAG/phorbol ester–binding domain, the C2 region is the putative calcium-binding domain, and the C3 and C4 regions constitute the kinase domain.¹ The novel PKC isoforms (δ, ε, ζ, δI, η, ι, ε, and ζ) are structurally related to the conventional PKC isoforms. However, they lack the C2 domain and do not require calcium for maximal activation.¹¹ ¹⁶ PKC-ζ also differs from other members of the PKC gene family in that it contains only one cysteine-rich zinc finger-like motif in the C1 domain. As a result, neither calcium nor DAG/phorbol ester activates this PKC isoform; the
mechanism by which it is activated in intact cells remains unknown. Distinct PKC isoforms differ in their tissue distribution, cofactor regulation, and susceptibility to phorbol ester–induced proteolysis/down-regulation. These differences have led to speculations that distinct PKC isoforms subserve discrete biologic functions. This hypothesis is supported by some recent experimental data.

There is a paucity of information regarding PKC isoform expression in rat ventricular myocytes. Although several isoforms of PKC have been detected in adult rat heart, most studies have been performed on extracts from the intact heart, precluding a distinction between PKC isoform expression in cardiac myocytes and PKC isoform expression in coronary vasculature, nerves, and connective tissue elements in the heart. Information regarding PKC isoform expression in neonatal myocytes is even more meager. Cultured neonatal rat ventricular myocytes have been reported to contain multiple isoforms of PKC. In one study, two of the isoforms were characterized as PKC-α and PKC-β, whereas the precise identity of a third isoform was not established.

In view of the uncertainties regarding the identity of the PKC isoforms expressed and their actions in the heart, the goal of the present study was to examine PKC isoform expression and to systematically consider the possibility that PKC isoform expression is regulated during normal cardiac development.

Materials and Methods

Preparation of Cardiac Myocytes

Cardiac myocytes were dissociated from the ventricles of 2-day-old Wistar rats by a series of trypsin digestions and were cultured according to a protocol described previously. Briefly, the isolated cells were pooled, centrifuged at 200g for 5 minutes, and resuspended in MEM (GIBCO Laboratories, Grand Island, NY) with 10% fetal calf serum, 5x10^-6 mol/L hypoxanthine, and 12 mmol/L NaHCO3. The cells were preplated for 40 minutes at 37°C to decrease fibroblast contamination. The muscle cells were then resuspended at a density of 5x10^6 cells per milliliter in MEM supplemented with 10% fetal calf serum and plated at 4 mL per dish in 60-mm plates that had been precoated with fibronectin (25 ng/mm²) for 45 minutes. Although the preplating step markedly reduces initial fibroblast contamination, in all studies an irradiation protocol was included after 24 hours of culture to effectively further eliminate contamination with any proliferating cells such as cardiac fibroblasts. In preliminary studies, we compared PKC isoform expression in neonatal ventricular myocyte cultures and myocytes freshly isolated from the neonatal ventricle. Except for the appearance of a lower molecular weight PKC-ε immunoreactive species during culture (see below), in all other respects the culture conditions did not introduce other differences in PKC isoform expression (data not shown). Where indicated, cells were cocultured with neurons dissociated from the paravertebral sympathetic chains or treated with 300 nmol/L TPA for the final 30 minutes or 24 hours of the culture interval. Fibroblasts were obtained during the differential plating protocol and were cultured for 4 days in MEM with 10% fetal calf serum according to methods published previously. Myocytes were enzymatically disaggregated from the adult rat ventricle as described previously. Hearts were isolated from 14-day-old fetuses (n=24), 2- to 15-day-old neonatal rats (n=5 to 12 per age group), and adult rats (n=6). These were immediately frozen in liquid nitrogen and stored at -80°C until use.

Protein Extraction

To prepare total protein extracts from 6- or 15-day-old neonatal ventricular myocyte cultures and isolated adult myocytes, cells were washed with phosphate-buffered saline, immediately lysed in preheated (95°C) homogenization buffer (20 mmol/L Tris-HCl, pH 7.5, 2 mmol/L EDTA, 2 mmol/L EGTA, 6 mmol/L β-mercaptoethanol, 50 μg/mL aprotinin, 48 μg/mL leupeptin, 5 μmol/L pepstatin A, 1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L sodium vanadate, and 50 mmol/L NaF) containing 1% sodium dodecyl sulfate (SDS), and homogenized by sonication. Soluble and particulate protein fractions also were obtained from irradiated neonatal cardiac cultures and isolated adult myocytes. For these studies, cells were transferred to ice-cold homogenization buffer (0.8 mL of homogenization buffer per 4x10^6 cultured neonatal rat ventricular myocytes or 2.2x10^6 isolated adult ventricular myocytes), lysed by sonication, and centrifuged at 100 000g for 1 hour. The supernatant was removed (soluble fraction), and the pellet was resuspended in 1 mL preheated homogenization buffer containing 1% SDS (particulate fraction). In experiments designed to determine whether individual PKC isoforms display calcium-dependent translocation to the particulate fraction, cells were sonicated in homogenization buffer containing 3 mmol/L CaCl2 instead of EGTA. Soluble and particulate fractions were then prepared as described above, except that EGTA was added back to the soluble fraction to achieve a final concentration of 3 mmol/L. For studies on intact cardiac tissues, preheated homogenization buffer containing 1% SDS was added to minced myocardium (12 mL/g tissue). After homogenization with a Polytron, total protein extract was prepared as described above. Protein content in the samples was determined according to a modified Lowry assay.

