Protein kinase C isoform expression and activity in the mouse heart

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Schreiber, Kathy L., Louise Paquet, Bruce G. Allen, and Hansjörg Rindt. Protein kinase C isoform expression and activity in the mouse heart. Am J Physiol Heart Circ Physiol 281: H2062–H2071, 2001.—The expression of protein kinase C (PKC) isoforms in the developing murine ventricle was studied using Western blotting, assays of PKC activity, and immunoprecipitations. The abundance of two Ca2+-dependent isoforms, PKCα and PKCβII, as well as two Ca2+-independent isoforms, PKCδ and PKCε, decreased during postnatal development to <15% of the levels detected at embryonic day 18. The analysis of the subcellular distribution of the four isoforms showed that PKCδ and PKCε were associated preferentially with the particulate fraction in fetal ventricles, indicating a high intrinsic activation state of these isoforms at this developmental time point. The expression of PKCs in cardiomyocytes underwent a developmental change. Although preferentially expressed in neonatal cardiomyocytes, this isoform was downregulated in adult cardiomyocytes. In fast-performance liquid chromatography-purified ventricular extracts, the majority of PKC activity was Ca2+-independent in both fetal and adult ventricles. Immunoprecipitation assays indicated that PKCδ and PKCε were responsible for the majority of the Ca2+-independent activity. These studies indicate a prominent role for Ca2+-independent PKC isoforms in the mouse heart.

ventricle; postnatal development; immunoprecipitation

Protein Kinase C (PKC) is a multimember family of lipid-dependent serine-threonine protein kinases commonly divided into three subgroups based on molecular structures and activation requirements (reviewed in Ref. 23). The conventional PKCs (α, βI, βII, and γ) are activated by diacylglycerol in a Ca2+-dependent manner. The novel PKCs (ε, δ, η, and θ) also require diacylglycerol but not Ca2+ for activation. The activation of the atypical PKCs (ζ and ξ) is independent of Ca2+ and of diacylglycerol. PKCs are regulators of numerous cellular events, including neuronal activity and memory, exocytosis, cell proliferation and differentiation, and contractility (26, 27). This multitude of effects, together with broadly overlapping substrate specificities in vitro, has made the determination of individual PKC isoform function challenging. Pharmacological tools to activate or inhibit PKC are, with very few exceptions, not isoform specific. This impedes the assessment of the family member physiological function in vivo. The selective translocation of PKC isoforms to different subcellular compartments on activation suggests isoform-specific activation and function. PKC translocation is generally viewed as an indirect indication of activation status. The specificity of these translocations is at least in part mediated by the type of stimulus and the interaction with isotype-specific receptors (25).

In the heart, different subsets of PKCs have been detected by immunological methods depending on the species studied (5, 9, 10, 30). The analysis of isoform translocation and overall PKC activity under different stimulation conditions has indicated a role for specific isoforms under physiological and pathological situations. For example, activation of PKC mediates a protective effect from ischemia-reperfusion injury (21, 45). This was demonstrated in conscious rabbits where ischemic preconditioning led to the translocation of the specific isoforms PKCe and PKCη (30). In samples from failing human myocardium, selective upregulation of the Ca2+-dependent isoforms, PKCα, PKCβI, and PKCβII were described (6). Evidence for isoform-specific functions in the heart in vivo has also been gathered by using transgenic approaches. Forced expression of PKCβII in cardiomyocytes resulted in hypertrophy and decreased left ventricular performance suggesting that overactivation of this isoform can cause cardiac dysfunction (43). Similarly, overexpression of a constitutively active mutant of PKCe also led to cardiac hypertrophy, whereas left ventricular function was largely maintained suggesting that the different PKC isoforms have specific functions within the myocardium (40).

Despite a more detailed knowledge of PKC abundance in other species, little is known about the presence and activity of PKC isoforms intrinsic to the murine heart. Because the mouse is becoming an increasingly important model system to study cardiac PKC signaling, we have initiated an analysis of the abundance and activity of Ca2+-dependent and Ca2+-independent isoforms in the murine heart. In this report, we show for the first time isoform-specific expression patterns during postnatal development in...
mouse ventricles. Measurements of phospholipid-stimulatable PKC activity after partial purification by fast-performance liquid chromatography (FPLC) on MonoQ affinity columns demonstrated a prevalence of Ca\(^{2+}\)-independent activity. Employing immunoprecipitations (IPs), we demonstrate that PKCe and PKCδ are the major isotypes underlying the total measurable PKC activity intrinsic to the adult mouse ventricle.

