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Transient Upregulation of Protein Kinase C in Pressure-Overloaded Neonatal Rat Myocardium

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Summary

Protein kinase C (PKC) appears to play a significant role in the signal transduction of cardiac growth and development. The aim of this study was to determine changes in the total PKC activity and the expression of PKC isoforms α, δ and ε in the rat heart that was affected by pressure overload imposed at postnatal day (d) 2. Three groups of Wistar rats were employed for the experiment: rats submitted to the abdominal aortic constriction (AC), sham-operated controls (SO) and intact controls. Animals were sacrificed at d2, d3, d5 and d10. The total PKC activity was measured by the incorporation of ^{32}P into histone IIIS and the expression of PKC was analyzed by immunoblotting in the homogenate of the left ventricular myocardium and in the cytosolic, membrane-enriched ($10^5 \times g$) and nuclear-cytoskeletal-myofilament-enriched ($10^3 \times g$) fractions. We observed the significant transient increase in both the total PKC activity and the expression of all isoforms at d5 (the 3rd day after the operation) in the cardiac homogenate of AC rats as compared with SO animals. Aortic constriction did not significantly affect the distribution of activity and isoform abundance among individual cellular fractions except for PKCδ, which increased significantly at d10 in the cytosolic fraction at the expense of the membrane-enriched fraction. It is concluded that PKCα, PKCδ and PKCε undergo transient upregulation associated with the accelerated cardiac growth induced by pressure overload imposed in the very early postnatal period.

Key words

Protein kinase C • Pressure overload • Postnatal development • Rat myocardium

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Introduction

The developing mammalian heart has to overcome an increased workload, which is associated with the rapid growth of the left ventricle during postnatal ontogeny. Just after birth, the myocardium continues growing by adding new cells (hyperplasia), but the further growth of the heart is due to the increase of cell volume (hypertrophy) as ventricular cardiomyocytes lose their capability of dividing soon (Clubb and Bishop 1984, Li *et al.* 1996), though limited proliferative activity may be restored under certain conditions even in adulthood (Beltrami *et al.* 2001, Bruel *et al.* 2007). It is well known that protein kinase C (PKC) is an important player in the complex regulation of cell proliferation and myocardial hypertrophy (Bogoyevitch and Sugden 1996, Sabri and Steinberg 2003, Buchner 2000). The Gq/PLC/PKC signaling pathway was proved to mediate both the normal cardiac postnatal growth and hypertrophic growth of pressure-overloaded hearts (Dorn and Force 2005, Wettschureck *et al.* 2001).

PKC consists of a family of at least 11 isozymes, which are divided into three groups. The members of a classical group (α, $\beta_{1/2}$ and γ) are Ca^{2+}-dependent and

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need 1,2-diacylglycerol (DAG) and phosphatidylserine (PS) for their activation; novel PKC isoforms (δ, ε, η and θ) are Ca^{2+}-independent and are activated by DAG and PS; atypical isoforms (ζ and ι/λ) are Ca^{2+} and DAG independent but require PS as a cofactor. PKC isozymes are expressed differentially in various organs and tissues (Wetsel *et al.* 1992). PKCα, PKCδ, PKCε and PKCζ were mostly identified in the neonatal rat heart and all of them diminished markedly until adulthood (Clerk *et al.* 1995, Rybin and Steinberg 1994, Hamplová *et al.* 2005). However, we have recently described rapid day-by-day developmental changes in the expression of PKCα, PKCδ and PKCε in the homogenate and the cellular fractions of rat heart during the first 10 postnatal days. We observed more than twofold transient increase in the content of PKCδ and PKCε associated with nuclear-cytoskeletal-myofilament structures between d3 and 5 (Hamplová *et al.* 2005). It is worth noting that this narrow developmental period is connected with the rapid transition from the hyperplastic to the hypertrophic growth (Li *et al.* 1996).

In the present study we were interested to find out whether the aortic constriction, an additional growth-promoting stimulus targeted just prior to period of hyperplastic-to-hypertrophic growth transition, can influence the developmental changes in the total activity and the expression and subcellular distribution of PKCα, PKCδ and PKCε. We used the experimental model of the neonatal pressure-overloaded heart that allows to observe a response of the myocardium which is still in the proliferative phase of the cardiomyocyte growth (Kolář *et al.* 1998, Sedmera *et al.* 2003).

