MEMBRANE PHOSPHOLIPIDS AND PROTEIN KINASE C IN THE HEART DURING POSTNATAL DEVELOPMENT

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Ph.D. thesis

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ABBREVIATIONS

d	postnatal day
DAG	diacylglycerol
DPG	diphosphatidylglycerol
FA	fatty acid
PC	choline phosphoglycerides
PE	ethanolamine phosphoglycerides
PI	phosphatidylinositol
PIP_2	phosphatidylinositol 3,4-bisphosphate
PL	phospholipid
PLC	phospholipase C
PLPC	choline plasmalogen
PLPE	ethanolamine plasmalogen
PKC	protein kinase C
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
RACK	receptor for activated C kinase
SM	sphingomyelin
T_3	triiodothyronine
T_4	thyroxine
TAG	triacylglycerol
TH	thyroid hormone

1. INTRODUCTION

Cardiovascular diseases kill about sixty thousand people in the Czech Republic every year. In other words, the heart and vascular diseases are the cause of almost 55% of all deaths, more than cancers, injuries and other diseases together.

One half of this threatening number of casualties is a result of the cardiovascular disease named ischemic heart disease, which can develop into myocardial infarction. It is well known that high cholesterol, smoking, stress, obesity and lack of exercise increase the risk of heart disease. Nevertheless, the cause of the disease appears to occur in earlier periods of ontogeny than we had supposed. It was demonstrated that an adverse intrauterine environment due to maternal low protein diet, premature birth or low weight of newborns could program cardiac dysfunction in adulthood. These observations shift the experimental research to a former stage of cardiac development, to understand the environment and conditions under which ischemic heart disease originates.

A further prominent subject of developmental experimental cardiology is the study of changes in heart physiology and function caused by congenital heart diseases. An adverse event occurring in the early part of pregnancy, when the heart is forming, can lead to congenital defects ranging from mild alternation that produce minimal symptoms until adult life to severe anomalies that can cause death in the perinatal or early postnatal period of ontogeny. Prenatal and newborn screening for congenital heart diseases helps to diminish infant mortality, as timely recognition and surgical removal of the defect is vital to prevent cardiovascular collapse. The understanding of the mechanism by which defects alter normal postnatal cardiac development could help to find new therapeutic approaches to diminish injuries of immature hearts after surgery.

To understand the changes in the developing heart under pathological conditions, we must primarily know the structure, function and regulation of the immature heart under physiological conditions and during normal postnatal ontogeny.

2. AIMS OF THE THESIS

The aims of my thesis were to determine:

1. developmental changes in the concentration of individual phospholipid species and their fatty acid composition in the membranes of left ventricular myocardium from the rat at several developmental stages between postnatal days (d)2 and 100;
2. the effect of hypo- and hyperthyroidism induced just after birth on the concentration and fatty acid composition of ventricular membrane phospholipids on d21;
3. phospholipid composition of cardiac membranes from the myocardium of children myocardium affected by normoxemic and hypoxemic congenital defects;
4. developmental changes in the amount and fatty acid composition of total diacylglycerol, the physiological activator of PKC, in the left ventricular myocardium from the rat on d2, 3, 5, 7 and 10;
5. developmental changes in the total activity and expression of prevalent PKC isoforms α, δ and ε in the cytosolic, membrane ($10^5 \times g$) and nuclear-cytoskeletal-myofilament fractions ($10^3 \times g$) and in the homogenate of ventricular tissue from the rat on d2, 3, 5, 7 and 10;
6. the effect of the pressure overload induced just after birth on the total activity and PKCα, PKCδ and PKCε expression in the cytosolic, membrane and nuclear-cytoskeletal-myofilament fractions of the ventricular homogenate from the rat on d3, 5 and 10.

3. REVIEW

An accelerated growth of the heart during early postnatal development is in response to mechanical, neural and endocrine changes at birth. The heart just after birth has to primarily overcome the switchover from a right ventricle dominance in the fetus to a postnatal left ventricular dominance that is accompanied by a corresponding alteration in the relative levels of ventricular myocardial blood flow. The altered postnatal hemodynamics initiates increased growth in the left ventricular tissue relatively to the right ventricular one (for review see *1*). The myocardium grows either by an increase in cell number (hyperplasia) or cell volume (hypertrophy). In most mammals, myocytes lose their capability of division shortly after birth, and further growth of the heart is due to cardiomyocyte hypertrophy and nonmyocyte cell hyperplasia. In the rat, three phases of postnatal myocardial growth has been described in a classic study of Clubb and Bishop (*2*). In the first phase, from birth to 4 days of age, there is sustained hyperplastic growth of myocardial cells. A second phase of postnatal growth, from 6 to 14 days of age, represents a transition from hyperplastic to hypertrophic growth, when the percentage of binucleated myocytes increases significantly. During the third phase, 14 to 21 days of age, the heart weight increases continually; there is little DNA synthesis, no change in percentage of binucleated cells and the tissue grows by hypertrophy. However, according to a recent study (*3*), the switch from hyperplastic to hypertrophic growth of rat cardiomyocytes occurs sharply as early as after d3. A comparable developmental profile of postnatal cardiomyocyte growth has been found in the mouse (*4*). On the other hand, different timing of the switch from hyperplasia to hypertrophy was observed in sheep, where Burrell et al. (*5*) found an increase in binucleated myocytes already before birth.

Similarly, binucleated myocytes appear by 32 weeks gestation in humans, which suggests that the cardiomyocytes' hyperplasia may cease before the birth of an individual (*6*).

Another dramatic perinatal change that the immature heart has to overcome is the switch in the energy substrate preference from lactate and glucose to fatty acids (FA) after birth (*7*). As the heart of the newborn begins to employ the oxidative metabolism, the expression of corresponding metabolic enzymes changes (*8*). Naturally, the number, structure and function of mitochondria change during early postnatal development as well (*9-11*). Since the fetus is almost exclusively dependent on anaerobic metabolism, its heart has a relatively high tolerance to oxygen deficiency that endures also shortly after birth (*12*). A possible explanation of the rapid loss of anaerobic tolerance is the depletion of glycogen stores from the prenatal period, the prevalent reliance on FA metabolism, the increased sensitivity to acidosis, the increased sensitivity to Ca^{2+} overload and the developmental changes in mitochondria (*13*).

From the nutritional point of view, the second important early ontogenetic period is the suckling-weaning transition, which takes place between the 3rd and 4th postnatal week in the rat. The animals start to eat a solid diet, which has a higher carbohydrate content and lower fat content than milk. Moreover, the FA composition of triacylglycerols (TAG) is different between milk and the solid diet. TAGs in maternal milk contain preferentially medium-long chain FAs that are almost absent in adult food (*14*). Weaning is also the time when the developmental changes in mitochondria mentioned above terminate (*9; 15*).

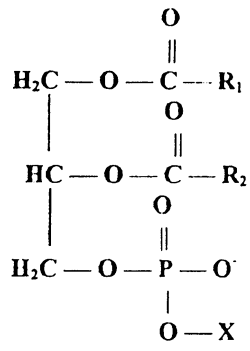
The newborn requires essential modifications in hormone secretion to withstand all these changes linked with birth and early ontogeny. The plasma level of the majority

of hormones alters radically during the immediate postnatal period (for review see *14*). Nevertheless, the early postnatal developmental process of the myocardium is under the thyroid hormones' (TH) control (*16*). Whereas in humans the thyroid function is mature at birth and the level of triiodothyronine (T_3) and thyroxine (T_4) rises rapidly (*17*), in rats, TH reach their peak levels as late as in the third postnatal week (*18*), though they appear in the plasma of rats at prenatal day 18 (*19*). In thyroid-responsive tissues like the heart, T_4 is deiodinated to the biologically active form T_3, which is transported to the nucleus of the cell, where it directly controls gene transcription (*20*). In the immature rat myocardium, TH regulate among others, the development of the coronary capillary network (*21*), early maturation of cardiac adrenergic signaling (*22; 23*), the maturation of Ca^{2+} handling (*24; 25*) and also the phospholipid (PL) composition of cardiac membranes (*26-28*).

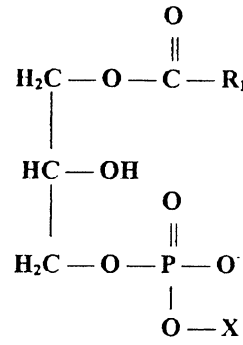
Since cardiac membranes create the environment for a number of membrane proteins involved in the excitation-contraction coupling, energy metabolism, signal transduction and other vital processes occurring in the heart, the ontogenetic PL remodeling contributes to the modification of the proteins' function, and thus might influence the proper heart maturation. Furthermore, the metabolic products of membrane PLs are known to fulfill the second messenger function in a variety of signaling pathways, from whence it follows that changes in the quantity and quality of cardiac PLs might have an indirect effect on the regulation of cardiac function and growth during postnatal development.

3.1. MEMBRANE PHOSPHOLIPIDS

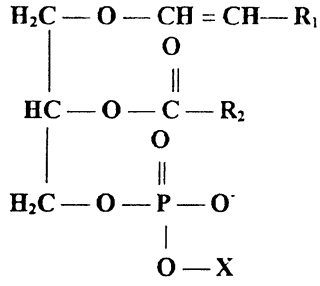
For many years PLs (Fig. 1) and other membrane lipids were thought to be evenly distributed structural components of biomembranes.



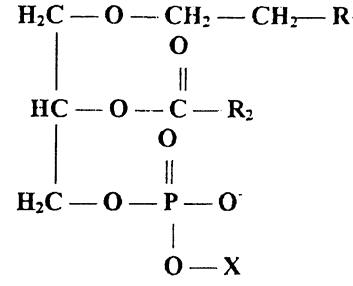
a) 1,2-diacyl PL



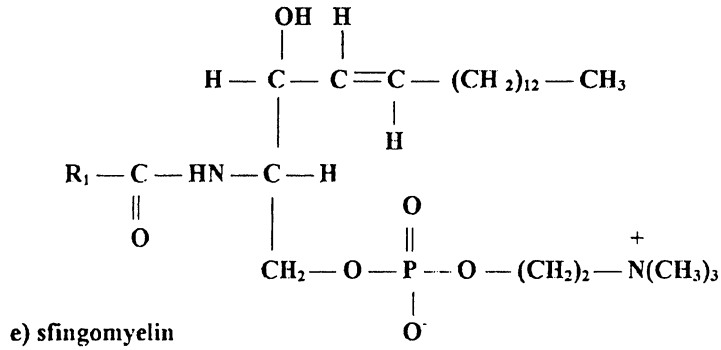
b) 2-lyso PL



c) 1-alkenyl-2-acyl PL (plasmalogen)



d) 1-alkyl-2-acyl PL



e) sfingomyelin

Fig. 1. The structure of individual phospholipid species. PL, phospholipid; $R_{1,2}$, fatty acyl chain; X, polar base (choline, ethanolamine, phosphatidylglycerol, inositol, serine). Adopted from (*29*).