**MATERIALS AND METHODS**

**Animals.** CD1 mice (Charles River Laboratories) of various ages were used throughout the study. Adult mice were 12-wk-old males. Fetal and neonatal mice were euthanized by 

**Reagents.** Chemical reagents were from J. T. Baker or Sigma-Aldrich (American Chemical Society or molecular biology grade). PKC isoform-specific polyclonal antibodies were purchased from Santa Cruz Biotechnology. Collagenase was obtained from Worthington. The PKC substrate, peptide ε (amino acid sequence ERMRPRKRQGSVRRRV) was obtained from the University of Calgary peptide synthesis core facility. Phosphatidylserine and 1,2-diolein were purchased from Serdary Research Laboratories. Membrane-grade Triton X-100 was purchased from Roche Molecular Biochemicals.

**Tissue fractionation.** Hearts were rapidly removed and washed free of blood with ice-cold PBS, and the atria were removed. Ventricles were then homogenized using a Polytron PT3000 (Brinkman) at 10,000 rpm for 3 × 10 s in ice-cold lysis buffer A composed of (in mmol/l) 20 K-HEPES (pH 7.4), 20 β-glycerophosphate, 20 NaF, 0.2 Na\(_3\)VO\(_4\), 5 EDTA, 5 EGTA, 1 benzamidine, 0.5 PMSF, 5 DTT, plus 10 \(\mu\)g/ml leupeptin. To obtain total, cytosolic, and particulate fractions the homogenate was divided in two equal portions and extracted. One portion was supplemented with an equal volume of buffer A and the other was supplemented with an equal volume of buffer A containing 2% (vol/vol) Triton X-100 (peroxide free). The detergent-supplemented homogenate was further extracted by 20 passes in a 2-ml Potter-Elvehjem hand homogenizer and incubated on ice for 20 min before centrifugation. Both homogenates were centrifuged at 100,000 g for 30 min. The supernatant of the detergent-supplemented homogenate was taken as the total fraction, whereas the supernatant of the detergent-free homogenate was taken as the cytosolic fraction. The resulting pellet from the latter was supplemented with an appropriate volume of buffer A containing 1% (vol/vol) Triton X-100 (peroxide free), homogenized by 20 passes in a 2-ml Potter-Elvehjem hand homogenizer, left on ice for 20 min, and then centrifuged at 100,000 g for 30 min. The resulting supernatant was taken as the solubilized particulate fraction.

**Partial purification of PKC by MonoQ FPLC.** Chromatographic purification of PKC was performed as described previously (44). Briefly, total ventricular extracts (4 mg of protein) were loaded onto a MonoQ HR5/5 column equilibrated with buffer A composed of (in mmol/l) 10 MOPS, 2 EDTA, 2 EGTA, 20 β-glycerophosphate, 1 benzamidine, 1 Na\(_3\)VO\(_4\) plus 5% glycerol, 0.03% Brij, 0.1% β-mercaptoethanol, and 10 \(\mu\)g/ml leupeptin (pH 7.4 at 4°C). The linearity of the column capacity was determined in preliminary experiments. For fetal extracts, ventricles from a whole litter (usually 10 to 12 animals) were pooled. After protein extract was applied, the column was washed with 5 ml of buffer A, and bound proteins were eluted with a biphasic NaCl gradient (0.0–0.4 M/25 ml; 0.4–1 M/17.5 ml in buffer A) at a flow rate of 0.1 to 0.4 ml/min. Forty fractions of 1 ml were collected.