Methods

Animal model

Newborn male Wistar rats were obtained from the animal care facility of the Institute of Physiology, Prague, Czech Republic. Under light ether anesthesia, pressure overload was induced by the constriction of the abdominal aorta (AC) at postnatal day 2 as described earlier (Kolář *et al.* 1998). Sham-operated (SO) littermates were used as the age-matched controls; their aorta was exposed, but not constricted. Intact littermates were also used. The animals were sacrificed at d2 (intact subgroup only), d3, d5 and d10. Their hearts were dissected free of an atrial tissue, large blood vessels and the right ventricle. The left ventricle including the septum (LV+S) was rinsed in cold saline (5 °C), weighed, frozen in liquid nitrogen and stored at –80 °C until use. This study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institute of Health (National Institutes of Health publication No. 85-23, revised 1996).

Tissue fractionation

A specimen for a fractionation contained ventricles from 4 (d10) to 14 (d2) animals. The tissue was minced using Turrax (30 s, twice) and then homogenized in 10 volumes of ice-cold homogenization buffer composed of (in mmol/l): 12.5 Tris-HCl (pH 7.4), 250 sucrose, 2.5 EGTA, 1 EDTA, 100 NaF, 5 dithiothreitol, 0.3 phenylmethylsulfonyl fluoride, 0.2 leupeptin, 0.02 aprotinin. The homogenate was fractionated according to the method described previously by Gu and Bishop (1994), with slight modifications. Briefly, the homogenate was centrifuged at 100 × g for 20 min to remove cellular debris and unbroken cells. A supernatant was centrifuged at 1000 × g for 10 min to produce a nuclear-cytoskeletal-myofilament-enriched fraction (for simplicity denoted as *nuclear fraction* in the text) followed by centrifugation at 100,000 × g for 60 min. The 100,000 × g pellet contained a membrane-enriched fraction and the supernatant was a cytosolic fraction. The homogenate and the pellets of nuclear and membrane fractions were resuspended in the homogenization buffer containing 1 % Triton X-100, held on ice for 60 min and centrifuged at 100,000 × g for further 60 min. The resulting detergent-treated supernatants were used for activity and immunoblotting analyses. Triton X-100 was also added to the cytosolic fraction to give the final concentration of 1 %. Protein content was determined according to Lowry, as modified by Peterson (1977).

Activity assay of total PKC

The PKC activity was determined as a difference in the incorporation of ^{32}P from γ[^{32}P]ATP into a histone IIIS in the presence and the absence of PS, 1-octanoyl-2-acetyl-glycerol (OAG) and Ca^{2+}. The required amount of PS and OAG dissolved in chloroform was dried in the stream of nitrogen and solubilized in 0.3 % Triton X-100 by vortexing and incubation at 30 °C for 5 min. A reaction mixture (0.1 ml) contained (in mmol/l): 20 Tris-HCl (pH 7.45), 10 $MgCl_2$, 1 dithiothreitol, 1 $CaCl_2$, 0.1 vanadate, 21 μg PS, 5.1 μg OAG and protein (in μg: 2.5 homogenate, 3 cytosolic, 2 nuclear, 1 membrane fractions). The reaction was started by the addition of 10 μl of 1 mM γ[^{32}P]ATP (50-100 cpm/pmol)

and terminated after 5 min at 30 °C by the addition of 1 ml ice-cold 25 % trichloroacetic acid. The resulting precipitates were filtered through pre-soaked nitrocellulose filters (Pragopor, Pragochema) and washed three times with 5 % trichloroacetic acid. The radioactivity was quantified using the Cerenkov decay.