Immunoblot Analysis

Samples were electrophoresed on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose. Prestained molecular weight markers were electrophoresed in parallel. After an incubation in 5% dry milk, 50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, and 0.1% Triton X-100 (blocking buffer I) for 1 hour at room temperature to block nonspecific binding, the nitrocellulose was probed with a 1:500 dilution of primary PKC isoform-specific antisera in 5% bovine serum albumin, 50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, 0.1% Triton X-100, and 0.02% NaN3 overnight at 4°C. Five PKC isoform–specific antisera were used. The antisera were generated against synthetic peptides corresponding to amino acids 313 to 326 for PKC-α, amino acids 313 to 329 for PKC-β, or unique sequences in the carboxy-terminal variable region of PKC-δ, PKC-ε, and PKC-ζ. The anti-PKC-α antisemur was affinity-purified. The anti-PKC-β antibody recognizes both the β1 and βII isoforms of the enzyme. The nitrocellulose was then washed five times, each with 50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, and 2% Nonidet P-40 and incubated in the same buffer containing 5% dry milk (blocking buffer II) for 30 minutes at room temperature. To detect bound primary antibody, blots were incubated for 1 hour at room temperature with 50 μCi/mL goat anti-rabbit IgG F(ab')2 fragment at a final dilution of 0.25 μCi/mL in blocking buffer II. The nitrocellulose was washed seven times as described above, dried, and autoradiographed with Kodak XAR film with intensifying screens at -70°C. For autoradiograms in which the densities of the bands linearly increased with loading of increased amounts of protein, the relative abundance of individual proteins identified was quantified by scanning densitometry.

To ensure the specificity of the immunoreactive proteins, Western blots were done in the presence and absence of competing immunizing peptide. Isoform-specific antisera (10 μL) were preincubated without or with 5 μg of the respective peptide antigen used for immunization in 50 μL of 20 mmol/L Tris-HCl, pH 7.5, 2 mmol/L EDTA, 2 mmol/L EGTA, 6 mmol/L β-mercaptoethanol, 50 μg/mL aprotinin, 48 μg/mL leupeptin, 5 μmol/L pepstatin A, 1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L sodium vanadate, and 50 mmol/L NaF containing 1% sodium dodecyl sulfate (SDS).
Tris-HCl buffer (pH 7.4) containing 1 mmol/L EDTA, 5 mmol/L EGTA, 20 μg/mL aprotinin, 200 μmol/L leupeptin, 0.5% Triton X-100, and 0.1% SDS for 1 hour and then diluted to working concentration as described above.

Materials

Polyclonal antibodies against PKC-α, PKC-β, PKC-δ, and PKC-ζ were purchased from Gibco BRL. Polyclonal anti–PKC-ε was the generous gift of Dr Doriano Fabbro, CIBA-GEIGY, Basel, Switzerland.13 Lysates of rat 6 embryo fibroblasts, which stably overproduce the βI isoform of PKC, were provided by Dr Bernard Weinstein.12 125I-labeled goat anti-rabbit IgG F(ab')₂ fragment was purchased from Du Pont NEN, Boston, Mass. TPA was purchased from LC Services, Woburn, Mass. All other chemicals were reagent grade.

Results

PKC Isoform Expression in Neonatal and Adult Ventricular Myocardium

We used polyclonal antibodies generated against synthetic peptides derived from unique sequences of individual PKC isoforms to identify the isoforms of PKC expressed in the heart. The results of Western blot analyses performed on total protein extracts from intact neonatal and adult myocardium, cultured neonatal ventricular myocytes, and acutely isolated adult ventricular myocytes are presented in Fig 1, top.

Specific PKC-α immunoreactivity was detected in total protein extracts from the neonatal and the adult heart. However, the amount of PKC-α immunoreactivity in the neonatal preparation was much greater than the amount in the adult preparation. Specific PKC-α immunoreactivity also was detected in total protein extracts from cultured neonatal ventricular myocytes; it was not detected in the isolated adult ventricular myocyte preparation. These results suggest that neonatal myocytes express PKC-α; the small amount of PKC-α immunoreactivity detected in total protein extracts from adult myocardium is likely to represent PKC-α expressed by noncardiac muscle cells in the heart. Indeed, PKC-α immunoreactivity was detected in lysates of cardiac fibroblasts (Fig 1, bottom).

One could argue that there might be limitations to an approach that compares PKC isoform expression in an equivalent amount of extract from neonatal and adult cardiomyocytes. Although PKC is conventionally expressed per microgram protein in studies that examine developmental changes in PKC isoform expression33,34 or survey PKC isoform expression in different tissues,23 myocytes from the neonatal and adult heart differ in size and protein composition. These differences could confound the interpretation of the data. Therefore, we also performed additional experiments to probe for PKC-α under conditions in which extracts from an equivalent number of cultured neonatal and acutely isolated adult ventricular myocytes were loaded onto the gel. We easily detected PKC-α immunoreactivity in extracts from 60 000 neonatal myocytes but could not detect any PKC-α immunoreactivity in the approximately sixfold greater amount of protein derived from the identical number of acutely isolated adult ventricular myocytes (35 and 220 μg, respectively; results not shown). These results further support our conclusion that PKC-α is expressed by neonatal rat ventricular myocytes, whereas PKC-α resides primarily in the nonmyocyte elements of the adult ventricle.