Singer and Nicholson (*30*) proposed the "fluid mosaic" model of the plasmalemma, which argued that the membrane is a homogenous fluid bilayer in which transmembrane proteins drift regardless of lipids. However, it turned out that the lipid membrane components are rather organized, into discrete regions or domains with particular lipid compositions that are specialized for specific functions (*31*). Recent studies have demonstrated the existence of domains in the plasmalemma named "lipid rafts" or "caveolae", depending on the presence of the protein caveolin. They both are enriched in cholesterol, sphingolipids and long, saturated FAs in PLs, and provide the structural integrity and the appropriate environment for the regular functioning of membrane proteins (*32; 33*). Furthermore, these domains are rich in signaling proteins and are intimately involved in the signal transduction linked with the cardiovascular system (*34; 35*) (Fig. 2).

Despite the fact that the PL composition of cardiac membranes can markedly influence heart function, very little information is available about how the content of cardiac PLs and their FA profile changes during ontogeny. Knowledge of developmental changes in cardiac PLs could help to understand the signaling machinery that regulates the energy metabolism and the growth of the heart.

3.1.1. Developmental changes in the content of phospholipids

The mass of cardiac membrane structures rises dramatically during early postnatal development: the biosynthesis of PL predominates over its degradation, whereas both processes are more in dynamic equilibrium in the adult heart under physiological conditions (*14*). We have recently shown that the content of cardiac total PLs grows

much more rapidly especially in two critical postnatal periods, the neonatal period (d2-5) and the suckling-weaning transition (d20-40). During the rest of rat ontogeny it rises instead proportional to ventricular growth (*37:* ***Supplement 1***) (Fig. 3).

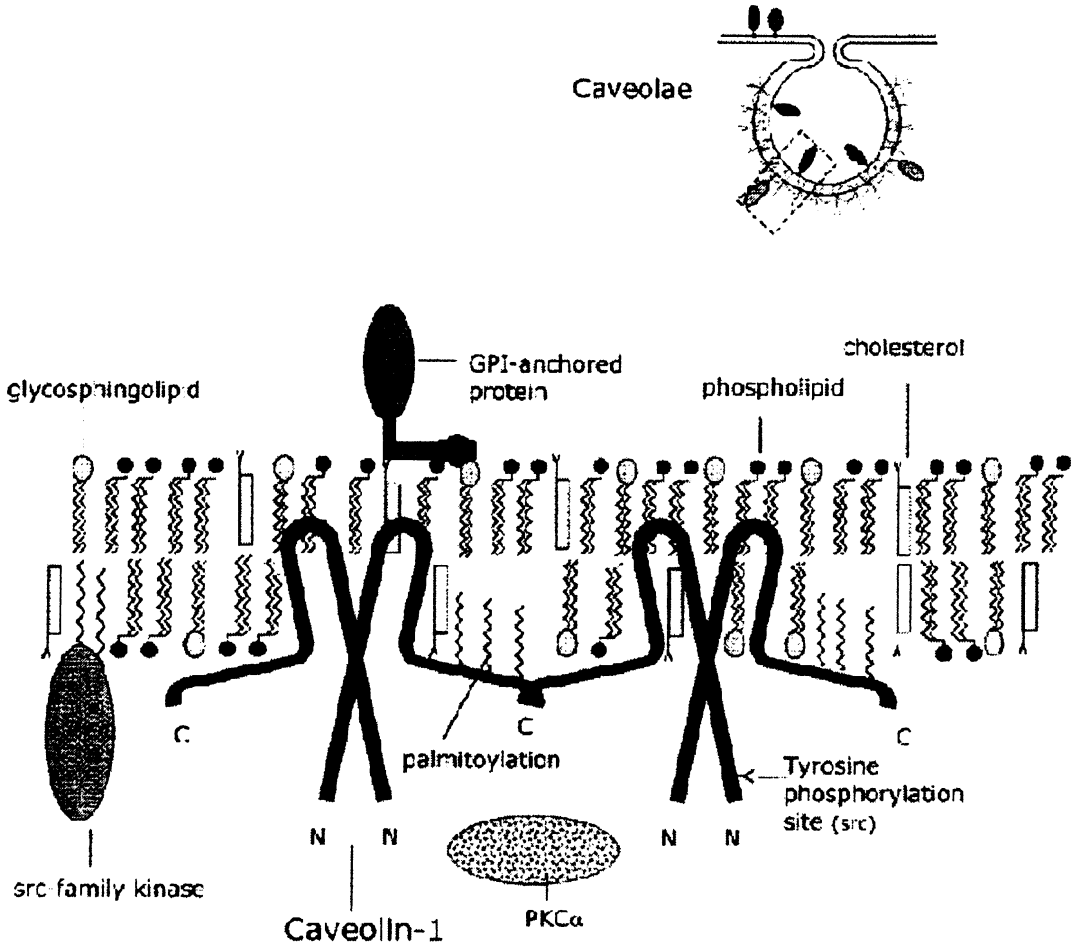


Fig. 2. Schematic representation of caveolae. The caveolae are small invaginations of the plasma membrane (50-100 nm) with the particular lipid content. They are enriched in proteins associated with the membrane via lipid anchors, such as glycosylphosphatidylinositol (GPI)-anchored proteins and non-receptor tyrosine kinases of the src-family, many receptors (not shown) and cytosolic signaling proteins, such as PKCα. Caveolins are important for the structure of caveolae, thanks to their ability to oligomerize and bind cholesterol, and serves as a docking site for binding signaling molecules. However, they are not the essential components of the membrane domains. Adopted from (*36*).

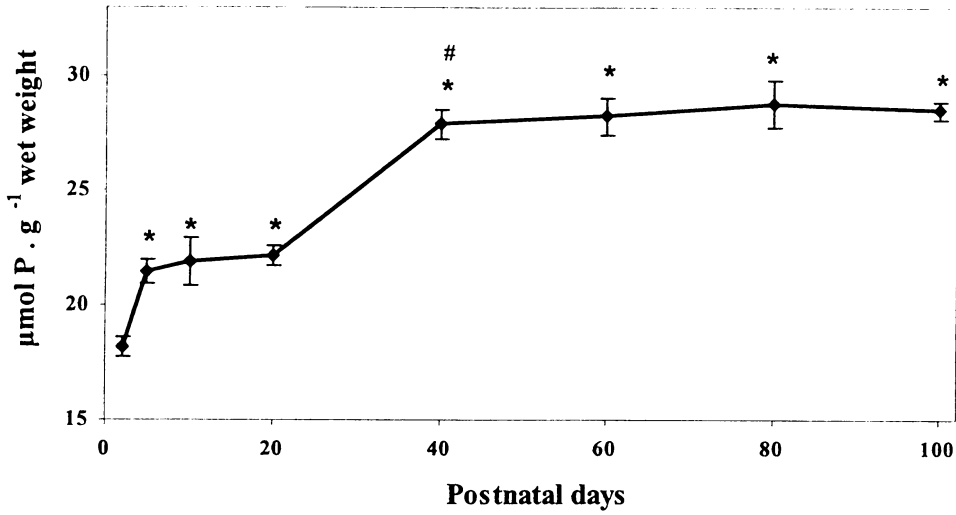


Fig. 2. Concentration of total phospholipids in left ventricular myocardium on postnatal days 2, 5, 10, 20, 40, 60, 80 and 100. Values are mean ± S.E.M. from six experiments. * $p < 0.05$, significant difference compared with d2; # $p < 0.05$, significant difference compared with the previous stage. Adopted from (*37:**Supplement 1***) and modified.

The detailed inspection of changes in individual PL species demonstrated that mainly choline phosphoglycerides (PC) and ethanolamine phosphoglycerides (PE) are responsible for this non-continual rise during early postnatal development, since these two major PLs account for almost 80 % of total PLs in the neonatal rat heart (*37: **Supplement 1***). Kumar and Chaudhuri (*28*) published a similar increase in the amount of total PLs, PC and PE, but with a different time course. They observed a slight increase by d7 and then transient pronounced elevation by d14 followed by a fall by d21 and almost no change by d28. Gudmunsdottir and Gudbjarnason (*38*), who followed the same developmental period, did not observe this barely explicable increase in the concentration of major PLs by d14. They found a more or less gradual increase in the content of PC through d21 and in that of PE even through the 2nd postnatal month.

Amazingly, Tappia et al. (*39*) observed the tendency toward stepless decrease in the content of major PLs during the whole time from birth until d21. Considering the hyperplastic growth of the myocardium and huge enlargement in the mass of membrane structures occurring in early rat ontogeny, the increase in the amount of major cardiac PLs seems instead to be the more probable observation. PE is thought to be crucial during cardiomyocyte proliferation: there is evidence that this aminophospholipid plays a pivotal role in cytokinetic process (*40*). PE resides in the inner leaflet of the majority of eukaryotic plasma membranes (*41*), inclusive of that of rat myocytes (*42*), but it was found exposed to the cell surface during cytokinesis (*43*). It is probably localized in PE-rich domains (*40*), which tend to form a nonbilayer hexagonal structure that has been shown to regulate various membrane-bound enzymes, such as phospholipase D (*44*) and protein kinase C (PKC) (*45*). On the other hand, PC is more disposed to maintain the lamellar organization of membranes due to its large polar headgroup, which provides an equilibrium of lateral pressures between the headgroup and acyl chain levels (*46*). Besides, PC plays an important role in PKC signaling (*47; 48*).

PC and PE occur not only as diacylglycerophospholipids but their *sn*-1 ether-linked analogues, plasmalogens, are present in relatively high amounts in the rat myocardium as well (*37; 42*). Ether PLs are less polar than their ester analogues because of the absence of the ester carbonyl dipole. This fact results in molecular conformation changes and consequently in the different physico-chemical properties of the lipid bilayer (*49*). There is a huge variety in plasmalogen content among animal cardiac membranes. The amount of choline plasmalogen (PLPC) is rather high in human, rabbit, dog and guinea pig myocardium (up to 20–40 % of PC), whereas in rat, mouse and hamster it

comprises only 3–8 % of PC (*50*). Ethanolamine plasmalogen (PLPE) concentration is reported approximately 5-fold higher than that of PLPC in rat heart (*37; 51*). As for developmental changes in PLPC, its proportion drops transiently to minimum by d5, then grows again by d10, and does not change dramatically after, except for the decrease during the suckling-weaning transition. PLPE proportion falls within the suckling-weaning transition (*37:* ***Supplement 1***). The dramatic changes in cardiac PLPC and PLPE proportion during early rat ontogeny might be in connection with the postnatal changes in intracellular Ca^{2+} transients that take place in the first postnatal weeks as well (*52*). This suggestion is supported by the preferential distribution of the plasmalogens in the inner sarcolemma (*42; 53*) and sarcoplasmic reticulum (*54*) and by their propensity for inverted hexagonal phase formation (*55*), which are indications of their participation on ion transports (*56*). In line with this, Ford and Hale (*57*) showed that the plasmalogens provide a critical lipid environment for the regulation of the trans-sarcolemmal sodium-calcium exchanger. The plasmalogens also play an important role as the source of second messengers: ether-linked diglycerides, the effective activators of PKC (*58*), and arachidonic acid (20:4n-6) (*53*), the precursor of eicosanoids and a potent signaling molecule (*59*).