Immunoblot analyses of PKC isoforms

Samples were electrophoresed on the 8 % bis-acrylamide polyacrylamide gel. SDS-PAGE was carried out at 20 mA/gel for 90 min on a Mini-Protean II apparatus (Bio-Rad). After the electrophoresis, resolved proteins were transferred to a nitrocellulose membrane (Amersham International). The membranes were incubated in 5 % dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 min at room temperature in order to block a nonspecific binding. After washing in TTBS buffer (3-times quickly, 3-times 5 min each) they were probed with the PKC isoform-specific primary rabbit antisera (1:8000 in TTBS) for 90 min at room temperature. The membranes were washed again and incubated with the secondary swine anti-rabbit IgG antibody labeled with the horseradish peroxidase (1:4000 in TTBS) for 60 min at room temperature. Before an enhanced chemiluminescence (ECL), the nitrocelluloses were washed as described above and stored in TTBS for at least 2 h. For ECL, we prepared ECL substrates A (Luminol solution) and B (H_2O_2 solution); they were mixed 1:1 and poured on the immunoblot. A specific signal was documented on an autoradiography film (Amersham Int). A scanning (Epson Perfection 1240U) and ImageQuant software were used for the quantification of a relative abundance of the individual PKC isoforms. To ensure the specificity of the immunoreactive proteins, Western blots were done in the presence and the absence of competing immunizing peptides (see Fig. 1 in Hamplová *et al.* 2005). The PKC δ isoform was identified as the doublet of proteins. Both proteins were recognized using the antibody mentioned above, blocked by the appropriate immunizing peptide and taken for the quantification in our study. Similarly, Ogita *et al*. (1992) immunodetected PKC δ as the doublet of proteins (78 and 76 kDa) in the preparation from rat brain; the 78 kDa protein was identified as the phosphorylated form of the 76 kDa protein by means of the protein phosphatase 2A treatment.

Chemicals

Chemical reagents including the PKC isoform-specific primary antisera and competing immunizing peptides were obtained from Sigma-Aldrich. The secondary swine anti-rabbit IgG antibody labeled with a horseradish peroxidase was from Sevapharma. Radioactive γ[^{32}P]ATP was obtained from ICN Biomedicals.

Statistical analyses

All results are expressed as means ± S.E.M. The statistical significance of differences was determined by one-way ANOVA and subsequent Newman-Keuls test ($p<0.05$).

Results

Weight parameters

Body weight was not significantly altered by either SO or AC in any age group of the animals. AC led to the mild left ventricle enlargement, as evident from increases of LV+S/BW ratio by 14 % and 23 % at d5 and d10 (i.e. 3 and 8 days after the surgery), respectively, as compared with the SO littermates (Table 1).

Total PKC activity

The total PKC activity in the homogenate of the intact ventricular myocardium during early postnatal development, and the effect of SO and AC on the total activity within the period of the study are shown in Figure 1A. The PKC activity declined gradually from d2 till d10 in intact rats. SO did not induce any changes. AC caused the transient increase in the activity (by 45 %) at d5 as compared with the SO rats. The changes after SO and AC in the total activity in cellular fractions are presented in Figure 1B. Compared to intact controls, we observed relative increase in the PKC activity at d3 in the nuclear fraction of SO group that occurred at the expense of the membrane and the cytosolic fractions.

PKC isoforms expression and distribution

The PKCα expression declined gradually from d2 till d10 in the homogenate of the intact myocardium. SO influenced the expression of PKCα neither in the homogenate nor in any cellular fraction. AC transiently increased the expression of PKCα by 55 % at d5 in the homogenate, but did not affect its distribution between fractions (Fig. 2).

The PKCδ expression declined from d2 till d10 in the myocardial homogenate of intact animals. SO had no effec, while AC transiently elevated the expression of

Table 1. Weight parameters of intact, sham-operated and aortic-constricted rats during early postnatal development.

Postnatal days	n	BW (g)	LV+S (mg)	RV (mg)	LV+S/BW (mg/g)
2 day intact	44	6.61 ± 0.40	21.89 ± 0.46	7.83 ± 0.26	3.22 ± 0.05
3 day intact	24	9.04 ± 0.49	30.11 ± 0.88	9.64 ± 0.30	3.43 ± 0.10
SO	21	9.44 ± 0.98	32.92 ± 1.15	9.78 ± 0.55	3.40 ± 0.05
AC	21	7.45 ± 0.50	32.44 ± 1.63	10.14 ± 0.51	3.56 ± 0.07
5 day intact	24	10.78 ± 0.97	31.56 ± 1.29	10.10 ± 0.53	3.03 ± 0.05
SO	18	14.52 ± 2.02	37.10 ± 1.86 *	13.04 ± 0.76 *	3.02 ± 0.04
AC	18	14.01 ± 1.00	42.32 ± 1.77	12.45 ± 0.61	3.45 ± 0.07 #
10 day intact	12	22.42 ± 0.98	61.23 ± 2.59	21.51 ± 1.09	2.71 ± 0.06
SO	12	23.71 ± 1.63	61.56 ± 2.50	21.13 ± 0.93	2.78 ± 0.05
AC	12	23.67 ± 2.03	72.02 ± 3.01 #	20.56 ± 1.04	3.42 ± 0.12 #