**PKC activity assay.** PKC activity was assayed in column fractions by quantifying \(^{32}\)P incorporation into peptide ε (a substrate for both Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent PKC isotypes) essentially as described (1). Briefly, assays were performed in triplicate at 30°C in a total reaction volume of 16 \(\mu\)l containing (in mmol/l) 20 HEPEs (pH 7.5), 10 MgCl\(_2\), 10 dithiothreitol, 0.1 CaCl\(_2\) (Ca\(^{2+}\)-dependent activity) or 10 EGTA (Ca\(^{2+}\)-independent activity), 0.1 \(\mu\)g Triton X-100, and 10 \(\mu\)g/ml leupeptin with or without 0.3 mg/ml phosphatidylserine and 62 \(\mu\)g/ml 1,2-diolein. Reactions were terminated after 10 min by adding 8 \(\mu\)l of 3% (wt/vol) trichloroacetic acid. 20 \(\mu\)l were then spotted onto Whatman P81 phosphocellulose paper, washed three times for 5 min in 0.5% H\(_2\)PO\(_4\), and air dried. \(^{32}\)P incorporation was quantified by Cerenkov counting. Preliminary experiments with fetal and adult preparations were performed to determine the linearity of the kinase assay with respect to reaction time and sample input. The specific PKC inhibitor bisindolylmaleimide 1 (BIM), was added as indicated in the figures at a final concentration of 500 nmol/l.

**Myocyte isolation.** Neonatal myocytes were isolated from 1.5-day-old mice by collagenase digestion as previously described (3) with slight modifications. Hearts were collected in MEM-Joklik’s medium (Life Technologies) and rinsed free of excess blood. Under a magnifying glass, ventricles were dissected free of atria, cut into small pieces, and transferred to fresh medium. Care was taken to remove red blood cells as completely as possible. The tissue was minced and collected in 1 ml of fresh MEM-Joklik’s medium to which 1 ml of 2 mg/ml collagenase type 2 (Roche Molecular Biochemicals) in MEM-Joklik’s medium was added. A total of seven rounds of 5-min digestions were performed at 30°C on a rocking platform. After each digestion, the tissue was gently aspirated up and down three times through a 10-ml pipette and allowed to settle for 1 min. The supernatants of the first three digestions were discarded, because they contained primarily dead cells and red blood cells. The supernatants collected from digestions 4–7 were collected, adjusted to 20% FCS, and centrifuged at 900 rpm for 5 min. The pelleted cells were immediately resuspended in 1 ml DMEM containing 10% FCS. After the final digestion, a small magnetic stir bar was added to the remaining tissue in digestion medium and gently agitated for an additional 5 min at 30°C. The myocytes thus liberated were collected as described above. The cell suspensions were pooled, filtered through a nylon mesh (200-μm pore size), and incubated twice for 30 min in a tissue culture grade petri dish at 37°C and 5% CO\(_2\) to allow for the attachment of fibroblasts. Each myocyte preparation was examined microscopically to assess cell yield. Viability, determined by exclusion of trypan blue, was usually 60–80%.

Adult myocytes were prepared by perfusion of collagenase solution as previously described (11). Briefly, hearts were excised and rinsed in Ca\(^{2+}\)-containing Tyrode’s solution composed of (in mmol/l) 130 NaCl, 5.4 KCl, 1 MgCl\(_2\), 0.33 Na\(_2\)HPO\(_4\), 10 HEPEs (pH 7.5), 5 glucose, 0.1 CaCl\(_2\). The heart was cannulated via the aorta and perfused as follows: 5 min with Ca\(^{2+}\)-containing Tyrode’s solution, 10 min with nominally Ca\(^{2+}\)-free Tyrode’s solution; 20 min with collagenase-containing, Ca\(^{2+}\)-free Tyrode’s solution; and 5 min with Kraftbrühe (KB) buffer. The concentration of collagenase was 0.3 mg/ml. KB buffer was composed of (in mmol/l) 100 K-glutamate, 10 K-aspartate, 25 KCl, 10 KH\(_2\)PO\(_4\), 2 MgSO\(_4\), 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, 5 HEPEs (pH 7.2),...
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RESULTS