Neonatal and adult rat myocardial preparations also were probed with antiserum raised against PKC-β. This antiserum detected immunoreactivity in total protein extracts from the neonatal and adult heart but failed to detect specific proteins in either of the pure cardiac cellular preparations tested. This antiserum also specifically recognized high levels of a 78-kD protein in lysates of rat 6 embryo fibroblasts, which stably overproduce the βI form of PKC (Fig 1, top, far right panel).32 It should be noted that PKC-β has been detected in extracts from cultured neonatal ventricular myocytes when PKC is partially purified by DEAE cellulose chromatography.26,27 Thus, it is possible that rat ventricular myocytes express low levels of PKC-β that are beneath the limits of detection in this assay. Nevertheless, our results argue that PKC-α is the major calcium-dependent PKC species expressed in neonatal rat ventricular myocardial tissue.

Three novel PKC isoforms were detected in rat myocardium. These novel PKC isoforms were detected both in ventricular myocytes and fibroblasts cultured from the neonatal heart (Fig 1). Antiserum to PKC-δ detected a single 73-kD species in total protein extracts from the neonatal and adult heart as well as lysates from cultured neonatal and isolated adult cardiomyocytes. However, we noted a striking difference in the abundance of PKC-δ in these preparations. PKC-δ is a major PKC isoform in neonatal myocardium or cultured neonatal ventricular myocytes. In contrast, considerably weaker immunoreactivity was detected in total protein extracts from adult myocardium or isolated adult cardiomyocytes (note that to optimally detect specific immunoreactivity, immunoblots for neonatal and adult tissues were exposed for 23 and 48 hours, respectively).

Immunoblots with antiserum to PKC-ε revealed a prominent protein band, which migrated with an apparent molecular mass of ~96 kD on SDS–polyacrylamide gel electrophoresis (PAGE), in all neonatal and adult preparations studied. PKC-ε was resolved as a protein doublet in some, but not all, experiments (see Fig 5, top, for better resolution of the doublet). The mobility of this protein is similar to the reported molecular mass of PKC-ε chromatographically purified from brain.13,35 In addition to this major higher molecular weight species, a minor 76-kD protein that cross-reacted with the PKC-ε–specific antibody was consistently detected in cultured neonatal ventricular myocytes but not in isolated adult cardiomyocytes or in preparations from the intact ventricle. It also was not detected in myocytes freshly isolated from the neonatal ventricle (data not shown). This 76-kD protein appears to represent a PKC-ε–like polypeptide, since it was not detected when antiserum was preincubated with peptide antigen. It is not likely to be an artifact due to proteolysis of PKC-ε during sample preparation, since it was detected when cultured neonatal myocytes were directly lysed in hot SDS-containing buffer.

Finally, PKC-ζ was detected as a protein doublet with an apparent molecular mass of ~78 kD. Although the predicted molecular mass of PKC-ζ is 67 kD,36 higher molecular weight PKC-ζ species have been detected in several other studies.17,18,22 PKC-ζ was easily detected in total protein extracts from the neonatal heart and cultured neonatal cardiomyocyte. Faint PKC-ζ immunoreactivity also was observed in total protein extracts from the adult heart but not from isolated adult ventricular cardiomyocytes. The observation that cardiac


**Fig 1.** Protein kinase C (PKC) expression in cardiac myocytes and fibroblasts. Top, Total protein extracts (150 μg) from intact day 2 neonatal and adult myocardium (ventricle) and cultured neonatal and acutely isolated adult ventricular myocytes (myocytes) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblot analysis was performed with PKC-specific antibodies that had not (−) and had (+) been preadsorbed with 5 μg of the corresponding antigen peptide. Rat 6 embryo fibroblasts, which stably overexpress PKC-β also were probed for PKC-β in the same experiment. The autoradiograph demonstrating specific PKC-β immunoreactivity in rat 6 embryo fibroblasts is depicted at the far right of the figure for the sake of presentation. Data are from a single experiment. Three separate sets of myocardial preparations yielded similar results. Autoradiography was performed at −80°C for 21 hours (PKC-α), 23 hours (neonatal PKC-δ and PKC-ζ), 48 hours (adult PKC-δ), 96 hours (PKC-β), or 137 hours (PKC-α) with intensifying screens. Positions of the molecular weight standards (in kilodaltons) are indicated on the right. Bottom, Total protein extracts (79 μg) from cultured neonatal fibroblasts were subjected to SDS-PAGE and immunoblot analysis with PKC isoform-specific antibodies. In each case, the immunoreactivity was specific, because it was blocked by competing peptide (data not shown). Data are from a single experiment and are representative of results in two separate preparations. Autoradiography was performed at −80°C for 20 hours (PKC-α) or 29 hours (PKC-δ, PKC-ζ, and PKC-ζ) with intensifying screens.

fibroblasts express PKC-ζ (Fig 1, bottom) suggests that PKC-ζ immunoreactivity detected in extracts of the intact adult heart might be attributable to PKC-ζ present in noncardiac cellular elements.