Values are means ± S.E.M. BW, body weight; LV, left ventricle; S, septum; RV, right ventricle; SO, sham operation; AC, aortic constriction; n, number of animals. *P<0.05, significant difference *vs.* intact controls; #P<0.05, significant difference *vs.* sham-operated controls.

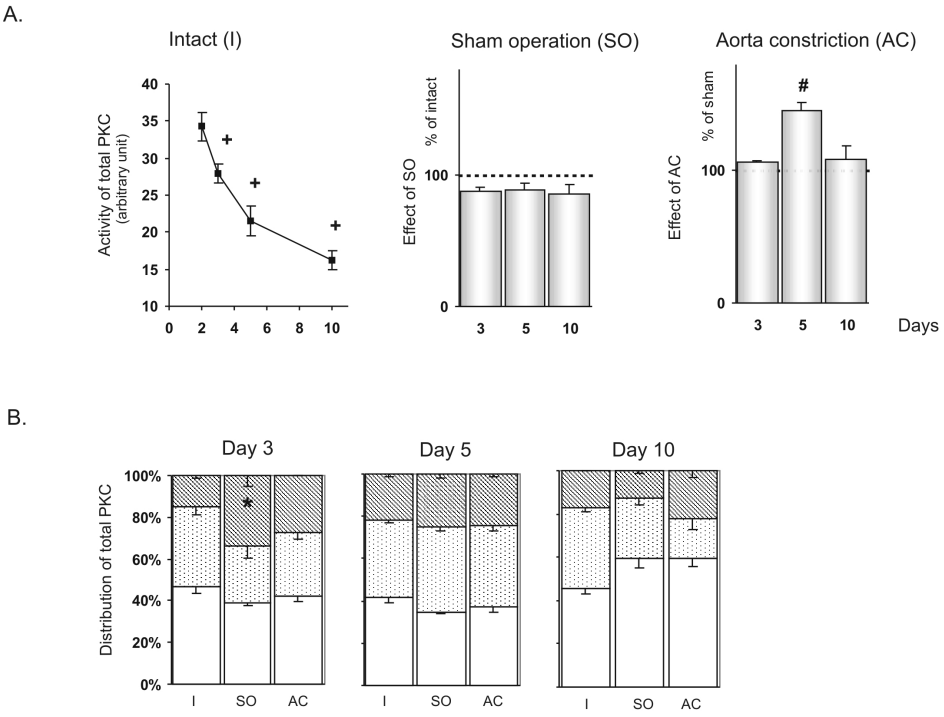

Fig. 1. The effect of sham operation and aortic constriction on developmental changes in the total protein kinase C activity **(A)** in the homogenate of left ventricular myocardium and **(B)** its distribution between cytosolic (white columns), membrane (dotted columns) and nuclear-cytoskeletal-myofilament fractions (cross-hatched columns) during the first 10 postnatal days. Values are expressed **(A)** as arbitrary units (the sum of specific radioactivity measured on postnatal days 2, 3, 5 and 10 is equal to 1) or % of corresponding control values, and **(B)** as mol % (the sum of all cellular fraction equals 100 %). Data are means ± S.E.M. from 3 fractionations in each age group. +P<0.05, significant difference *vs.* day 2; *P<0.05, significant difference *vs.* intact controls; #P<0.05, significant difference *vs.* sham-operated controls.

PKCδ by 67 % at d5 as compared with the SO group. As for the distribution of PKCδ, we observed the relative decrease in the nuclear fraction at d5 by 13 % and the increase in the membrane fraction at d5 by 8 % and at d10 by 11 % in SO rats as compared with intact controls. In the group of AC rats, we found the relative decrease of δ isoform in the membrane fraction (by 13 %) and the increase in cytosol at d10 in the comparison with SO animals (Fig. 3).