Differential regulation of PKC isoforms in the maturing mouse ventricles. Several PKC isoforms that have been implicated in pathophysiological processes in the heart were included in the present study. A member of the Ca$^{2+}$-dependent subgroup, PKCα was shown previously to be the most abundant isoform in the rabbit heart, and is translocated during ischemic preconditioning (22, 24). Another member of this subgroup, PKCβII, is implicated in cardiac hypertrophy in humans and transgenic models of cardiac-specific overexpression (6, 7, 43). Likewise, PKCe is the most abundant Ca$^{2+}$-independent isoform in rabbit heart and also plays a role in the induction of hypertrophy (30, 40). Both PKCe and PKCδ are implicated in the protection from ischemia-reperfusion injury in the rat (15). Expression of these selected PKC isoforms in murine ventricles between embryonic day 18 and 12 wk (adult) was determined by Western blotting. Identical amounts of protein from extracts prepared at the various time points were compared as shown in Fig. 1. Note that a direct comparison of the abundance between the four PKC isoforms cannot be made, because different amounts of protein were loaded on the gel for each isoform to optimize blotting conditions and because of the potentially different avidities of the primary antibodies. PKCδ was detected as two bands that likely reflect differential phosphorylation of the molecule (36). The abundance of all four isoforms was decreased in the adult to ~15% compared with the fetal ventricles. However, kinetics of this decrease differed between the isoforms. Although the abundance of PKCα, PKCβII, and PKCδ declined more rapidly in the early postnatal days, PKCe expression remained relatively high until at least postnatal day 9. This regulation suggests a specific functional role for PKCe in postnatal development and suggests that regulation of the expression of members of the PKC family during development is isoform-specific.

Intrinsic subcellular distribution of PKC. The translocation of PKC from the cytosol to the particulate fraction on stimulation is a hallmark of its activation. To test the intrinsic activation state of the four PKC isoforms, particulate and cytosolic fractions were prepared from fetal, postnatal day 4, and adult ventricles, and subcellular distribution was determined by Western blotting. As shown in Fig. 2, distribution of the two Ca$^{2+}$-dependent isoforms (PKCα and PKCβII) remained the same with increasing age, whereas the two Ca$^{2+}$-independent isoforms (PKCδ and PKCe) tended to be associated with the particulate fraction to a higher degree in fetal ventricles compared with the adult. This result suggests that the fetal environment has an intrinsically higher stimulatory effect for Ca$^{2+}$-independent PKC isoforms. In contrast to these studies using acutely isolated cells, analyses of cultured neonatal rat cardiomyocytes showed that the subcellular distribution of PKCα and PKCδ favored an almost exclusive cytosolic location that may represent an influence of culture conditions or point to species differences in PKC isoform location in the cardiomyocyte (9). The physiological significance for a preferential association in vivo of the Ca$^{2+}$-independent PKC isoforms with the particulate fraction, and hence, increased activation state, in fetal ventricles is at present unclear.
Fig. 1. Developmental profile of protein kinase C (PKC) isoform expression in murine ventricle. A: representative Western blot. Total protein extract was prepared as described in MATERIALS AND METHODS and separated by SDS-PAGE using a 10–15% polyacrylamide gradient. Equal amounts of protein were loaded for each developmental time point. Protein amounts were optimized for each PKC isoform to facilitate detection and quantitation. Postnatal days are indicated at the bottom. F, embryonic day 18; Ad, 10–12 wk. B: quantitation of PKC immunoreactivity. Signals from Western blots were visualized by enhanced chemiluminescence (ECL) and quantified using a Bio-Rad molecular imager. Value for embryonic day 18 was set to 100% for each isoform. Data are presented as means ± SE; n = 3 independent experiments.