These results indicate that the PKC isoform profile of neonatal and adult myocytes differs dramatically. Neonatal, but not adult, myocytes exclusively express calcium-dependent PKC-α and calcium-independent PKC-ζ.
Neonatal myocytes also are enriched in the δ isoform of PKC, relative to adult myocytes. In contrast, PKC-ε is expressed by both neonatal and adult cardiomyocytes, although a lower molecular weight PKC-ε immunoreactive species is uniquely expressed by myocytes cultured from the neonatal ventricle.

Subcellular Distribution of PKC Isoforms in the Heart

PKC immunoreactivity in soluble and particulate fractions from cultured neonatal rat ventricular myocytes and acutely isolated adult ventricular myocytes is presented in Fig 2. Although most studies designed to examine the subcellular localization of PKC probe an equivalent amount of protein derived from the soluble and particulate fraction, we were impressed that considerably less than 50% of total cell protein partitions to the soluble fraction of myocytes cultured from the neonatal ventricle or isolated from the adult ventricle. Rather, in preliminary studies, we found that 26±2% of the total cellular protein from cultured neonatal rat ventricular myocytes partitions to the soluble fraction. When isolated adult ventricular myocytes are processed in an identical fashion, 11±1% of the total cellular protein partitions to the soluble fraction (n=3 for each). We reasoned that protein loading of the gel should reflect these differences. Thus, Fig 2 presents PKC immunoreactivity that partitions to the soluble and particulate fractions from an aliquot of neonatal or adult ventricular myocytes (the details of the experimental approach are described in the legend to Fig 2).

Consistent with previous studies in other cell types, we found that PKC-α was detected in the soluble fraction when cells were lysed in buffers containing EGTA. In contrast, all three novel PKC isoforms partitioned to the particulate fraction in the absence of stimulation by agonists. This was most striking for PKC-δ, which was found almost exclusively in the particulate fraction of neonatal and adult ventricular myocytes (~80% and 90%, respectively); only a minor fraction of this isoform was detected in the soluble fraction. PKC-ε also resided primarily in the particulate fraction. Nevertheless, ~35% of this isoform was detected in the soluble fraction of both cultured neonatal rat ventricular myocytes and acutely isolated adult ventricular myocytes. Finally, PKC-ζ partitioned to both the soluble and particulate fractions of cultured neonatal rat ventricular myocytes. It is noteworthy that the higher molecular weight PKC-ζ species partitioned exclusively to the particulate fraction; the lower molecular weight PKC-ζ species was relatively evenly distributed between the soluble and particulate fractions.

Subcellular Redistribution of PKC Isoforms in Response to Calcium and TPA

PKC translocation from the soluble to the particulate compartment has been used as an indicator of its enzymatic and physiological activation. We examined the effect of lysis in excess calcium or acute exposure to the phorbol ester TPA (300 nmol/L) on the subcellular distribution of distinct PKC isoforms to determine whether conditions for selective activation could be established. We also stimulated neonatal myocyte cultures with TPA for prolonged intervals to determine whether PKC isoforms differ in their susceptibility to phorbol ester–induced downregulation.

Fig 3 illustrates the results of experiments on neonatal ventricular myocyte cultures. PKC-α resided in the soluble fraction when cell lysis was performed in EGTA but completely shifted to the particulate fraction when cell lysis was performed in the presence of excess calcium. In contrast, the subcellular localization of all three novel PKC isoforms was not influenced by calcium. TPA profoundly influenced α, δ, and ε isoforms of PKC. Exposure of cells to 300 nmol/L TPA for 30 minutes resulted in complete translocation of PKC-α and the small amount of PKC-δ that resides in the soluble fraction to the particulate fraction. A prolonged TPA treatment was associated with the complete loss of PKC-α and PKC-δ immunoreactivity from both cellular compartments. Treatment of 300 nmol/L TPA for 30 minutes also resulted in the extensive translocation of the 96-kD immunoreactive PKC-ε species. With more prolonged TPA treatment, particulate PKC-ε immunoreactivity decreased to ~20% to 30% of control levels. However, it is significant that some PKC-ε immunoreactivity persisted in the particulate fraction of cells exposed to 300 nmol/L TPA for 24 hours. Finally, the subcellular distribution of PKC-ζ was not affected by either calcium or TPA.
Fig 3. Subcellular redistribution of protein kinase C (PKC) isoforms in neonatal rat ventricular myocytes in response to calcium and 12-O-tetradecanoylphorbol 13-acetate (TPA). Soluble and particulate fractions were prepared from untreated neonatal rat ventricular myocytes lysed in homogenization buffer depleted of calcium (EGTA) or in the presence of excess calcium (Ca\(^{2+}\)) or from cultures pretreated with 300 nmol/L TPA for 30 minutes or 24 hours and lysed in the presence of EGTA (TPA). The soluble fraction (72 µg per lane) or the particulate fraction (150 µg per lane) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with the indicated antisera as described in "Materials and Methods." Note that the amount of soluble protein per lane exceeds that used in Fig 2 in order to improve detection of PKC isoforms that partition preferentially to the particulate compartment. Arrows identify specific immunoreactive bands. Positions of the molecular mass standards (in kilodaltons) are indicated on the right.