The expression of PKCε declined from d2 till d10 with the sharpest drop by d3 in the cardiac homogenate of intact rats. SO had no effect on PKCε

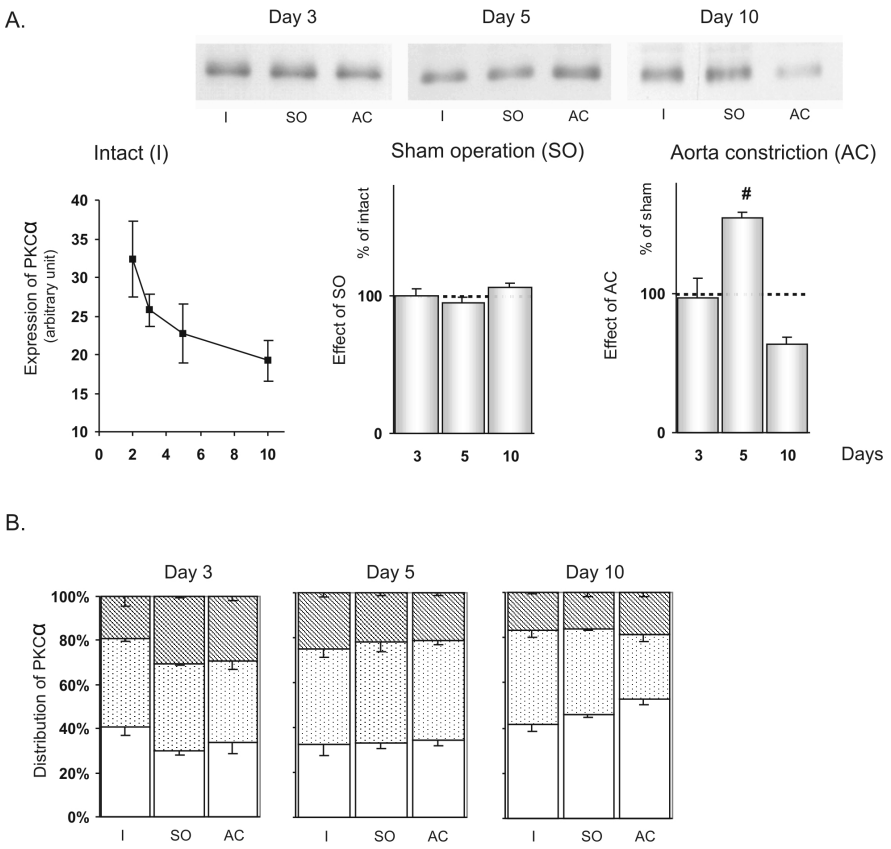

Fig. 2. The effect of sham operation and aortic constriction on developmental changes in the expression of protein kinase Cα **(A)** in the homogenate of left ventricular myocardium (representative imuno-blots are shown) and **(B)** its distribution between cytosolic (white columns), membrane (dotted columns) and nuclear-cytoskeletal-myofilament fractions (cross-hatched columns) during the first 10 postnatal days. Values are expressed **(A)** as arbitrary units (the sum of densitometry volumes measured on postnatal days 2, 3, 5 and 10 is equal to 1) or % of corresponding control values and **(B)** as mol % (the sum of all cellular fraction equals 100 %). Data are means ± S.E.M. from 3 fractionations in each age group. $^{\#}P<0.05$, significant difference *vs.* sham-operated controls.

expression and AC transiently increased it at d5 by 39 % as compared with SO controls. Neither SO nor AC influenced the distribution of PKCε among cellular fractions (Fig. 4).