The concentration of diacylglycerol (DPG), which is a marker of the inner mitochondrial membrane, increases markedly by d40. This almost 3-fold increase suggests that membranes of mitochondria are the predominant structures accounting for the growth of total PL concentration during the first five weeks of the postnatal period (*37:* ***Supplement 1***). It has been shown that the interval between d1 and d4 was associated with the rapid and large accumulation of mitochondria and myofibrils in rabbit

left ventricular myocardium. Mitochondria were packed more densely with cristae and the area of mitochondrial inner membrane per unit of myofibrillar volume increased progressively throughout the perinatal period (*60*). The developmental changes in DPG distribution also indicate that mitochondrial membranes grow disproportionately compared with other membrane structures from d5 till d60; their relative proportion increased at the expense of PC and all minor PL proportion (*37:* ***Supplement 1***). On the contrary, Rogers (*61*) found lower proportion of DPG in adult rats as compared with newborns, although the distribution of other PLs did not considerably differ from our results in both developmental stages (*37:* ***Supplement 1***). This observation may be disregarded, as Rogers reported unusually low DPG proportion in adult cardiac membranes (less then 3% of total PLs), which is inconsistent with the observation that the area of mitochondrial inner membrane exceeds by 16-fold and almost 41-fold that of the sarcoplasmic reticulum and sarcolemma, respectively, in adult rat myocytes (*62*). The rapid increase in DPG amount in the neonatal rat heart is confirmed by several incorporation studies using radioactive precursors. Kako et al. (*63*) showed that the synthesis of phosphatidic acid, the precursor of glycerophospholipids, is 1.3-fold higher in mitochondrial and 4-fold higher in microsomal fractions from newborn rat hearts than in adults. The rate of DPG biosynthesis was 4.5-fold higher in mitochondria isolated from neonatal rat hearts than in that from adults (*64*). The elevated DPG biosynthesis in neonatal mitochondria reflects its importance for the incorporation and regulation of proteins in mitochondrial membranes during maturation. DPG is known to be associated with several inner mitochondrial membrane proteins (*65*), the best characterized is DPG interaction with cytochrome c oxidase. Paradies et al. (*66*) demonstrated the close linkage

between the content of DPG in the mitochondrial membrane and cytochrome c oxidase activity in adult myocardium. In agreement with the rise of DPG during early postnatal ontogeny (*37:* ***Supplement 1***), a 2-fold increase in the specific content of cytochrome c oxidase was found between birth and d30 in neonatal mitochondria (*15*).

Although phosphatidylinositol (PI) constitutes below 6% of total PLs in the ventricular myocardium, this quantitatively minor PL plays a major role in signal transduction. We observed maximal PI concentration in ventricular homogenate on d5 (*37:* ***Supplement 1***), which is in line with the finding that the rate of PI biosynthesis is 2-fold higher in the microsomal fraction isolated from 5-day-old rat hearts as compared with adults (*64*). Together with the increase in PI concentration on d5 (*37:* ***Supplement 1***), we observed a significant drop in the concentration of myocardial DAG, the product of phosphoinositol 3,4-bisphosphate breakdown after G protein-coupled receptor stimulation (*67:* ***Supplement 4***). Thus, the results suggest a blockade of G protein/PLC/PKC signaling between d2 and d5, within the narrow period of the hyperplasia-to-hypertrophy switch (*3*).

As for developmental changes in phosphatidylserine (PS), its concentration tends to rise by d5; then it falls significantly by d10 and does not change further on (*37:* ***Supplement 1***). This minor aminophospholipid is distributed primarily in the inner leafleat of the plasma membrane (*42*), where it serves as the cofactor of PKC. Besides this, the exposure of PS on the plasma membrane surface is one of the earliest events in apoptosis (programmed cell death) (*68*).

The last minor but still detectable PL component in cardiac membranes is sphingomyelin (SM), the content of which is about 4 % in newborns and declines to half

of that figure in adulthood (*37:* ***Supplement 1***). In contrast to PI and PS, SM is distributed mainly in the outer monolayer of the sarcolemma (*42*) and can be concentrated in lipid rafts and caveolae, as was mentioned above. Upon various stressful stimuli, cells metabolize SM to sphingosylphosphorylcholine or ceramide, which can be further metabolized first to sphingosine and then sphingosine-1-phosphate. All of these SM metabolites are able to alter intracellular Ca^{2+} release in the heart, and thus reduce cardiac function in most cases (*69*). Sphingosine was reported as a potent inhibitor of PKC (*70*). During the past two decades, data have emerged that support an important role for ceramide and sphingosine-1-phosphate in the regulation of cell proliferation, cell-cycle arrest, apoptosis and angiogenesis (*71*).

3.1.2. Developmental changes in the fatty acid composition of phopsholipids

Although individual myocardial PLs have a relatively different composition of FAs, their profiles undergo similar changes during ontogeny, as revealed by our developmental study (*37:* ***Supplement 1***). In brief, the proportion of saturated FAs grows during the first 10 postnatal days and then declines until adulthood; meanwhile, the proportion of monounsaturated FAs declines from birth until d10 and rises subsequently, with the exception of DPG, in which the gradual decline endures until adulthood. As for polyunsaturated FAs (PUFA), the marked decrease in the n-6 class, and conversely, the increase in the n-3 class, occur during the suckling period, with the opposite tendency after weaning in both classes. The developmental shifts in the PUFA profile of DPG differ from the other PLs. There is a slight decrease and increase in n-6 and n-3 classes by d10, respectively, followed by more pronounced inversed changes lasting into adulthood.

It should be stressed that the FA profile of DPG shifts the most remarkably among membrane PLs during heart ontogeny. In newborns, the profile of DPG resembles that in other PLs, but it becomes very different in later stages of ontogeny. All FA classes are predominated by n-6 PUFAs, namely by linoleic acid (18:2n-6), since d60 (*37: **Supplement 1***) (Fig. 4).

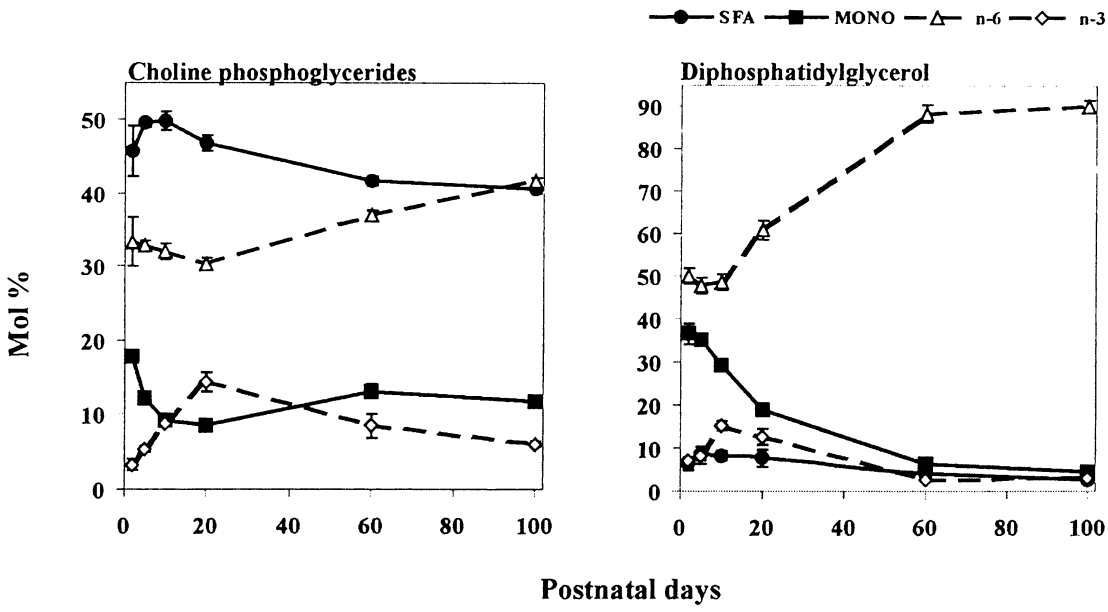


Fig. 4. Composition of main fatty acid (FA) classes in choline phosphoglycerides and diphosphatidylglycerol in left ventriclar myocardium of rats on postnatal days 2, 5, 10, 20, 40, 60, 80 and 100. SFA, sum of saturated FA; MUFA, sum of monounsaturated FA; n-6, n-3, sum of corresponding polyunsaturated FA. Values are means ± S.E.M. from six experiments. $*p<0.05$, significant difference compared with d2; $^{\#}p<0.05$, significant difference compared with the previous stage. Adopted from (*37: **Supplement 1***) and modified.

Lee et al. (*72*) demonstrated that the FA composition of DPG alters dramatically again with aging: the amount of 18:2n-6 decreases in favor of 20:4n-6 and docosahexaenoic acid (22:6n-3) in 24-month-old rats as compared with 4-month-old ones. The acyl chain

specificity of DPG is believed to occur *via* remodeling, as the enzymes responsible for its *de novo* synthesis are not acyl group specific (*73*). The functional significance of the unique DPG profile still remains unknown. However, the coincident changes in mitochondrial cytochrome c oxidase activity (*66*) and DPG remodeling (*37:* ***Supplement 1***) suggest the importance of the high 18:2n-6 proportion in DPG for the proper function of this key mitochondrial enzyme. In line with this, cytochrome c oxidase was stimulated most effectively by DPG rich in 18:2n-6 in reconstituted vesicles (*74*). On the other hand, the least expressive developmental changes in FA profile were observed in PS. This remains highly saturated during all postnatal ontogeny (*37:* ***Supplement 1***) as well as under various pathological states of the heart, including hyperthyroidism, chronic hypoxia and constricted abdominal aorta (*27; 75; 76*). This exclusive constancy in FA composition seems to be connected with the direct participation of PS in cell signaling. As noted, this PL is the most well explored "eat me" signal, which is exposed to the cell surface very early after the onset of apoptosis (*68*), the apoptotic cell is then recognized and removed by phagocytes (*77*). Likewise, highly saturated PS is specifically recognizes by PKC in the membrane (*78*).

Several factors may play a role in developmental changes in the FA profile of cardiac membrane PL, including nutrition, metabolic transition, hormonal changes and increasing workload. The results of dietetic studies demonstrate that the FA composition of rat heart and other organs are highly responsive to dietary n-3 and n-6 long chain PUFAs during the postnatal life (*79; 80*). Additionally, it was shown that reduced maternal protein intake during pregnancy in rats alters the FA composition of membrane PLs in offspring after weaning (*39; 81*). Thus, only the analyses with the same diet can

be taken into comparison. In the beginning of postnatal life, rats are solely sustained by their mother's milk; the FA profile of heart PLs is thereby substantially influenced by the quality and quantity of milk fat (*82; 83*). The composition of breast milk changes during lactation (*84; 85*) and the FA profile of milk TAGs shows marked interspecies differences for the chain length and saturated FA/PUFA ratio: whereas human milk contains a large proportion of long-chain PUFA (*84*), in other species (cows, rats) the amount of myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1n-9) and 18:2n-6 predominates (*38; 85*). However, in our developmental study and that of Gudmundsdottir and Gudbjarnason (*38*), a high amount of PUFAs incorporated into myocardial PC and PE of suckling rats was observed, which is not in line with the FA composition of rats' milk. As the standard diet was used in both studies, the high content of PUFAs in PLs suggests the involvement of endogenous sources in myocardial PL turnover during early ontogeny. Considering a limited ability for FA synthesis (*86*) and desaturation (*87*) in the myocardium, PUFAs in PLs must be derived from plasma TAGs and/or nonesterified FAs originating from shorter chain precursors by the desaturation-elongation process in the liver (*88*). In conformity with this, a marked quantitative increase in long-chain FAs in TAGs was observed in rat liver during the first postnatal week (*89; 90*). This unique accumulation of long-chain TAG species during early postnatal development, especially in the liver, indicates their important role as a pool of PUFAs for membrane PL synthesis in different tissues. Schroedl et al. (*91*) underlined the significance of exogenous nonesterified FAs (NEFA) in the membrane biosynthesis of the developing heart. They showed that most of the NEFA incorporated into cultured neonatal cardiomyocytes were directed towards polar rather than neutral lipid stores. These results contradict the

classical view, derived from adult hearts, that exogenous NEFAs are directed primarily to the pathways of oxidation or to intracellular deposition in TAG form.