Fig. 2. Association of PKC isoforms with the particulate fraction from fetal, neonatal, and adult ventricles. Cytosolic and particulate fractions were prepared from ventricles of fetal (embryonic day 18), postnatal day 4, and adult mice. After electrophoresis and Western blotting, signals were quantitated as described in MATERIALS AND METHODS. Data are expressed as ratio of particulate fraction divided by the sum of particulate plus cytosolic fractions. Data are means ± SE; n = 3 to 4 independent experiments. *P < 0.05 compared with fetal; †P < 0.05 compared with day 4 (Student's t-test).
Developmental changes of PKC expression in cardiomyocytes. Cardiomyocytes comprise ~80% of total cellular protein content of the heart while representing ~25% of the total cell number (42). Because noncardiomyocytes such as fibroblasts also express PKC, we determined the relative amount of immunologically detectable PKC in acutely isolated neonatal (postnatal day 1.5) and adult cardiomyocytes. Whole ventricles from the two developmental stages were also used. Comparison of PKC abundance in equal amounts of ventricular and cardiomyocyte extracts allows an estimate of the relative amount of PKC present in the cardiomyocyte compartment. Quantitation of the immunoreactive bands is shown in Fig. 3. For purposes of comparison, the signal obtained from whole ventricles for each isoform was set to one and used to express the relative signal strength obtained from isolated cardiomyocytes. In neonatal mice, the majority of the Ca\textsuperscript{2+}-dependent isoforms PKC\textalpha and PKC\textbeta II were in the cardiomyocytes (relative abundance close to one). In contrast, the Ca\textsuperscript{2+}-independent isoforms PKC\textdelta and PKC\textepsilon were expressed in noncardiomyocyte compartments as well (relative abundance <1). Developmental differences were observed in the expression of PKC\textalpha in myocytes versus nonmyocytes. Although almost exclusively expressed in cardiomyocytes in the neonate, only ~20% of total ventricular PKC\textalpha immunoreactivity was found in adult cardiomyocytes. This was in contrast to the partitioning of PKC\textbeta II, which remained predominantly expressed in the cardiomyocyte compartment in the adult. No significant developmental changes were observed for PKC\textdelta and PKC\textepsilon. These results suggest that PKC\textalpha expression is specifically downregulated in adult cardiomyocytes. Because the developmental regulation of PKC expression in cardiomyocytes differs from that of nonmyocytes, this downregulation indicates a more prominent role of PKC\textalpha in the neonatal cardiomyocytes. Evidence for the role of PKC\textalpha in cell proliferation comes from work with cultured epithelial cells lines where the activation of endogenous PKC\textalpha, or the inducible expression of PKC\textalpha, led to growth inhibition (37). Similar results were reported in bovine aortic endothelial cells (32) as well as intestinal epithelial cells (12). Whether PKC\textalpha has a similar function in the neonatal cardiomyocyte remains to be elucidated.

PKC activities in the murine ventricle. The detection by Western blotting provides important information on the presence and subcellular localization of PKC isoforms. However, some but not all PKC isoforms remain in a low activity conformation, or are inactive, unless phosphorylated themselves at “priming” sites (28). Therefore, immunoreactivity may not accurately reflect the pool of PKC responsive to activating stimuli. We sought to complement the analyses of immunoreactivity by determining the PKC activities intrinsic to the mouse heart using fetal and adult ventricles. To measure phospholipid-activatable PKC, total ventricular extracts were partially purified by chromatography on a MonoQ HR 5/5 column because lipid-dependent

**Fig. 3.** Expression of PKC isoforms in cardiomyocytes vs. whole ventricles. Total protein extracts from acutely isolated neonatal (postnatal day 1.5) and adult cardiomyocytes were subjected to SDS-PAGE and Western blotting to detect PKC isoforms. Neonatal and adult ventricles were analyzed as well. Bar graph shows ratio of PKC abundance in cardiomyocytes vs. ventricles at neonatal and adult stage, respectively. Signals for ventricles were set to one, and data are expressed as relative ratio of immunoreactivity in myocytes at neonatal and adult stages. Data are means ± SE; n = 3 to 4 independent experiments.

**Fig. 4.** PKC activities in fetal (A) and adult (B) mouse ventricle. Total protein extracts were partially purified by MonoQ fast-performance liquid chromatography and resulting chromatography fractions were assayed for PKC activity. Incorporation of \textsuperscript{32}P into peptide \textepsilon was measured in the presence of Ca\textsuperscript{2+} or EGTA. Nonspecific activity was determined in the absence of lipids. PKC-specific activity was determined by inclusion of bisindolylmaleimide 1 (BIM) in activity reactions. Representative elution profiles from fetal (A, embryonic day 18) and adult (B) ventricles is shown for those fractions containing substantial activity. Note different y-axis scale in the two panels.
kinase activity is inhibited or masked in crude myocyte extracts (44). PKC activities within selected fractions were determined in the presence or absence of Ca$^{2+}$ to assess the contribution of the Ca$^{2+}$-independent PKC pool. Representative elution profiles for fetal and adult ventricles are shown in Fig. 4. Both Ca$^{2+}$-dependent and Ca$^{2+}$-independent activities were detected with elution in an early peak around fractions 11 and 12 and in a late peak around fraction 15. In the fetal preparation, the bulk of the PKC activity was eluted in the early peak, whereas in the adult most of the activity was eluted in the late peak. None of the other fractions contained any appreciable activity. *Fraction 2* represents the column flow-through and was included as a reference point.