Discussion

The aim of this study was to examine the effect of pressure overload on the pattern of developmental changes in the total PKC activity and the expression of PKCα, PKCδ and PKCε in the rat heart during the first 10 postnatal days. Although several studies investigated the role of the PKC family in the regulation of the cardiac hypertrophy induced by the aortic constriction (Gu and Bishop 1994, Bayer *et al.* 2003, Braun *et al.* 2002), none of them examined the cardiomegaly induced before d5, i.e. in the proliferative phase of the rat cardiomyocyte growth (Li *et al.* 1996). It should be stressed that the myocardium affected at this period of development responds differently to pressure overload as compared with the adult heart. When the aortic constriction was performed early after birth (d2), the increase of the left ventricular mass resulted first from the stimulated cellular hyperplasia followed later by the enlargement of cardiomyocytes (Sedmera *et al.* 2003). Moreover, cardiomegaly was associated with capillary proliferation in proportion to the increase of ventricular mass in this experimental model, and exhibited neither increased rates of apoptosis nor myocardial fibrosis characteristic for adult pressure-overloaded hearts (Kolář *et al.* 1998, Sedmera *et al.* 2003, Oliviero *et al.* 2000). Thus, aortic constriction in neonates results in a unique cardiac phenotype that markedly differs from that induced in the adulthood, and accordingly the PKC isoforms might be differently involved in its regulation.

In adult pressure-overloaded myocardium, PKCα and PKCδ were markedly upregulated, the latter one even as early as the first day after aortic constriction (Bayer *et al.* 2003, Braun *et al.* 2002). These results provide indirect evidence that PKCδ might be involved in

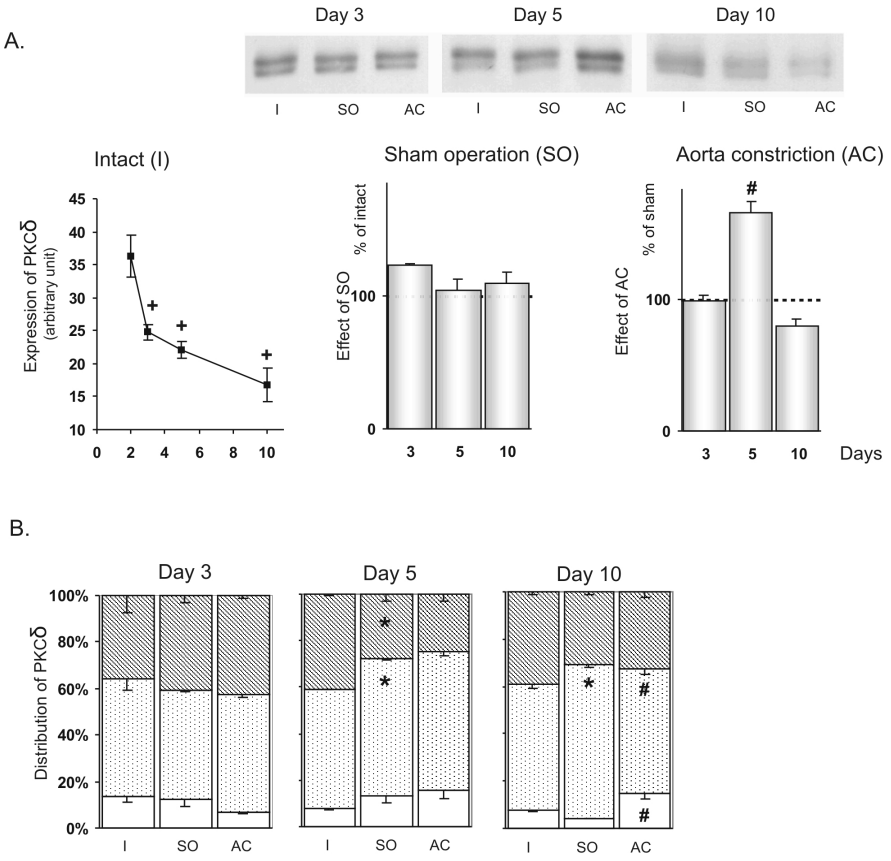

Fig. 3. The effect of sham operation and aortic constriction on developmental changes in the expression of protein kinase Cδ **(A)** in the homogenate of left ventricular myocardium (representative imunoblots are shown) and **(B)** its distribution between cytosolic (white columns), membrane (dotted columns) and nuclear-cytoskeletal-myofilament fractions (crosshatched columns) during the first 10 postnatal days. Values are expressed **(A)** as arbitrary units (the sum of densitometry volumes measured on postnatal days 2, 3, 5 and 10 is equal to 1) or % of corresponding control values and **(B)** as mol % (the sum of all cellular fraction equals 100 %). Data are means ± S.E.M. from 3 fractionations in each age group. $^{+}$P<0.05, significant difference *vs.* day 2; *P<0.05, significant difference *vs.* intact controls; $^{\#}$P<0.05, significant difference *vs.* sham-operated controls.