Fig 3 also emphasizes the differences in the subcellular distribution and TPA responsiveness of the 96-kD and 76-kD immunoreactive PKC-ε proteins. The 96-kD protein partitioned primarily to the particulate compartment, whereas the 76-kD protein was exclusively detected in the soluble fraction. The soluble component of the 96-kD PKC-ε immunoreactivity protein was translocated and downregulated by TPA. In contrast, the 76-kD protein did not undergo translocation or downregulation in response to TPA. The significance of this 76-kD PKC-ε immunoreactive protein expressed exclusively in cultured neonatal myocytes is uncertain. The antisera used in these studies was raised against the carboxyl terminus of PKC-ε and would be expected to recognize carboxy-terminal fragments generated by proteolytic degradation. Therefore, it is possible that this 76-kD protein is derived from the 96-kD PKC-ε protein by in vivo proteolysis. Alternatively, the 76-kD protein may represent the product of a distinct gene or an alternatively spliced mRNA of the PKC-ε gene. Indeed, a truncated version of PKC-ε, which differs only in the amino terminus and therefore would be recognized by the carboxy-terminal–specific PKC-ε antibodies used in the present study, has been isolated from rat brain.\(^{11}\) It is significant that this truncated PKC-ε species contains only one cysteine-rich sequence in the C1 domain and therefore might not effectively translocate to the particulate fraction in response to TPA.

Finally, the effect of calcium and TPA on the subcellular distribution of the two isoforms of PKC expressed in isolated adult cardiomyocytes was determined (Fig 4). The subcellular distribution of PKC-δ and PKC-ε was similar when cell lysis was performed either in EGTA-containing buffers or in the presence of excess calcium. In contrast, both isoforms were recruited to the particulate fraction after exposure to 300 nmol/L TPA for 30 minutes.

### Developmental Changes in PKC Isoform Expression

The contractile function of the heart changes substantially during postnatal cardiac development in association with changes in the expression of contractile proteins as well as components of receptor signaling complexes. We probed whole-protein extracts from 14-day-old fetal, 2- to 15-day-old neonatal, and adult hearts with antibodies directed against the α, δ, ε, and ζ isoforms of PKC to determine whether the time course for the developmental changes in PKC isoform expression in rat myocardium coincides with these other events. A representative autoradiogram is illustrated in Fig 5, top, and data for 14-day-old fetal, 2-day-old neonatal, and adult preparations are quantified in Fig 5, bottom.

Specific PKC-α immunoreactivity in the 14-day-old fetal and 2-day-old neonatal heart is similar and more than three times greater than the level detected in the adult heart. Similarly, PKC-ε expression is equivalent in extracts from 14-day-old fetal and 2-day-old neonatal myocardium. PKC-δ immunoreactivity in the adult heart is modest by comparison (25% of that measured in
the 2-day-old neonatal heart). For both the α and δ isoforms of PKC, the decline in immunodetectable protein occurs during the first 2 weeks of postnatal life. As previously established in Fig 1, PKC-δ is expressed at low levels in adult ventricular myocytes, whereas the faint PKC-α immunoreactivity detected in whole-protein extracts from the adult heart is presumed to represent PKC-α in noncardiac cellular elements.

The time course for the developmental decline in PKC-ζ expression is quite different. PKC-ζ is most abundant in 14-day-old fetal myocardium. PKC-ζ expression in 2-day-old neonatal myocardial tissue is only 32% of the level detected in the fetal sample. In extracts from the adult ventricle, PKC-ζ expression is reduced to a level that is only 9% of that detected in the fetal sample. This low level of PKC-ζ expression in extracts from the intact adult ventricle is likely to be attributable to PKC-ζ in contaminating noncardiac cellular elements (see Fig 1). The observation that PKC-ζ is expressed at high levels in fetal myocardium and diminishes rapidly around the time of birth is of considerable interest. First, the decrease in PKC-ζ precedes the decrease in PKC-α and PKC-δ, suggesting that the expression of these proteins is controlled by distinct mechanisms that are regulated differently during development. Second, it is particularly intriguing that immature cardiac tissue that retains the capacity for proliferative growth is enriched in PKC-ζ. It remains to be determined whether the presence of this unique phorbol ester/DAG-insensitive member of the PKC family in the growing fetal heart is indicative of a specific role for PKC-ζ in the signal transduction pathway leading to myocyte proliferation or is simply happenstance.

Finally, we noted modest age-dependent differences in PKC-ε expression. Compared with the level of expression in extracts from the 2-day-old neonatal ventricle, immunoreactive PKC-ε was reduced by 30% in extracts from the 14-day-old fetal ventricle and 46% in extracts from the adult ventricle. In addition, we noted an age-dependent difference in PKC-ε mobility in SDS-PAGE. PKC-ε appeared as a doublet in all postnatal samples studied but migrated as the single higher molecular weight immunoreactive species in total protein extracts from the fetal heart. This higher molecular weight PKC-ε species in the fetal heart may represent a fully phosphorylated form of the enzyme, which has been reported to be more resistant to degradation and exhibit higher kinase activity than lower molecular weight PKC-ε species.10