To quantify the activity measured, the area under the activity curves was determined. Preliminary experiments were performed to ensure that experiments were conducted within the linear range of this kinase assay. Total activity was determined using peptide e as substrate in the presence of Ca$^{2+}$ and represents the sum of Ca$^{2+}$-dependent and Ca$^{2+}$-independent activities. Fetal preparations contained ~4.3-fold more total kinase activity than the adult ventricle. In the fetal heart, 88.4 ± 4.8% of the activity was Ca$^{2+}$-independent. Similarly, in the adult heart, Ca$^{2+}$-independent activity comprised 80.5 ± 4.0% of the total activity (Fig. 5). Nonspecific (background) activity was determined in the absence of lipids in the reactions (Fig. 4, “no lipids”). To determine the relative amount of total, lipid-stimulatable activity attributable to PKC, parallel sets of reactions were performed in the presence of BIM, a specific PKC inhibitor (Fig. 4, triangles). The calculation of the area under the curves showed that 68.0 ± 1.9% and 70.4 ± 7.3% of the total fetal and adult activity, respectively, was inhibitable by BIM, indicating a contribution of non-PKC-related activity.

Identification of PKC isoforms underlying Ca$^{2+}$-independent activity. Because the majority of PKC activity in the adult murine ventricle was Ca$^{2+}$-independent, we wanted to determine further which Ca$^{2+}$-independent isoforms contributed specifically to the observed activity. To identify the PKC isoforms responsible for the Ca$^{2+}$-independent activity, the abundance of the four Ca$^{2+}$-independent isoforms in the active chromatography peaks was determined by Western blotting. In the fetal preparation (Fig. 6, left), PKC$\delta$ and PKC$\epsilon$ were readily detectable. Although PKC$\epsilon$ eluted preferentially in the early peak, PKC$\delta$ was almost exclusively restricted to the late peak. PKC$\eta$ showed a slightly wider elution profile. In the adult, the majority of PKC$\epsilon$ was distributed between the early and the late peaks, whereas PKC$\delta$ seemed relatively less abundant and restricted to the late peak. PKC$\eta$ eluted as a broader peak with a maximum in fraction 17 in which no activity had been detected. The fourth Ca$^{2+}$-independent isoform, PKC$\theta$, was undetectable by immunoblotting in the chromatography fractions containing PKC activity. This was not due to technical limitations, because the antibody recognized PKC$\theta$, which is abundant in lymphocytes in spleen extracts (data not shown). These results suggest the abundance of PKC$\delta$ and PKC$\epsilon$ correlates well with the distribution of Ca$^{2+}$-independent PKC activity (Fig. 4), and this activity is in large part attributable to these two isoforms.

Isoform-specific contribution to Ca$^{2+}$-independent PKC activity in the ventricle. Neither specific pharmacological activators nor inhibitors for individual Ca$^{2+}$-independent isoforms are available. We therefore adopted an IP protocol to deplete specific PKC iso-
forms. Extracts from adult mouse ventricles were partially purified by MonoQ FPLC. The fraction containing the highest amount of activity (fraction 15 of the elution profile) was used in these experiments. Aliquots from freshly prepared fractions were incubated with specific antibodies against each of the Ca\(^{2+}\)-independent PKC isoforms, followed by incubation with protein A/G agarose beads. The PKC bound to the beads was removed from the sample and the resulting depleted lysate was tested for PKC activity. The efficiency of depletion was determined by Western blotting using aliquots taken before and after IP and was \(\geq 85\%\) (Fig. 7A). No cross reactivity was detected against the Ca\(^{2+}\)-dependent isoform PKC\(\alpha\) (data not shown) indicating the specificity of the depletion reactions. Note that PKC\(\theta\) was not present in sufficient amounts to be detected by Western blotting.