the induction of compensatory hypertrophy in the adult heart after aortic constriction, whereas both PKCα and PKCδ might be responsible for the transition to heart failure. The studies in transgenic mice show that the function of PKCδ depends on the strength of hypertrophic stimuli (Hahn *et al.* 2002). A modest increase in PKCδ expression was shown to result in so called "physiological" hypertrophy, but on the other hand, high chronic expression of PKCδ evoked cardiomyocyte necrosis and contractile dysfunction (Chen *et al.* 2001, Hahn *et al.* 2002). The role of PKCε in the regulation of cardiac hypertrophy appears to be parallel with that of PKCδ during compensatory hypertrophy (Chen *et al.* 2001, Mochly-Rosen *et al.* 2000, Takeishi *et al.* 2000). Besides, Heidkamp *et al.* (2001) showed that just PKCδ and PKCε act as a switch between cardiomyocyte hypertrophy and apoptosis, and PKCε selectively activates the MAPK cascade implicated in growth response and cell survival, whereas PKCδ preferentially activates stress-activated protein kinase cascades implicated in detrimental changes of the heart. Thus, it seems that the fate of cardiomyocytes in pressure-overloaded hearts depends mostly on the balance of novel PKC isoforms to downstream signaling cascades.

In this study, we have found the transient increase in PKC activity and upregulation of all isoforms (PKCα, PKCδ and PKCε) at d5 (it means the 3rd day after the operation) in the myocardium of AC rats. Already at this early stage, a slight but significant increase in the relative left ventricular mass was observed. It can be speculated that the delay in the normal postnatal decline in PKC activity/expression caused by aortic constriction is related to transitory stimulation of myocyte hyperplasia and a delayed transition to hypertrophic growth. As for the redistribution of the PKC isoforms between cellular fractions, we did not observe any major changes except of the decreased proportion of PKCδ in the membrane fraction and, conversely, the increased proportion of this isoform in the cytosol in hearts of pressure-overloaded rats at d10 only. Noteworthy, PKCα and PKCε had a