These studies do not provide precise quantification of the amounts of distinct PKC isoforms expressed by myocardial tissue. Such information can only be obtained by quantitative immunoblot analysis to correct for any potential differences in titer and/or hybridization efficiency between individual antisera. However, Fig 5 emphasizes that, under identical experimental conditions, PKC-ε appears to be expressed at a lower level in neonatal myocardium than in fetal myocardium, whereas PKC-δ and PKC-ζ are expressed at similar levels in fetal and neonatal myocardium. These results suggest that PKC-ε may play a different role in the developing heart than PKC-δ and PKC-ζ, which are expressed at similar levels in fetal and neonatal myocardium.
conditions (dilutions of antisera, exposure time for autoradiographs), immunoreactivity for PKC-ε vastly exceeds that detected for other PKC isoforms in the adult ventricle. These results agree with recent evidence from another laboratory that PKC-ε is the most abundantly expressed PKC isoform in the adult rat heart.24

**Developmental Changes in PKC Isoform Expression Are Not Dependent on Sympathetic Innervation of the Heart**

Several laboratories have recently presented compelling evidence that sympathetic innervation of the heart plays an important role in the developmental maturation of certain aspects of myocardial cell function. For example, studies in our laboratory established that developmental changes in α1-adrenergic receptor responsiveness are induced by sympathetic innervation.23,27 In vitro sympathetic innervation of previously noninnervated neonatal rat ventricular myocytes also has been shown to increase the expression of functional L-type calcium channels,38 alter the gating of the voltage-gated sodium channel,39 and modulate contractile function.40 Since the age-dependent decline in PKC-α and PKC-δ expression coincides with the onset of sympathetic innervation of the heart, we tested the hypothesis that sympathetic innervation regulates PKC isoform expression. Two experimental paradigms were used. First, we assessed PKC isoform expression in neonatal ventricular myocytes cultured alone or cocultured with and innervated by sympathetic neurons.2 Fig 6 illustrates that in vitro sympathetic innervation of previously noninnervated neonatal ventricular myocytes does not appreciably alter PKC isoform expression, even if the cultured interval is prolonged to 15 days. These results further emphasize the finding that the repression of PKC-α and PKC-δ isoform expression observed in vivo by postnatal day 15 is lost when ventricular myocytes are maintained in culture for an identical time interval. Second, we altered sympathetic neural development in vivo by repeatedly injecting animals with 6-hydroxydopamine according to a treatment regimen that achieves a high degree of chemical sympathectomy.41 We detected comparable levels of each isoform of PKC in total protein extracts from myocardium of 11-day-old vehicle-injected and 11-day-old 6-hydroxydopamine–denervated animals (data not shown). These results further support the conclusion that sympathetic innervation is not the dominant influence regulating the developmental changes in PKC isoform expression in the heart.

**Discussion**

The goal of the present study was to examine the pattern of PKC isoform expression in the context of cardiac development. Our data indicate that fetal, neonatal, and adult rat ventricular myocytes express multiple isoforms of PKC. Moreover, we demonstrate that development is associated with dramatic changes in calcium-dependent and calcium-independent PKC isoforms expression. The present study begins to clarify some of the discrepancies that have arisen from a literature that characterizes PKC isoforms in the heart without regard for possible age-dependent or cell-specific expression. For example, PKC-α was detected in two studies performed on cultured neonatal ventricular myocytes, consistent with its presence in greater abundance in neonatal than in adult ventricular myocardial tissue. Although two recent studies also detect PKC-α in the adult heart,23,25 it is important to note that these studies were performed on extracts from the intact adult heart, where the interpretation of data will be confounded by the presence of nonmyocyte elements. Moreover, PKC-α immunoreactivity in whole tissue extracts could signal the presence of atrial myocardium, since we and others have detected PKC-α in the atria of the adult rat heart (Reference 24 and authors’ unpublished observation). In contrast, we and Bogoyevitch et al.24 do not detect PKC-α in preparations that are restricted to ventricular myocytes from the adult heart. Similarly, reports that PKC-β is detectable in extracts from the adult heart23,25 but not in preparations highly enriched with ventricular myocytes24 suggest that it too might be present in the nonventricular myocardial cell elements of the adult heart. In support of this conclusion, a recent study that used a reverse-transcriptase polymerase chain reaction method to amplify transcripts from rat ventricular myocytes failed to detect PKC-β.42 However, as noted in results, PKC-β has been detected in extracts from cultured neonatal rat ventricular myocytes when PKC is partially chromatographically purified.26,27 Thus, we cannot exclude the possibility that PKC-β is expressed at a low level, beneath the limits of detection in our assay, in neonatal and/or adult ventricular myocytes.