To determine the contribution of the Ca\(^{2+}\)-independent isoforms to PKC activity, we measured Ca\(^{2+}\)-independent PKC activities in immunodepleted and mock-depleted fractions. The mock-depleted fractions were treated in an identical fashion except that the anti-PKC antibody was replaced by preimmune rabbit IgG. As shown in Fig. 7B, 78% of PKC activity remained after depletion of PKC\(\delta\), whereas 63% remained after depletion of PKC\(\epsilon\). No reduction in activity was seen after depletion of PKC\(\eta\) or PKC\(\theta\). When determining the contribution of the individual PKC isoforms to the total Ca\(^{2+}\)-independent activity, two technical considerations have to be taken into account. Our measurements after MonoQ chromatography indicated that in the adult preparation, \(\sim 35\%\) of the activity was not inhibited by the PKC inhibitor BIM. In addition, the efficiency of PKC depletion by IP was routinely around 85%. Thus activity measurements underestimate the relative contribution of PKC\(\delta\) and PKC\(\epsilon\) which, after adjustment for the two above parameters, is an estimated 39 and 56%, respectively. In addition, because PKC\(\eta\) and PKC\(\theta\) activities were not detected, PKC\(\delta\) and PKC\(\epsilon\) must be considered the major Ca\(^{2+}\)-independent isoforms within the mouse heart.

**DISCUSSION**

The distribution of PKC isoforms in the heart has previously been studied to varying extents in several species, most prominently in the rat. In the rat heart, PKC\(\delta\), PKC\(\epsilon\), and PKC\(\xi\) were detected reproducibly, whereas other isoforms, most notably PKC\(\alpha\), were detected in some, but not all studies (5, 34, 39). Discrepancies also exist for the detection of PKC\(\beta\) which may be restricted to the neonatal rat heart (9, 39), although its upregulation was reported in hypertrophy (13). However, others failed to detect this isoform in the adult heart (5, 39). These inconsistencies may, in part, be due to differences in the antisera used. In addition to these technical considerations, species differences also seem to exist. In the rabbit heart, all PKC isoforms except PKC\(\theta\) were found with varying abundances in a recent study by Ping et al. (30), including PKC\(\gamma\), which is not found in other species and was believed to be restricted to neuronal tissue. Other investigators have described a smaller subset of PKC including PKC\(\gamma\) in the rabbit heart (4, 33). In humans, PKC\(\beta\) was expressed in normal ventricular tissue and was increased in failing hearts (6). PKC\(\alpha\) and PKC\(\beta\)II were detected in canine and bovine ventricular tissue (2). In our study, these two Ca\(^{2+}\)-dependent isoforms were also readily detectable in mouse heart and cardiomyocytes. Three members of the Ca\(^{2+}\)-independent group, PKC\(\delta\), PKC\(\epsilon\), and PKC\(\eta\) were found as well, whereas PKC\(\theta\) was undetectable after partial purification by MonoQ chromatography. Taken together, these findings indicate the existence of species differences in the cardiac PKC expression profile.

The developmental progression of PKC isoform expression has previously been studied in the rat heart (9, 34). The overall decrease between the fetal and adult stage was similar to the one observed in the mouse in the present study. However, the kinetics of this down-regulation was markedly faster in the mouse. The abundance of PKC\(\alpha\), PKC\(\beta\)II, and PKC\(\delta\) at postnatal day 2 had decreased to \(<40\%\) relative to embryonic day 18 (F in Fig. 1B). In contrast, the decrease in PKC\(\alpha\) and PKC\(\delta\) was more gradual in the rat.
heart (9) and slight increases at day 2 have been reported as well (34). In both mouse and rat hearts, the abundance of PKCε declined much slower than the other isoforms examined to date. PKCβII, which has been reported to be expressed at very low levels in some preparations (44) or to be absent in others (39), was not examined in previous developmental studies. Given the importance of PKC in the regulation of cellular growth and differentiation, the seemingly faster downregulation in the mouse may be related to differences in the time of onset or rate of cardiomyocyte withdrawal from the cell cycle. In the mouse cardiomyocyte, DNA synthesis, an index of cell cycle exit, is drastically reduced between embryonic day 18 and postnatal day 0.04 (38), whereas cardiomyocyte numbers in the rat still increased 68% between postnatal days 1 and 3 (20). Although it is tempting to speculate about the involvement of PKC in these regulatory processes, the potential role of individual PKC isoforms in postnatal cardiomyocyte maturation remains unclear. Nevertheless, mechanisms governing the overall decrease of PKC during postnatal heart development seem to be specific, because the expression levels of a number of other kinases, including cAMP-dependent protein kinase, cGMP-dependent protein kinase, as well as members of the mitogen-activated protein kinase family are unchanged or even increased in the rat heart (17, 18).