18:2n-6 and 20:4n-6, the main n-6 PUFAs in myocardial PLs, have quite different levels at birth: the relative content of 18:2n-6 is low, whereas that of 20:4n-6 is high. During suckling-weaning transition, 18:2n-6 starts to increase, probably due to its high content in solid food. In contrast, 20:4n-6 declines gradually in all PLs after weaning (*37:* ***Supplement 1***). Gudmundsdottir and Gudbjarnason (*38*) observed a marked increase in 20:4n-6 between d1 and d7 in PC and PE; however, more detailed analysis in our study showed that the maximal proportion of 20:4n-6 is already reached on d2, and then until d10 the level is maintained or declines slightly in all PLs (*37:* ***Supplement 1***). Ghebremeskel et al. (*92*) also did not detect the maximal elevation of the 20:4n-6 content, as they analyzed the FA profile of PC and PE in rat liver and heart at delivery and on d15 only. The substantial rise of 20:4n-6 in rat myocardium after birth in PLs is in line with the results of Decrok et al. (*93*), who showed that the main PUFA in myocardial PLs was 20:4n-6 on d2 post-hatch in king penguin, although there was a preponderance of n-3 PUFAs in the yolk. Considering that the shift of 18:2n-6 and 20:4n-6 in PC, and the replacement of both by 22:6n-3 in PE observed just after birth, copy closely the alternations in FA composition in cardiac membranes after the administration of catecholamines (*94; 95*), this membrane modification reflects an early response of the heart to the stress connected with the transition from fetal to neonatal life. As follows from the study of Matos et al. (*96*), the FA composition of membranes from neonatal hearts does not dramatically differ from that of neonatal cardiomyocytes. However, there is an exception which just regards the ratio of 18:2n-6 and 20:4n-6. The relative

proportion of these n-6 PUFAs in major PLs is shifted as older animals were analyzed, which imitates exactly the state of the heart without neonatal stress. During aging, from the 3rd until the 23rd month, the level of 18:2n-6 decreases in PC and that of 20:4n-6 increases in PC and PE again in rat heart (*97*). Gudbjarnason and Benediktsdottir (*95*) illustrated that the specific developmental changes in the PUFA profile of the cardiac membrane, the replacement of more unsaturated FAs by shorter and less unsaturated FAs in both major PLs, coincided with the down-regulation of β-adrenoreceptors.

The gradual elevation of 22:6n-3 content in all PLs during the suckling period observed in our and other studies (*38; 92*) cannot be explained by nutritional intake only. Its content in mothers' milk even decreases during the development of rats (*98*) and human neonates (*99*). The process of hormonal maturation during postnatal development offers more acceptable enlightenment for 22:6n-3 increase. As was mentioned above, the plasma level of TH in the rat increases gradually until the third postnatal week, which resembles the time course of the increase in 22:6n-3 in cardiac PLs. The growth of 22:6n-3 in the neonatal heart can even be accelerated by the hyperthyroid state (*27: **Supplement 2***). The developmental rise of this n-3 PUFA can be also related to increasing adrenergic responsiveness in the developing rat heart (*22*). The stimulation of the heart by catecholamines leads to an increase of 22:6n-3 in myocardial PLs of adult rats (*95; 97*). Benediktsdottir and Gudbjarnason (*100*) even demonstrated that epinephrine administration induced the analogous changes in major PL composition despite large differences in initial levels of individual PUFAs in rats fed various diets. Similar remodeling of FA composition was observed in the myocardium in response to different stress conditions, such as exposure of immature (*101*) or adult rat heart to

chronic hypoxia (*75*) and induction of pressure overload (*102*). Gudbjarnason et al. (*103*) described the positive correlation between heart rate of mammals, ranging from mice to whales, and 22:6n-3 content in their myocardial PLs. Similarly, Hulbert et al. (*104*) reported substantial allometric decline in 22:6n-3 content in PLs of the heart, skeletal muscle, liver and kidney with increasing body mass. These trends support the hypothesis that the relative content of 22:6 n-3 can acts as a membrane pacemaker for metabolic activity. Although its mode of action is not quite clear, the fundamental role of 22:6n-3 in membranes of many tissues is evident. This unique PUFA is known to readily incorporate into PLs and thus significantly alter the basic properties of membranes, including fluidity and permeability (*105*). Recently, 22:6n-3 in PLs has appeared to play an important role in the formation of lipid rafts (*106*) and thus modify the function of proteins for which these respective regions provide a platform (*107*). Many dietetic studies suggest that the replacement of n-6 by n-3 PUFA in membrane PL might have beneficial effects on the heart and reduce the risk of sudden death (*108; 109*).

3.1.3. Phospholipid composition under thyroid hormone control

As was mentioned above, heart maturation is under TH control. The majority of studies has used a hypothyroid experimental rat model treated with propylthiouracil, the inhibitor of the T_4–to-T_3 conversion, and a hyperthyroid one treated with T_4 or T_3 for the observation of TH effect on developing myocardium. It has been well documented that hypothyroidism induced just after birth inhibits postnatal maturation of ventricular tissue whereas hyperthyroidism accelerate this process (*24; 25; 25; 110*).

The changes in PL composition of membranes from immature rat heart are described in our study (*27:* ***Supplement 2***) and that of Vasdev et al. (*26*). In both, the concentration of major PLs decreased in the hypothyroid myocardium, and it increased in the heart after T_3 administration during the early postnatal period. This is in line with the observation that T_4 stimulates biosynthesis of DPG and PC (*111; 112*) in the adult heart. When the thyroid state of rats was altered in the prenatal period through their mothers, the amount of total PLs, PC and PE decreased in cardiac membranes of hypothyroid rats in both fetal and neonatal age. However, only the values by postnatal d7 are available because of the huge mortality of offspring born to hypothyroid mothers (*28*). Surprisingly, similarly decreased concentrations of total myocardial PLs in fetuses and of PC in 14-day-old neonates were observed in the hyperthyroid group of rats in this study (*28*), in contrast to our results (*27:* ***Supplement 2***) and those of Vasdev et al. (*26*).

A decreased amount of major PLs in hypothyroid young rats suggests that normal maturation of cardiac membranes is delayed compared to euthyroid controls. There are several factors that might play a role in slowing down this process. Firstly, the stimulative effect of THs on the expression and activity of enzymes involved in PL metabolism is lacking in hypothyroid rats (*113; 114*). The nutrition deficit might be another reason for the reduction of PL amount in membranes of immature hearts, as hypothyroid mothers are hypolactating (*115*), thus, neonates do not receive an adequate amount of important substrates for PL biosynthesis *via* milk (*116*). The cardiac hypofunction with low heart rate, low velocity of contraction and low output resulting from hypothyroidism can be an additional cause of restricted membrane PL maturation. On the other hand, the maturation of ventricular membrane structures is accelerated in hyperthyroid rats, as was

demonstrated in the morphological study of Jarkovska et al. (*117*). The membrane growth is unbalanced in myocardium treated with THs during postnatal development, unlike in euthyroid animals (*118*). The contribution of mitochondria to the cell volume increases disproportionately, while the volume of myofibrils remains constant at its normal value (*119*). Page and McCallister (*120*) found elevated numbers and sizes of mitochondria as well as an increased number of cristae in the cardiac muscle of chronically hyperthyroid adults in comparison with controls. Accordingly, we observed a markedly increased relative proportion of DPG, though the concentration was elevated in all major PL, which is the marker of preferential growth of mitochondrial structures in hyperthyroid immature myocardium (*27:* ***Supplement 2***).

FA composition of cardiac PLs is under TH control as well. Hypothyroidism retards FA remodeling of membranes. The FA profile of PC and PE that we observed in 21-day-old hypothyroid rats is like that found in the ventricles of euthyroid newborns (*37; 38; 92*). They differ markedly only in their 18:2n-6 proportion, the content of which is higher in all PLs of hypothyroid rats as compared with euthyroid ones. In a similar way, the content of 18:2n-6 rises in cardiac mitochondria of hypothyroid adult rats (*121*). One possible explanation for this observation is the inhibition of Δ-6 desaturase activity under hypothyroid state, which is illustrated by the decreased 20:4n-6/18:2n-6 ratio. Diminished Δ-6 desaturase activity, the rate limiting step in the conversion of 18:2n-6 to 20:4n-6, was observed in the liver of hypothyroid adult rats (*122*). Aside from this, propylthiouracyl decreased dramatically the proportion of 22:6n-3 in all cardiac PLs, by reason of the unsaturation index decreased in PC, PI and PS (*27:* ***Supplement 2***). Pehowich (*123*) observed the similar decrease of 22:6n-3 content accompanied with a

reduced ratio of n-6/n-3 PUFA in the mitochondria of hypothyroid adult rats. Interestingly, this effect of hypothyroidism was diminished with a n-3 PUFA enriched diet. Decreased unsaturation index was also observed in cardiac mitochondria from hypothyroid adult rats (*121*).

A hyperthyroid state induced just after birth alters the redistribution of PUFAs in favor of the n-3 group in all membrane PLs of the immature rat heart. Hyperthyroidism decreases the proportion of 20:4n-6 in all PLs (*27:* ***Supplement 2***), which might be caused by the enhanced conversion of 20:4n-6 to prostaglandins (*124*). On the contrary, the percentage of 22:6n-3 was found to be markedly higher in membranes of hyperthyroid rats (*27:* ***Supplement 2***). The increased heart rate of hyperthyroid animals might be responsible for this change, as the positive correlation between 22:6n-3 and heart rate of various animal species has been already reported (*103*). The accumulation of 22:6n-3 in cardiac PLs may also result from the altered catecholamine sensitivity of the immature rat heart, because hypothyroidism decreases and hyperthyroidism increases alpha- and beta-adrenergic stimulation in the developing rat heart (*22; 23*). It has been well documented that the stimulation of the heart by increased doses of catecholamines leads to the enhanced accumulation of n-3 PUFAs in heart PLs of adult rats (*100; 125*). Aside from this, the increased proportion of 22:6n-3 can relate to an accelerated remodeling process of PLs, in which the stimulating effect of THs on enzymes of deacylation-reacylation cycle may play a role (*113*). T_4 treatment results in a 1.8-fold increase in cardiac microsomal lysophosphatidylethanolamine acyltransferase activity (*126*) and a 1.6-fold increase in mitochondrial monolysocardiolipin acyltransferase in adult myocardium (*127*). Surprisingly, the FA composition of DPG was found unaltered

in hyperthyroid adult rats (*127; 128*), and the proportion of normally represented 18:2n-6 remained unchanged in young hyperthyroid rats as well (*27:* ***Supplement 2***). As the enzymes of *de novo* DPG synthesis are FA unspecific (*73*), it seems that the elevated DPG biosynthesis (*111*) in hyperthyroid adult rats is accompanied by increased monolysocardiolipin acyltransferase activity (*127*) to maintain the appropriate DPG composition in cardiac mitochondrial membranes. As noted, the specific FA profile of DPG in cardiac membranes is supposed to be important for the proper function of cytochrome c oxidase.