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**Fig 6.** Protein kinase C (PKC) isoform expression in neonatal rat ventricular myocytes cultured alone or cocultured with sympathetic neurons. Two experimental paradigms were used. First, we assessed PKC isoform expression in neonatal ventricular myocytes cultured alone or cocultured with and innervated by sympathetic neurons.2 Fig 6 illustrates that in vitro sympathetic innervation of previously noninnervated neonatal ventricular myocytes does not appreciably alter PKC isoform expression, even if the cultured interval is prolonged to 15 days. These results further emphasize the finding that the repression of PKC-α and PKC-δ isoform expression observed in vivo by postnatal day 15 is lost when ventricular myocytes are maintained in culture for an identical time interval. Second, we altered sympathetic neural development in vivo by repeatedly injecting animals with 6-hydroxydopamine according to a treatment regimen that achieves a high degree of chemical sympathectomy.41 We detected comparable levels of each isoform of PKC in total protein extracts from myocardium of 11-day-old vehicle-injected and 11-day-old 6-hydroxydopamine–denervated animals (data not shown). These results further support the conclusion that sympathetic innervation is not the dominant influence regulating the developmental changes in PKC isoform expression in the heart.
The present study and that of Bogoyevitch et al. conclude that PKC-ε is a major immunoreactive PKC isoform expressed by adult ventricular myocytes. Our conclusions are consistent with reports that PKC-ε mRNA can be detected in rat heart. However, two other laboratories failed to detect PKC-ε in cardiac myocytes using an immunoblotting technique with isoform-specific antibodies. Of note, one of these laboratories localized PKC-ε to myocytes in immunohistochemical studies, suggesting that technical factors may have contributed to their failure to detect PKC-ε immunoreactivity by Western blot analysis. For PKC-δ, protein or mRNA is consistently detected in studies of neonatal rat ventricular myocyte cultures (References 26 and 42 and the present study), whereas the results of studies on adult ventricular myocytes are more variable. In the setting of the relatively lower abundance of PKC-δ in the adult heart, it is possible that various technical factors, including differences in antisera (i.e., titer, specificity, and/or affinity) as well as susceptibility of PKC to proteolytic degradation, contribute to the inconsistent results. In this regard, results reported herein emphasize the need to consider cell and/or age-specific differences in studies of PKC isoform expression.

One could argue that the multiple isoforms of PKC expressed in cardiac myocytes are functionally redundant and, therefore, that the identified changes in PKC isoform expression are inconsequential physiologically. Indeed, in support of this hypothesis, PKC-α and PKC-β acting equivalently to stimulate translocation through atrial natriuretic factor and myosin light chain-2 promoters. Moreover, both isoforms have been shown to stimulate AP-1 and AP-2 acclerophenyl acetyltransferase fusion gene expression in cultured neonatal rat ventricular myocytes. However, evidence that the copresence of distinct isoforms of PKC in a given myocyte imparts specificity to the receptor signaling pathway also has been presented. Kariya et al. used a transfection paradigm to demonstrate that PKC-β preferentially stimulates β-myosin heavy chain gene expression in neonatal rat ventricular myocytes. Although PKC-α also was stimulatory, its effect was substantially more modest than that associated with PKC-β. Given our observation that the major calcium-dependent isoform of PKC in neonatal cardiac myocytes is PKC-α and not PKC-β, the physiological implications of these observed differences in PKC-dependent regulation of gene transcription are uncertain. Nevertheless, evidence that PKC actions in cardiac myocytes are isoform specific supports the notion that the age-dependent differences in PKC isoform expression identified in the present study are likely to be associated with important alterations in biologic responses.

The copresence of at least four different isoforms of PKC in the neonatal heart and two different isoforms of PKC in the adult heart might be expected to impart a high degree of specificity and flexibility to the signal-transduction mechanism. Although a recent study reports a similar translocation of PKC-ε from the soluble to the particulate fraction of adult rat ventricular myocytes by two agonists that activate phosphoinositide hydrolysis (epinephrine and endothelin-1), it is possible that different PKC isoforms could be activated selectively by different receptor agonists. For example, in neonatal myocytes, calcium, and by implication agonists that increase intracellular calcium ion concentration, selectively translocates PKC-α, but not PKC-δ, or PKC-ε, from the soluble to the particulate fraction. In contrast, the calcium-insensitive isoforms, which are detected in greater abundance in the neonatal heart and are the only isoforms of PKC detected in the adult heart, might be expected to assume greater importance under conditions in which DAG is formed in the absence of calcium. Such a mechanism could involve receptor-activated phosphatidylincholine hydrolysis, which is stimulated by many agonists that promote phosphoinositide hydrolysis and results in the selective and sustained accumulation of DAG without the calcium-mobilizing second messenger IP₃. Moreover, because phosphoinositides and phosphatidylincholine characteristically have different fatty acyl moieties, phosphatidylincholine hydrolysis would be predicted to lead to the production of a distinct pool of DAG, which might differentially activate different isoforms of PKC. At present, the evidence that agonists stimulate the hydrolysis of phosphatidylincholine in cardiac tissue is indirect, and the relative importance of phosphatidylincholine as a source for DAG in the heart is uncertain. However, at least theoretically, such a mechanism might be particularly advantageous in cardiac tissue, where elevations in intracellular calcium accompany normal contractile activity.

The multiple isoforms of PKC expressed in the heart are predicted to phosphorylate distinct sets of intracellular target proteins, thereby conferring an additional dimension of complexity to this signal-transduction pathway. PKC isoforms are known to differ intrinsically in their substrate specificity. In addition, consistent with the known effects of activated PKC to phosphorylate proteins throughout the cell, recent studies indicate that distinct PKC isoforms translocate to different intracellular sites on exposure to phorbol esters. These sites include the plasma membrane as well as the cytoskeleton and the nuclear envelope. PKC isoform-specific translocation to distinct intracellular structures could lead to differences in substrate accessibility and thereby lead to PKC isoform-specific actions. Evidence that such a phenomenon is operative in cultured neonatal rat ventricular myocytes has been presented. An isoform putatively identified as PKC-β has been reported to translocate to the sarcolemma and perinuclear area on exposure to phorbol esters, whereas a different, and as yet unidentified, PKC isoform translocates to the myofibrils under the same experimental conditions. More recently, Mochly-Rosen and colleagues identified receptors for activated C kinase (RACKs) in the cytoskeletal-rich fraction of neonatal rat hearts and presented evidence that a protein-protein interaction between PKC and RACKs is required for PKC translocation and PKC-mediated cell activation. Taken together, these studies raise the possibility that, on activation, distinct isoforms of PKC translocate to the distinct intracellular sites of their protein substrates and that isoform-dependent differences in substrate specificity as well as isoform-dependent differences in intracellular compartmentalization might determine functional responsiveness. In this context, results reported herein suggest that the subcellular compartmentalization of PKC isoforms, as assessed by cell fractionation techniques, is similar in neonatal and adult ventricular myocytes. Accordingly, future studies to identify the physio-
logical substrates of individual PKC isoforms and determine whether the profile of target proteins phosphorylated by individual PKC isoforms differs between neonatal and adult cardiac myocytes will be essential.