Both PKC abundance, as judged by Western blotting, and PKC activity declined during postnatal development to a similar extent. Although all four PKC isoforms analyzed decreased in the adult to ~15% of their fetal expression levels (6.6 times) PKC activity decreased ~4.3 times. Although neither measurement is absolute, both Western blots and PKC activity assays are based on identically prepared extracts and normalizations to the amount of total protein, and the relative changes are, therefore, comparable. It is possible that not all of the immunologically detectable PKC pool can be activated by phospholipids and/or Ca2+, and, consequently, the activity levels are lower than predicted based on abundance levels only. In fact, it is increasingly evident that PKC requires phosphorylation by phosphoinositide-dependent protein kinase and/or tyrosine kinases to render it activatable by phospholipids (8, 19, 28, 41). This phosphorylation occurs on the PKC activation loop and seems to be required for subsequent autophosphorylation and the generation of a catalytically active conformation (31). Thus measurements of the apparent abundance of PKC may overestimate the fraction of PKC available for activation on stimulation in the fetal heart.

Standard Western blotting approaches allow a qualitative assessment of the presence of PKC isoforms. Because of different avidities of isoform-specific antibodies, it is difficult to quantitatively compare abundances between PKC isoforms. Quantitative assessments of PKC isoform abundance have been performed in the rabbit where Ca2+-dependent PKCs, most notably PKCα and PKCγ, were predominant (30). In contrast, a different study by the same team showed a prevalence of Ca2+-independent activity (71% of total activity) (29). This discrepancy suggests that PKC activity cannot be predicted merely from expression patterns. Nevertheless, it seems that the relative portion of Ca2+-independent PKC activity is similar between species. Using extracts from rat ventricles, Clerk et al. (9) found exclusively Ca2+-independent activity when using peptide ε, a substrate for both Ca2+-dependent and Ca2+-independent isoforms (16). However, with the preferred substrate for Ca2+-dependent PKC, histone IIIS (35), some Ca2+-dependent PKC activity was detected in both neonatal and adult rat ventricles (9). These studies were extended by Wientzek et al. (44) who found Ca2+-dependent PKC activity in isolated rat cardiomyocytes after partial purification by MonoQ FPLC. With the use of a quantitative Western approach on the human atria, PKCδ, PKCε, and atypical isoforms were detected in greater abundance (10). However, the PKC activity measured after DEAE sepharose and phenylsepharose chromatography was predominantly Ca2+-dependent. Thus the PKC activity content of human atria may differ from that of other species or the ventricular compartment.

The combination of isoform-selective IP and determination of PKC activity allows for measuring the contribution of a single PKC isoform to the intrinsic, activatable PKC pool. We have demonstrated the feasibility of this approach for the Ca2+-independent isoforms. Our approach was based on measuring the PKC activity left after IP and consequent depletion from the sample of a specific isoform. It should also be possible to measure the activity of the PKC retained on the beads after IP. We detected the retention of PKC on the beads by Western blotting but were unable to measure PKC activities (data not shown). Because the antibodies employed are directed against the COOH-terminal end of PKC that contains the catalytic domain, they may interfere with or block the catalytic site. Antibodies raised against other parts of the PKC molecule may be more suitable in this respect. Chemical compounds modulating the activity of individual PKC isoforms are rare, which impedes the assessment of isoform functions. Recently, an inhibitor specific for PKCβ has been described (14). The majority of inhibitors, or activators, are, however, not isoform specific. In addition, there is growing evidence that phospholipid-stimulatable kinases other than PKC exist and may contribute to the cellular responses to pharmacological agents previously considered to be PKC specific (31).

In summary, several lines of evidence suggest an important role for intrinsic PKCδ and PKCε in the murine heart. In the fetal heart, both isoforms were predominantly located in the particulate fraction, indicating an increased activation status (Fig. 2). In both fetal and adult hearts, Ca2+-independent PKC activity was prevalent (Fig. 5). In addition, after MonoQ FPLC, two activity peaks were detected that coincided with the presence of PKCδ and PKCε (Fig. 4). A shift in the elution profile of PKCε in the adult from early to late peak was accompanied by relative increase of activity in the late peak and a parallel decline in the early peak.

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(Fig. 6). Finally, the most direct evidence came from the IP experiments that indicated PKCδ and PKCe were the predominant, if not exclusive, basis of the total measurable Ca\(^{2+}\)-independent activity (Fig. 7).

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