Apparently, THs are essential for the maturation of the PL component in rat myocardial membranes during early postnatal development. The changes in the PL environment might consequently modulate the function of some membrane-associated proteins as was well documented in studies where PL composition was specifically altered (*123; 129-131*).

3.1.4. Phospholipid composition in human heart

The PL composition of rat cardiac membranes changes dramatically during postnatal ontogeny, as noted. I have tried to demonstrate how this remodeling is related to heart maturation. The question is to what extent these changes are similar to that occurring in the human heart, and whether some of these conclusions might be generalized. It should be stressed that there are not many analyses of membrane PLs from the human heart. The specimens are usually collected during open-heart surgeries, which means not from intact hearts and not under the same strict conditions as in laboratories. Nevertheless, all results

from human myocardium are extremely valuable, as they could help to encode the details of heart function and to reduce a threatening number of cardiovascular diseases.

As for ontogenetic remodeling of PL composition in humans, the PL analyses of heart membranes from infants (*132*), children (*133:* ***Supplement 3***) and adults (*134*) showed that the developmental changes are comparable with those observed in rat myocardium (*37:* ***Supplement 1***), although they are not as pronounced. The proportion of PC declines and that of DPG increases gradually during ontogeny of humans, as in rats. Nevertheless, the lowest proportion of SM was found in infants, at variance with rats. This difference might relate to diseases the patients suffered from, since the formation of SM metabolites increases under various stressful stimuli, as was mentioned above. Another discrepancy, the abnormally low percentage of PE in adult patients (only about 20%) (*134*), could be connected with the heart failure of operated patients, as the increased release of 20:4n-6 from PE and other PLs is a usual consequence of cardiac injury (*135; 136*).

The FA profile of human cardiac membrane PLs almost does not alter from birth till adulthood, as can be discerned from the comparison of total PL composition from infant (*132*), children (unpublished data, see Table 1) and adult cardiac membranes (*136; 137*). In all these studies, the proportions of 18:2n-6 and 20:4n-6 were found comparable (both about 20 %), and the proportion of 22:6n-3 tended to increase during postnatal development; nevertheless, no significant correlation was observed between FA remodeling and age in either children or adult patients. This finding indicates that the human heart is more mature in human newborns than in newborn rats. Bruce (*138*) followed developmental changes in FA composition of PLs from human skeletal muscle

membranes, and also found changes only in the prenatal, and not in the postnatal, period of human ontogeny. The comparison of the FA profile of individual PL species from human infant and rat hearts (*37; 132*) demonstrates the higher maturation of the human heart just after birth as well. Although the FA profile of individual PL species is essentially similar in infant and neonatal rat cardiac membranes, the proportion of 18:2n-6 and 20:4n-6 from infants corresponds to that observed in adult rats. The most prominent shift in the developmental curve was observed in DPG, where the percentage of 18:2n-6 in infant myocardium equals to almost 80% of the total FA. Besides, there is a higher proportion of 18:1n-9 and lower proportion of 22:6n-3 in all PLs extracted from infant hearts as compared with rats. The difference in 22:6n-3 is in agreement with the observation that the amount of this PUFA in heart PLs declines with the increasing body mass (*104*).

FATTY ACIDS	***NORMOXEMIC***	***HYPOXEMIC***
16:0	16.3 ± 0.90	15.4 ± 0.70
18:0	17.6 ± 0.88	15.6 ± 0.57
18:1n-9	14.2 ± 0.31	14.9 ± 0.75
18:1n-7	2.30 ± 0.79	1.32 ± 0.28 *
18:2n-6	21.5 ± 0.74	21.5 ± 0.50
20:3n-6	0.85 ± 0.04	1.11 ± 0.08 *
20:4n-6	19.9 ± 1.21	22.4 ± 1.12
22:5n-3	1.07 ± 0.21	1.16 ± 0.10
22:6n-3	2.92 ± 0.40	2.90 ± 0.24
SFA	34.5 ± 1.77	31.8 ± 1.07
MUFA	17.6 ± 0.26	17.2 ± 0.94
n-6	43.1 ± 1.38	46.2 ± 1.35
n-3	4.76 ± 0.58	4.89 ± 0.34

Table 1. Fatty acid (FA) composition of total phospholipids in ventricular myocardium from children with normoxemic and hypoxemic congenital heart diseases. SFA, sum of saturated FA; MUFA, sum of monounsaturated FA; n-6, n-3, sum of corresponding polyunsaturated FA. FA reaching at least 1 mol% are shown only. Values are means ± S.E.M. from five experiments. *$p<0.05$, significant difference compared with normoxemic group.

3.2. PROTEIN KINASE C

PKC is a family of serine-threonine kinases, which are PL dependent; some of them are also Ca^{2+} dependent. There are at least 12 different isoforms, which can be divided into three main groups according to the structure and requirement of activators. Members of the classical group (α, $\beta_{I,II}$ and γ) are Ca^{2+}-dependent and require DAG and 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. Recently, two additional Ca^{2+}- and DAG-independent PKC isoforms (μ and ν) has been identified, however, they differ in the structure from atypical isoforms (Fig. 5) (*139*).

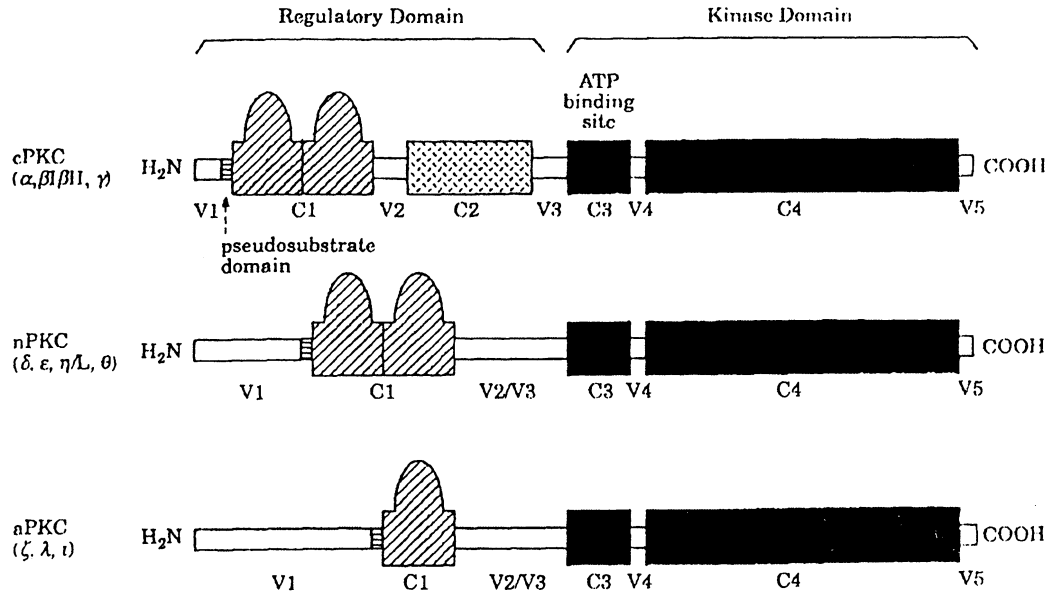


Fig. 5. Schematic representation of the structure of PKC isoforms. The enzymes consist of four conserved domains (C1-C4) and five variable domains (V1-V5). The C1 domain contains two (in case of atypical PKC only one) cystein-rich zinc finger-like sequences. PKCμ/ν contain two cystein-rich sequences, which are unusually widely spread. Adopted from (*140*).

Newly synthesized PKC isoforms remain associated with the plasma membrane until they are phosphorylated on three highly conserved positions within the catalytic domain. The upstream 3-phosphoinositide-dependent protein kinase-1 is responsible for the initial phosphorylation at the activation loop, which is followed by two auto-phosphorylations (Fig. 6). In case of atypical isoforms, the phosphorylation at the activating loop is sufficient for maximal kinase activity. Conversely, the activation of classical and novel isoforms is dependent on the interaction of DAG and PS with the regulatory domain (*141*).

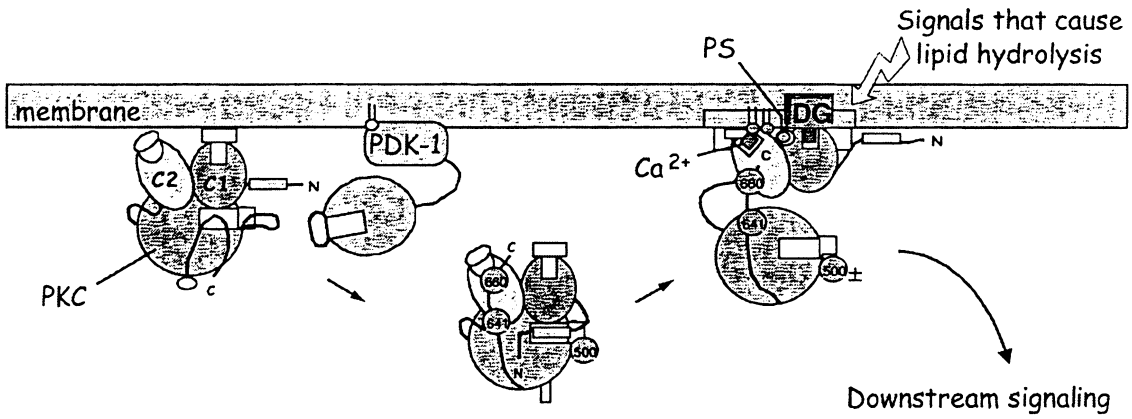


Fig. 6. Schematic representation of two-step activation of classical PKC isoforms. The membrane-associated PKC is phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK-1) on its activation loop, which is immediately followed by the autophosphorylation of two positions in C1 and C2 domains. The enzyme is then released into cytosol where it maintains in the inactive conformation until it is "attracted" by an increased concentration of diacylglycerol (DG) in the membrane after receptor stimulation. The specific binding of diacylglycerol and phosphatidylserine (PS) provides the energy to release the pseudosubstrate domain from the substrate-binding cavity and thus activate PKC for downstream signaling. Adopted from (*142*).

Membrane PLs and their metabolites can modulate PKC activity in two ways – by direct binding into the protein or by the incurrence of a change in the membrane

environment. Both effects are independent, but they can act synergistically. PKC inserts into the membrane hydrophobic region through the C1 domain. DAG increases the affinity of the enzyme for PS and Ca^{2+} (*78*). PUFAs, which could be produced concomitantly after cell stimulation under phospholipase A_2 catalysis (*143*), act in synergy with DAG, and together almost fully activate PKC at the basal level of Ca^{2+} concentration (*144*). DAG and PS bind to specific sites in the C1 domain, providing the energy to release the autoinhibitory pseudosubstrate domain from the active site of the enzyme (*78*). The activation of PKC is stereospecific for 1,2-sn-DAG (*145*), although Ford et al. (*146*) demonstrated that PKC could be activated by ether-linked diglycerides as well. FA chain length and unsaturation are also indispensable factors in PKC activation; DAG with long, saturated FAs are poorer activators (*147*). Furthermore, the dissimilar affinity of DAG kinase, the enzyme terminating DAG action, to particular DAGs play an important role in PKC activation (*148*).