PKC heterogeneity also provides a mechanism for differential rates of proteolytic inactivation. Our studies in neonatal rat ventricular cultures indicate that PKC-ε is more resistant to downregulation than are PKC-α and PKC-δ; cultures were not entirely depleted of PKC-ε immunoreactivity even after a prolonged treatment with a relatively high dose of TPA. Thus, it would be erroneous to assume that TPA-treated cultures can routinely be used to discriminate PKC-dependent from PKC-independent cellular events. However, specific TPA treatment protocols could be designed to produce neonatal ventricular myocytes enriched in the ε isoform of PKC. These cultures then might be used to identify PKC-ε isoform–specific function. This is of particular interest in view of the presumed in vivo difference in substrate specificity between PKC-ε and the calcium-dependent PKC isoforms.12,35,43

We have undertaken this detailed study of PKC isoform expression in the developing rat heart in an effort to elucidate further the mechanisms that underlie developmental changes in cardiac autonomic responsiveness. We previously demonstrated that α1-agonists stimulate phosphoinositide hydrolysis and enhance IP3 and DAG accumulation in myocardial tissue.3 This response is associated with important changes in myocellular contractile function.3,51,52 However, the precise cellular actions of IP3 and DAG in cardiac tissue remain uncertain. Most studies designed to identify the consequences of activation of the DAG/PKC limb of the signaling pathway have used phorbol esters. Phorbol esters have been reported to modulate calcium current,53 increase myofibrillar responsiveness to calcium,8 decrease calcium transport by the sarcoplasmic reticulum,54 and increase10 or decrease inotropy in the heart.7,9,55 However, phorbol esters are extremely potent and irreversible activators of PKC that may not precisely mimic the actions induced by receptor agonists. Moreover, the inability of phorbol esters to distinguish between different isoforms of PKC further compromises their ability to delineate the role of PKC in receptor responses. Results reported herein establish the presence of more than one species of PKC in the heart introduce further complexity to this analysis. In future studies, it will be necessary to consider the actions of both calcium-dependent PKC and novel PKC isoforms to determine the precise role of DAG activation of PKC in the cellular response to extracellular stimuli, such as hormones and neurotransmitters. Moreover, given that certain PKC isoforms are expressed in the heart in an age-dependent fashion and that these developmental changes in PKC isoform expression are presumed to be associated with important alterations in PKC biologic activity, it will be important to determine whether changes in PKC isoform expression underlie developmental differences in cardiac autonomic responsiveness. Evidence that activation of PKC fulfills different roles at different developmental stages would have broad implications regarding the role of PKC in cellular signaling.

The mechanism(s) responsible for the developmental changes in PKC isoforms expression is totally unknown at present. Studies reported herein indicate that the age-dependent decline in PKC isoform expression cannot be attributed to sympathetic innervation of the newborn heart. Therefore, other hormonal and physiologic changes that occur during the perinatal period must be considered. Another obvious potential physiologic regulator of gene expression in the heart is thyroid hormone. The surge in circulating thyroid hormone levels shortly after the time of birth is known to play a fundamental role in the regulation of cardiac β-myosin heavy chain expression and, thereby, influence cardiac function.55,56 Studies to determine whether changes in thyroid hormone levels also modulate PKC isoform expression currently are in progress.

Finally, the finding that PKC-ζ is most abundant in fetal myocardium is particularly intriguing. The mode of PKC-ζ activation within cells currently is not known, since PKC-ζ does not undergo translocation or downregulation after in vivo exposure to phorbol esters. The speculation that PKC-ζ may function in a distinct fashion in cell signaling has gained support recently from the finding that purified PKC-ζ is activated in vitro by phosphatidylinositol-3,4,5-trisphosphate (PIP3)19. PIP3, a putative signaling molecule formed on activation of phosphatidylinositol 3-kinase by certain oncogenes and growth factors, is believed to be involved in the control of cell proliferation.57 Proof that PKC-ζ is regulated by PIP3, in vivo would strongly support the hypothesis that PKC-ζ plays a role in a signaling cascade for growth factors. In this context, it will be important to consider the possibility that PKC-ζ contributes to the regulation of cardiac cell growth and division and is not merely a marker for a dividing myocyte. Evidence that cardiac cell growth can be regulated by PKC-ζ could provide a mechanism to manipulate myocardial cell growth and would be of great clinical significance.

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Protein kinase C isoform expression and regulation in the developing rat heart.
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