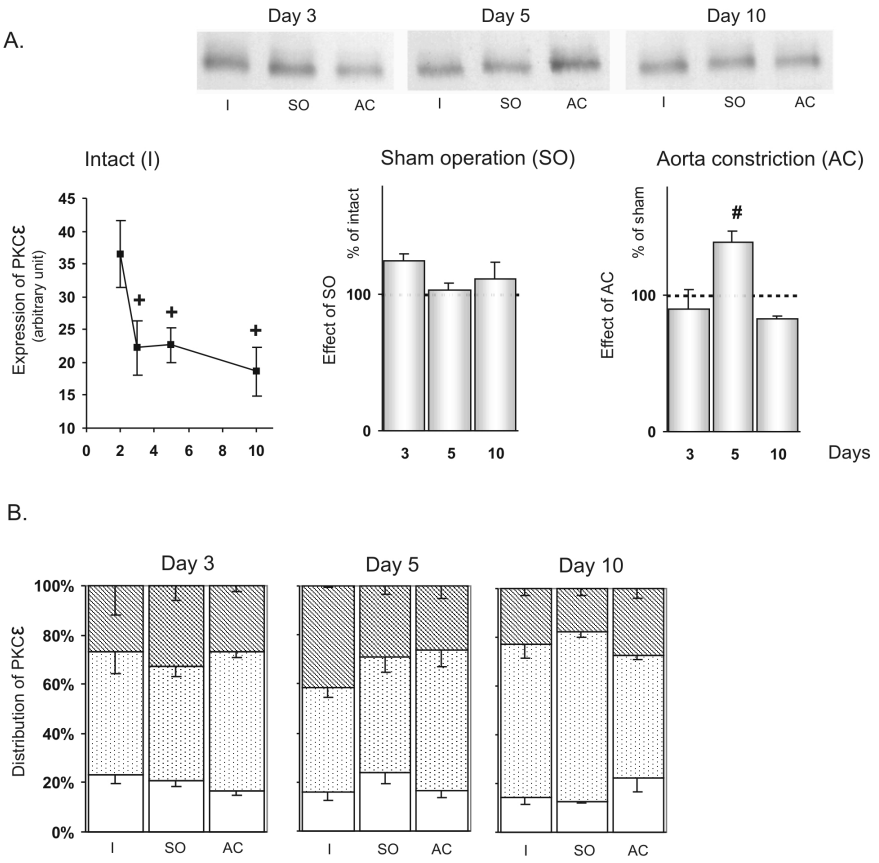

Fig. 4. The effect of sham operation and aortic constriction on developmental changes in the expression of protein kinase Cε **(A)** in the homogenate of left ventricular myocardium (representative imunoblots are shown) and **(B)** its distribution between cytosolic (white columns), membrane (dotted columns) and nuclear-cytoskeletal-myofilament fractions (cross-hatched columns) during the first 10 postnatal days. Values are expressed **(A)** as arbitrary units (the sum of densitometry volumes measured on postnatal days 2, 3, 5 and 10 is equal to 1) or % of corresponding control values and **(B)** as mol % (the sum of all cellular fraction equals 100 %). Data are means ± S.E.M. from 3 fractionations in each age group. *P<0.05, significant difference *vs.* day 2; #P<0.05, significant difference *vs.* sham-operated controls.

tendency to copy these changes in PKCδ distribution. Altogether, these data suggest that individual PKC isoforms may act in synergy during the early phase of pressure overload in this model. However, it is preliminary to speculate about possible implication of these changes for an accelerated myocardial growth response.

PKC isoforms can be activated by any membrane receptor that couples to Gq. It is unknown which of these receptors contribute to PKC activation and acceleration of cardiac growth in our experimental model of neonatal pressure-overloaded hearts. The only data obtained on the same model concern angiotensin II (Ang II) and its receptor types AT_1 and AT_2. It was demonstrated that Ang II content in sera increased transiently already 2 h after aortic constriction. At the receptor level, a transient increase in both AT_1 and AT_2 was observed one day after the surgery. Moreover, AT_2 transcript levels were linearly correlated with the left ventricular mass in the AC group (Oliviero *et al.* 2000). The rise in Ang II level preceding the increase in Ang II binding capacity and PKC activation/upregulation might suggest that this peptide is one of the triggers implicated in the PKC-mediated early response of neonatal myocardium to pressure overload. Interestingly, in the normal rat heart, the expression of AT receptors is maximal just after birth (Hunt *et al.* 1995) decreasing rapidly thereafter together with PKC activity and isoform expression (Hamplová *et al.* 2005). However, these correlative studies do not allow to make any clear conclusion because PKC is not the only signaling pathway activated by Ang II and a variety of other factors might also contribute to accelerated neonatal cardiac growth in response to pressure overload (Novák *et al.* 2009), either mediated by PKC or not.

While the operation itself did not influence the total PKC activity and isoforms expression in the cardiac homogenate, it is necessary to note that it had significant

effects on the distribution of PKC activity and PKCδ abundance among cellular fractions compared with intact littermates. This finding is in line with the view that PKC may play a role in myocardial inflammatory response to stress and injury. For example, Tan *et al.* (2007) demonstrated a marked increase in PKCα, PKCδ and PKCε abundance in membranes and PKC-dependent secretion of proinflammatory cytokines by cardiomyocytes induced by burn injury. In our study, only PKCδ redistributed to the membrane fraction in the SO group suggesting that the surgical intervention itself was a relatively mild stimulus. Moreover, an effect of anesthesia on PKC distribution cannot be excluded.

In conclusion, we demonstrated a transient increase in PKC activity and isoform expression at d5 in the rat ventricular myocardium subjected to pressure overload induced by aortic constriction at postnatal day 2. These effects were not accompanied by major changes in subcellular distribution of individual PKC isoforms. Although it is likely that PKC signaling is involved in mediating the early hyperplastic/hypertrophic myocardial growth response, further studies are needed to prove this hypothesis.

Conflict of Interest

There is no conflict of interest.

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