In addition to the role of DAG as the direct endogenous PKC activator, it exerts a variety of effects on the lipid bilayer. There are three major propositions about how DAG-induced changes in the membrane might influence PKC activity. First is the effect of PL head group spacing in the membrane. DAG added into PL vesicles is able to increase the spacing between PL polar groups, which correlates positively with the enhancement of PKC activity. The enhanced access to the bilayer hydrocarbone core probably simplifies the insertion of PKC into the membrane. Second, PKC activity can be stimulated by the formation of nonlamellar phases in the membrane. However, it is not the presence of these phases by themselves, but rather the DAG-induced propensity of the membrane to form nonlamellar structures that is decisive in the activation. The last

suggestion clarifying PKC activation due to changes in the membrane environment is related to lateral phase separation. DAG is not randomly distributed in the membrane, but is concentrated in "domains". The coexistence of DAG-rich and DAG-poor domains is important for the activation of PKC, which is proposed to bind the interfaces between these domains with high affinity (*46*). Together with DAG, other lipid membrane components can modulate PKC activity. PE has similar properties in the membrane to DAG, as it also has a tendency to minimize the head group packing and form non-bilayer phases (*149*). LysoPLs are supposed to induce the formation of DAG-rich domains in the membrane, and thus activate PKC (*150*). Increased PC unsaturation has been demonstrated to activate PKC in PL vesicles (*48*). By contrast, the higher proportion of unsaturated FA in PS decreased PKC activity, which is in line with the priority of PKC to highly saturated PS (*78*).

PKC plays a key role in the regulation of numerous biological processes. In cardiac tissue, PKC appears to modulate ion transport, contractility, cell proliferation and differentiation, hypertrophy and apoptosis (*139; 151-153*). It is apparent that the processes like cell proliferation or differentiation must ultimately be regulated in the nucleus. The transduction of signals to the nucleus was shown to occur either through the mitogen-activated protein kinase pathway, which can be activated by PKC on several levels, or directly by PKC isoforms. PKC can translocate to the nucleus after activation in the cytoplasm, or the isoforms localized in the nuclear matrix can be activated by nuclear activators (*154*) (Fig. 7). It has been shown by several laboratories that the nucleus contains many enzymes involved in PKC signaling, including kinases required for synthesis of PIP_2, phospholipases generating DAG either from PIP_2 or PC, and DAG-

kinase (*154; 155*). The presence of PKC isoforms inside the nucleus allows the enzyme to phosphorylate not only the proteins co-localized with the membrane, but also those localized further inside the nucleus, such as lamin, DNA topoisomerase, DNA polymerase and transcription factors (*156*).

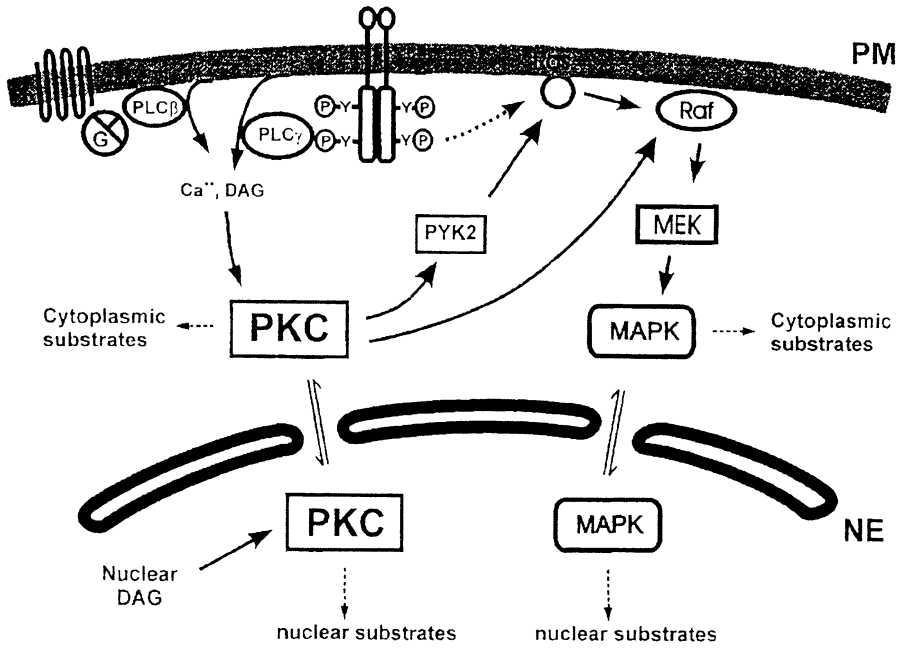


Fig. 7. Signal transduction toward the cell nucleus mediated by protein kinase C (PKC). PKC isoforms can either trigger mitogen-activated protein kinase (MAPK) pathway or they can translocate to the nucleus after the activation, or they may be activated directly in the nucleus. PM, plasma membrane; NE, nuclear envelope; PLC, phospholipase C; DAG, diacylglycerol. Adopted from (*154*).

3.2.1. Developmental changes in protein kinase C isoforms

Different PKC isoforms were immunochemically detected in rat myocardium depending on the age of the animals. In most cases, PKCα, PKCδ, PKCε and PKCζ were identified

in neonates and these isoforms were markedly downregulated during development (*67; 157; 158*). Rybin and Steinberg (*158*) reported that the most prominent decline in PKCα and PKCδ expressions occurred during the first two postnatal weeks. Since we followed the early postnatal development in more detail, our results revealed that PKCδ expression dropped predominantly between d2 and d3, whereas the decline in PKCα was gradual until d10 (*67:* ***Supplement 4***). PKCε expression almost did not differ between fetal and adult myocardium, but it increased transiently on d2 (*158*). We observed that it fell down again between d2 and d3, like PKCδ expression (*67:* ***Supplement 4***). PKCζ, the amount of which was relatively high in fetal myocardium, diminished rapidly after birth and was almost undetectable in adult myocardium (*157; 158*). The lack of immunoreactivity of this atypical isoform in adult isolated cardiomyocytes (*157; 158*), together with its detection in cardiac fibroblast (*158; 159*), suggest that PKCζ resides primarily in nonmyocyte elements.

Since levels of several hormones change dramatically just after the birth (*18; 160; 161*), it appears that the humoral regulations might potentially contribute to developmental changes in the expression and activity of PKC isoforms in rat myocardium. Although the maturation of sympathetic innervation in rat myocardium coincides with the age-dependent decline in the amount of PKC isoforms, its dominant influence has been excluded (*140; 158*). On the contrary, postnatal changes in thyroid state are supposed to play a role in the regulation of the decline of PKC isoforms during ontogeny, as PKCα and PKCε expressions were shown to be repressed by THs in neonatal ventricular myocytes (*162*).

The activity of individual PKC isoforms *in vivo* is dependent not only on their expression level, but also on their subcellular localization and access to isoform-specific substrates and activators (for review see *139*). Inactive PKC isoforms were thought to be present mainly in the cytosol, whereas their activators should be present in membranes. However, immunocytochemical studies revealed that inactive isozymes are localized mostly to subcellular structures as well, and upon activation translocate to new distinct sites (*163*). The rank of association with the particulate structures in rat ventricles is PKCδ > PKCε > PKCζ > PKCα and this association has been found weaker in adult myocardium as compared with neonates (*67; 157; 164*). By contrast, in mouse ventricles, PKCδ and PKCε tended to be more associated with the particulate fraction with increasing age, whereas classical PKC isoforms distribution remained the same throughout ontogeny (*165*). We reported a detailed time course of developmental changes in the subcellular distribution of PKCα, PKCδ and PKCε during the first ten postnatal days (*67:* ***Supplement 4***). The changes in PKCδ and PKCε distribution between particulate and cytosolic fractions were more variable compared with those in PKCα. The association of PKCδ with particulate fractions decreased markedly between d5 and d7, and increased again by d10. PKCε was relatively highly associated with microsomal membranes just after birth, it tended to gradually release from these membranes, and to translocate to nuclear-cytoskeletal-myofilament fraction by d5 (*67:* ***Supplement 4***) (Fig. 8).

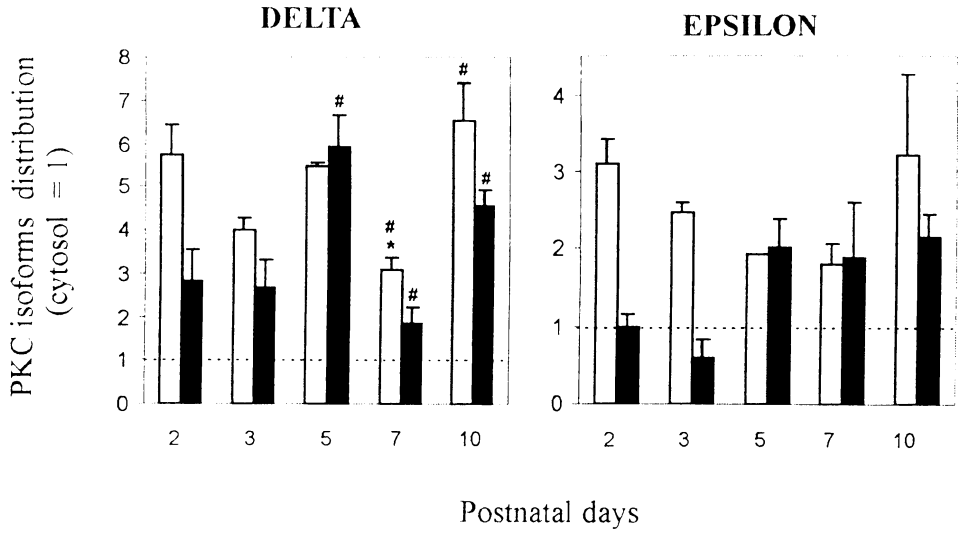


Fig. 8. Changes in the subcellular distribution of protein kinase C (PKC) delta and epsilon in rat ventricular myocardium during the first 10 postnatal days. Values are expressed as membrane-to-cytosol ratio (white columns) and nuclear-to-cytosol ratio (black columns) of PKC isoform abundance. Data are means ± S.E.M. from 3 independent experiments. *p<0.05, significant difference compared with d2; # p<0.05, significant difference compared with the previous day. Adopted from (*67;* ***Supplement 4***) and modified.

The diverse developmental changes in the expression and subcellular localization of PKC isoforms suggest that each of the different PKC isoforms plays a specific and distinct regulatory role in cellular signal transduction during postnatal ontogeny. It appears likely that the isoform-selective phosphorylation of downstream targets should be the basis of isoform-selective function *in vivo*. However, *in vitro* studies did not indicate a large degree of substrate specificity (*166*) as K_m and V_{max} for different substrates did not differ more than 3-fold. For this reason, focus has been turned to subcellular localization to explain the selective phosphorylation *in vivo*. The compartmentalization of PKC isoforms close to the localization of specific substrates appears to determine their function. This is achieved by the isoform anchoring at its specific binding protein called receptor for activated C kinase (RACK) (*163; 167; 168*).

The appropriate compartmentalization of PKC isoforms within the cell seems to be an important factor also for the activation of PKC by DAG. It is well known that after receptor stimulation DAG is rapidly generated by PIP_2 breakdown. This is, at a relatively later phase of cellular response, followed by the second generation of DAG, but this time from PC by the phosholipase D pathway (*169*). PC-derived DAG was thought to provide sustained PKC action; however, a number of studies showed that only DAG derived from PIP_2 is capable of activating PKC *in vivo,* and the function of PC-derived DAG is unknown (*170; 171*). The different FA composition of these DAG pools suggests itself to be the proper explanation, as DAG generated from PC was shown to be rather monounsaturated and saturated, whereas that from PI polyunsaturated (*171*), even though polyphosphoinositides, the direct source of DAG, are high-saturated according to Lamers *et al.* (*172*). Nevertheless, the results from *in vitro* studies have demonstrated that PC-derived DAGs have also been able to activate PKC isoforms (*173*). Thus, different locations of PI- and PC-derived DAGs within the cell appear to be the reason for the inefficiency of PC-derived DAG to activate PKC in vivo, they are probably not co-localized (*174*). It should also be noted that only PI-derived DAG is utilized by a DAG kinase (*148*), which means that only polyunsaturated DAGs are metabolized to PA. This fact could explain the relatively low proportion of PUFA in total DAG that we observed in neonatal myocardium within the first week after birth (*67:* ***Supplement 4***).

The diverse regulatory roles of individual PKC isoforms in the developing myocardium seem to be apparent. Nevertheless, their proper specification is rather complicated. Gene knockout models, which are highly informative in many instances, fail in the case of myocardial PKC due to their diversity, parallel activation, substrate

promiscuity and overlapping function. Likewise, transgenic expression approaches can be misleading because of impaired stoichiometry between the overexpressed enzyme and the endogenous substrates (for review see *175*). On the other hand, transgenic models with the isoform-specific translocation modifiers, which are based on the affinity of isoform-specific binding to RACKs, seems to be highly isoform-specific, mainly due to the maintenance of a normal expression level and the minimally altered basal activity of individual PKC isoforms (*176*). The combination of experiments with these transgenic models has suggested isoform-specific roles of PKC, among others, in the control of myocardial growth.

3.2.2. Protein kinase C isoforms in the control of myocardial growth

During normal postnatal development there is a linear relationship between the increase in body weight and heart weight (*37:* ***Supplement 1***). It is well known that the increase in heart weight is largely attributed to the enlargement of myocytes during ontogeny, as the proliferation of cardiomyocytes ceases soon after birth (*3*). However, what drives the proliferative growth of neonatal myocardium and by which mechanisms it is switched to hypertrophic growth are not well understood. Several signal transduction pathways regulate cardiac growth and/or proliferation (for review see *177*). Recent evidence suggests that normal cardiac growth is largely regulated via signaling through the phosphoinositide-3-kinase/Akt pathway, whereas, the pathological or reactive one is controlled through the G protein/PLC pathway leading to PKC activation (for review see *175*). Nevertheless, i*n vivo* experiments with transgenic isoform-specific PKC translocation modifiers have evidenced essential roles of PKCδ and PKCε in normal

cardiac growth as well (*176; 178*). We found parallel expressive downregulation of these novel isoforms by d3 (*67;* ***Supplement 4***), which provide indirect evidence that they regulate cardiomyocyte proliferation during fetal and early postnatal development.

The pathological hypertrophic growth of myocardium is characterized as an adaptation response of cardiomyocytes to hemodynamic overload produced during pathological states such as hypertension, valvular insufficiency, ischemic heart diseases and/or congenital heart diseases. Mechanical stretch is the initial factor for cardiac hypertrophy in response to hemodynamic overload; however, circulating or locally produced neurohumoral factors induce cardiac hypertrophy as well. At first, the reactive hypertrophy connected with ventricular wall thickening and cardiomyocyte enlargement helps to decrease wall stress and improve contractility, however, when the pathological stimulus is not removed, it leads to maladaptive hypertrophy and heart failure (for review see *179*). Although the pathological hypertrophy is more intensively studied than normal cardiac growth during development, the signaling pathways that are responsible for the regulation of beneficial adaptive hypertrophy and the mechanism of transition to contractile dysfunction still remain incompletely understood. Pressure-overloaded myocardium induced by aortic banding is a well-characterized experimental model suitable for investigation of the development of cardiac hypertrophy and its conversion to heart failure. The G protein/PLC/PKC pathway was proven to be activated to produce this kind of hypertrophy (*180*). It was shown that the myocardium responds differently to pressure overload depending on the time of the intervention. When the aorta constriction is performed just after birth, in the proliferate stage of cardiomyocyte growth, cellular hyperplasia contributes significantly to increased heart mass aside from cardiomyocyte

enlargement (*181*), and the hypertrophied hearts of this unique experimental model do not show the fibrosis characteristic of adult pressure-overloaded models (*182*). Likewise, different PKC isoforms have been found upregulated or activated in pressure-overloaded heart in an age-dependent manner (*183-187*). We observed an increased expression of PKCα, PKCδ and PKCε in the cardiac homogenate of 5-day-old rats banded on d2 (*187*). Since there is probably a functional synergism between normal cardiac growth and pathological growth (*188*), it is hard to interpret this observation. We can only speculate that the temporary upregulation of these isoforms might be a reaction of immature myocardium, which has to overcome further increase of hemodynamic load within the key developmental period of the hyperplasia to hypertrophy transition (*3*). Furthermore, the contribution of PKC isoforms expression from cardiac fibroblasts cannot be excluded, as they comprise two third of cardiac cells (*189*), and isoforms α, δ and ε have been detected there (*159*). Moreover, we found transient translocation of PKCδ and PKCε to particulate fractions as early as on d3 (*186:* ***Supplement 5***). This rapid translocation of novel isoforms is consistent with the shift of PKC to membranes observed immediately after ANG II receptor activation (*190*), and ANG II has been reported to play a role in cardiac hypertrophy due to pressure overload (*191; 192*). In three-week-old rats, Gu and Bishop (*183*) found activated PKCε and upregulated PKCβ two weeks after aortic banding. The function of PKCβ in cardiac hypertrophy is controversial. Although PKCβ overexpression in myocardium led to cardiac hypertrophy and heart failure in an age-dependent manner (*193*), PKCβ-knockout mice demonstrated that this isoform was not necessary for the development of cardiac hypertrophy (*194*). In all accounts, increased PKCβ expression was found in failed human hearts (*195*), which strongly supports its

important role in the development of pathological hypertrophy. As for a role of PKCε, studies on transgenic mice have demonstrated that PKCε activation in pathological cardiac hypertrophy is more compensatory than the pathological event (*176; 196; 197*). In adult pressure-overloaded myocardium, PKCα and PKCδ were markedly upregulated, the latter one even as early as the first day after aortic banding (*184; 185*). These results provide indirect evidence that PKCδ might be involved in the induction of the compensatory hypertrophy in the adult heart after aorta constriction, whereas both PKCα and PKCδ might be responsible for the transition to heart failure. In agreement with this, recent studies with transgenic mice revealed that PKCα is a more important regulator of myocardial contractility (*198*) than cardiac hypertrophy as former analyses proposed (*199*), and the function of PKCδ depends on the strength of hypetrophic stimuli (*178*). A modest increase in PKCδ expression was shown to result in compensated hypertrophy, but on the other hand, high chronic expression of PKCδ evoked cardiomyocyte necrosis and contractile dysfunction (*178; 200*). In line with this, a moderate level in G protein signaling induced compensated cardiac hypertrophy, whereas high-level G protein activation induced cardiomyocyte apoptosis (*201*). These latter observations clearly demonstrate that only the extent of G protein/PLC/PKC pathway stimulation is critical for the transition from compensated hypertrophy to heart failure. Heidkamp et al. (*202*) showed that just PKCδ and PKCε act as a switch between cardiomyocyte hypertrophy and apoptosis, and PKCε selectively activates the mitogen-activated protein kinase 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 depends mostly on the balance of novel isoforms to downstream signaling cascades.

I have tried to review the recent observations about developmental changes in myocardial PKC isoforms and their multiple roles in the regulation of myocardial growth. There is number of *in vivo* and *in vitro* studies, but to keep the text compact and in reasonably broad scope, I have discussed only the most prominent and relevant results of this relatively diverse field of research. The observations regarding PKC in the heart during postnatal ontogeny under physiological and pathological conditions have often been controversial. Various technical factors, including differences in the process of cellular fractionation, antisera or data evaluation, can contribute to the inconsistency of the results. Nevertheless, the main reason for this inconsistency perhaps lies in the difficulty of ensuring the same experimental conditions in all of the studies. The critical factors that can influence PKC acting in the heart are the experimental model and the developmental stage of animals and/or isolated cardiomyocytes. Under pathological conditions, additional factors are the nature and strength of pathological stimuli, and the severity of hypertrophy. Furthermore, the changes in the concentration of individual PLs and their FA profiles in cardiac membranes can markedly affect the expression and activity of PKC isoforms. As was described above, the membrane PLs, which can modify PKC function on several levels, follow dramatic developmental changes and are highly sensitive to the nutritional and humoral changes. For example, an altered diet or thyroid state might take effect on the expression and/or activity of PKC isoforms in the heart.

The use of isolated cardiomyocytes that allow the analysis of PKC under controlled settings diminishes the mentioned irreproducibility of the results; however, the observations from *in vivo* experiments contribute more to cardiovascular research. Determination of multiple actions of individual PKC isoforms in the complex signaling network in the myocardium, resolution of their beneficial and detrimental actions, and elucidation of their signaling in time and space might contribute to the development of new therapeutic approaches to attenuate or reverse heart injury.

3.3. REFERENCES

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4. PUBLICATIONS

1. Novak, F., Tvrzicka, E., **Hamplova, B.,** Kolar, F., and Novakova, O. (2006) Postnatal development of phospholipids and their fatty acid profile in rat heart, *Mol. Cell. Biochem. 293*, 23-33.

2. **Hamplova, B.,** Novakova, O., Tvrzicka, E., Pelouch, V., and Novak, F. (2003) Effect of hypo- and hyperthyroid states on phospholipid composition in developing rat heart, *Mol. Cell. Biochem. 252*, 295-303.

3. **Hamplova, B.,** Pelouch, V., Novakova, O., Skovranek, J., Hucin, B., and Novak, F. (2004) Phospholipid composition of myocardium in children with normoxemic and hypoxemic congenital heart diseases, *Physiol. Res. 53*, 557-560.

4. **Hamplova, B.,** Novakova, O., Tvrzicka, E., Kolar, F., and Novak, F. (2005) Protein kinase C activity and isoform expression during early postnatal development of rat myocardium, *Cell. Biochem. Biophys. 43*, 105-117.

5. **Hamplova, B.,** Novakova, O., Kolar, F., and Novak, F. (2007) Effect of pressure overload on protein kinase C in rat myocardium during early postnatal development, *manuscript in preparation*.

5. SUMMARY

The studies discussed in my thesis deal with the changes, function and regulation of membrane PLs and PKC isoforms during postnatal ontogeny.

The results that we achieved can be summarized as follows:

1. The concentration of total myocardial PLs increases proportionally to ventricular growth in rats, except for the critical developmental periods just after birth and within the suckling-weaning transition, when the increase in total PL concentration is greater than that of ventricular weight. Mainly PC, PE and DPG are responsible for the elevated gain in total PLs during the early postnatal development of rat heart. The concentration of minor PLs changes modestly until d20 and remains unchanged further. The proportion of PLPC and PLPE decreases during ontogeny, with a dramatic time course in the period just after birth and within suckling-weaning transition. ***(Supplement 1)***
2. Every PL species has its characteristic FA profile, which likely coheres with its specific structural and signaling role in cardiac membranes. Nevertheless, the FA profile of any given PL species undergoes qualitatively similar changes during postnatal ontogeny. ***(Supplement 1)***
3. The altered thyroid states induced just after birth influence the maturation of the PL component of cardiac membranes in rats during early postnatal development. Hypothyroidism decreases the concentration of PC, PE and DPG, and increases the proportion of PLPE, whereas hyperthyroidism increases the concentration of PC, PE and DPG, and decreases the proportion of PLPE in the myocardium of 21-day-old rats as compared with euthyroid controls. The FA composition of individual cardiac

PLs also underlies the TH control. Hypothyroidism decreases the ratio of saturated/unsaturated FA in PE and increases the ratio of n-6/n-3 PUFA in PC, PE and PI; on the contrary, hyperthyroidism has opposite effect on the FA composition in these PLs. Both hypo- and hyperthyroidism decrease the ratio of 20:4n-6/18:2n-6 in the majority of PLs. ***(Supplement 2)***

4. The concentration of PLs in ventricular membranes from children with normoxemic and hypoxemic congenital heart diseases does not differ much from that observed in neonatal rats. Membranes of the atrium have a lower content of PC and PE as compared with ventricles in both hypoxemic and normoxemic groups of children. In the ventricular tissue, the concentration of PE and PS is higher in children with a hypoxemic defect; in the atrium, only the PE amount is higher as compared with normoxemic ones. ***(Supplement 3)***
5. The changes in the concentration of cardiac DAG follow a biphasic pattern during the first 10 postnatal days. It declines rapidly by d5 and then increases again. The FA profile of total DAG is rather saturated and does not significantly change in the period of study. ***(Supplement 4)***
6. The total PKC activity measured by the incorporation of ^{32}P into histone IIIS decreases transiently between d2 and d3 and again between d7 and d10 in the homogenate and cytosolic, membrane and nuclear fractions essentially in a similar manner. PKCα, PKCδ and PKCε are markedly downregulated in the early postnatal period, and the expression of PKCδ and PKCε decreases mostly between d2 and d3, whereas, that of PKCα declines gradually until d10. PKCδ and PKCε are predominantly associated with particular fractions, whereas PKCα is more in the

cytosolic fraction. In the membrane and nuclear fractions, the amount of PKCδ and PKCε decrease markedly by d3, return to or close to the initial level immediately on d5 and do not change or decrease slightly later. PKCα exhibits the changes in the nuclear fraction, but is associated with the membrane fraction by d7. In the cytosolic fraction, all isoforms tend to diminish by d10, but with the transient marked increase on d7. ***(Supplement 4)***

7. The pressure overload induced on d2 affects in particular PKCδ and PKCε in the developing heart. The constriction of the abdominal aorta provoked a marked elevation in the amount of PKCδ in the membrane fraction as soon as on d3 as compared with sham operated rats. This effect gradually diminished until d10. The cardiac PKCε associated with the membrane fraction changed essentially with the same trend during the first 10 postnatal days in the pressure-overloaded heart. ***(Supplement 5)***

6. SUPPLEMENTS

SUPPLEMENT 1

Table 1. Weight parameters of intact, sham-operated and aorta-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
SH	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
SH	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
SH	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; SH, sham operation; AC, aorta constriction; n, number of animals.

Figure legend

Fig. 1

The effect of sham operation and aorta constriction on developmental changes in the total protein kinase C activity in cytosolic (white columns), membrane (crosshatched columns) and nuclear-cytoskeletal-myofilament fractions (dotted columns) of rat myocardium during the first 10 postnatal days. Values are expressed as arbitrary units (the sum of specific radioactivity measured on postnatal days 2, 3, 5, 7 and 10 is equal to 1) and as percent of corresponding control values. Data are means ± S.E.M. from 3 fractionations in each age group. [a]$P<0.05$, significant difference *vs.* day 2; [b]$P<0.05$, significant difference *vs.* previous age group; [c]$P<0.05$, significant difference *vs.* intact controls; [d]$P<0.05$, significant difference *vs.* sham-operated controls.

Fig. 2

The effect of sham operation and aorta constriction on developmental changes in the expression of protein kinase C α in cytosolic (white columns), membrane (crosshatched columns) and nuclear-cytoskeletal-myofilament fractions (dotted columns) of rat myocardium during the first 10 postnatal days. Values are expressed as arbitrary units (the sum of specific radioactivity measured on postnatal days 2, 3, 5, 7 and 10 is equal to 1) and as percent of corresponding control values. Data are means ± S.E.M. from 3 fractionations in each age group. [a]$P<0.05$, significant difference *vs.* day 2.

Fig. 3

The effect of sham operation and aorta constriction on developmental changes in the expression of protein kinase C δ in cytosolic (white columns), membrane (crosshatched columns) and nuclear-cytoskeletal-myofilament fractions (dotted columns) of rat myocardium during the first 10 postnatal days. Values are expressed as arbitrary units (the sum of specific radioactivity measured on postnatal days 2, 3, 5, 7 and 10 is equal to 1) and as percent of corresponding control values. Data are means ± S.E.M. from 3 fractionations in each age group. [a]$P<0.05$, significant difference *vs.* day 2; [b]$P<0.05$, significant difference *vs.* previous age group; [c]$P<0.05$, significant difference *vs.* intact controls; [d]$P<0.05$, significant difference *vs.* sham-operated controls.

Fig. 4

The effect of sham operation and aorta constriction on developmental changes in the expression of protein kinase C ε in cytosolic (white columns), membrane (crosshatched columns) and nuclear-cytoskeletal-myofilament fractions (dotted columns) of rat myocardium during the first 10 postnatal days. Values are expressed as arbitrary units (the sum of specific radioactivity measured on postnatal days 2, 3, 5, 7 and 10 is equal to 1) and as percent of corresponding control values. Data are means ± S.E.M. from 3 fractionations in each age group. [a]$P<0.05$, significant difference *vs.* day 2; [b]$P<0.05$, significant difference *vs.* previous age group; [c]$P<0.05$, significant difference *vs.* intact controls; [d]$P<0.05$, significant difference *vs.* sham-operated controls.

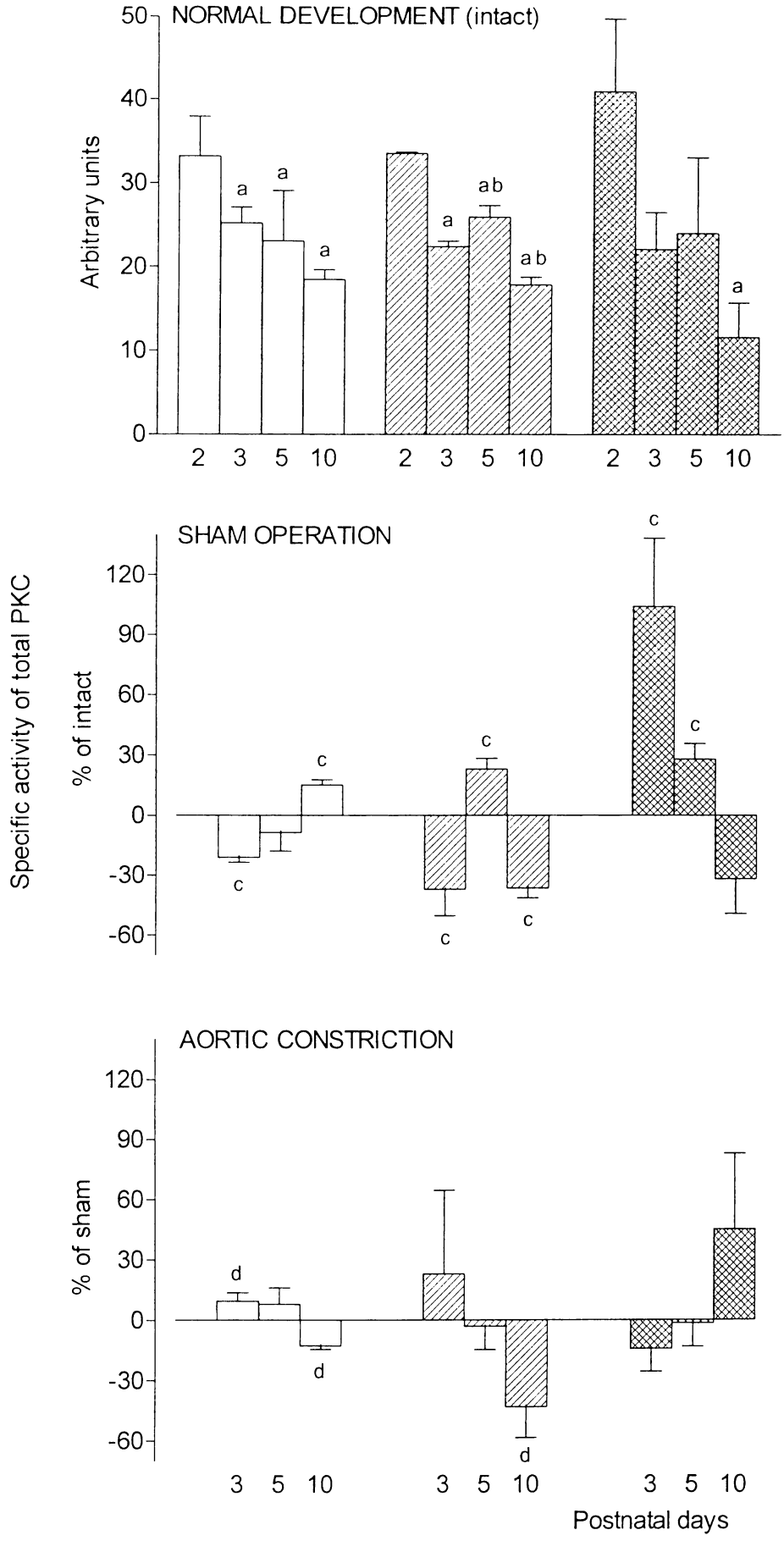
NORMAL DEVELOPMENT (intact)
Arbitrary units
50
40
30
20
10
0
a
a
a
a
ab
a
ab
a
2 3 5 10 2 3 5 10 2 3 5 10
SHAM OPERATION
AORTIC CONSTRICTION
Specific activity of total PKC
% of intact
% of sham
120
90
60
30
0
-30
-60
c
d
3 5 10 3 5 10 3 5 10
Postnatal days

Fig. 1

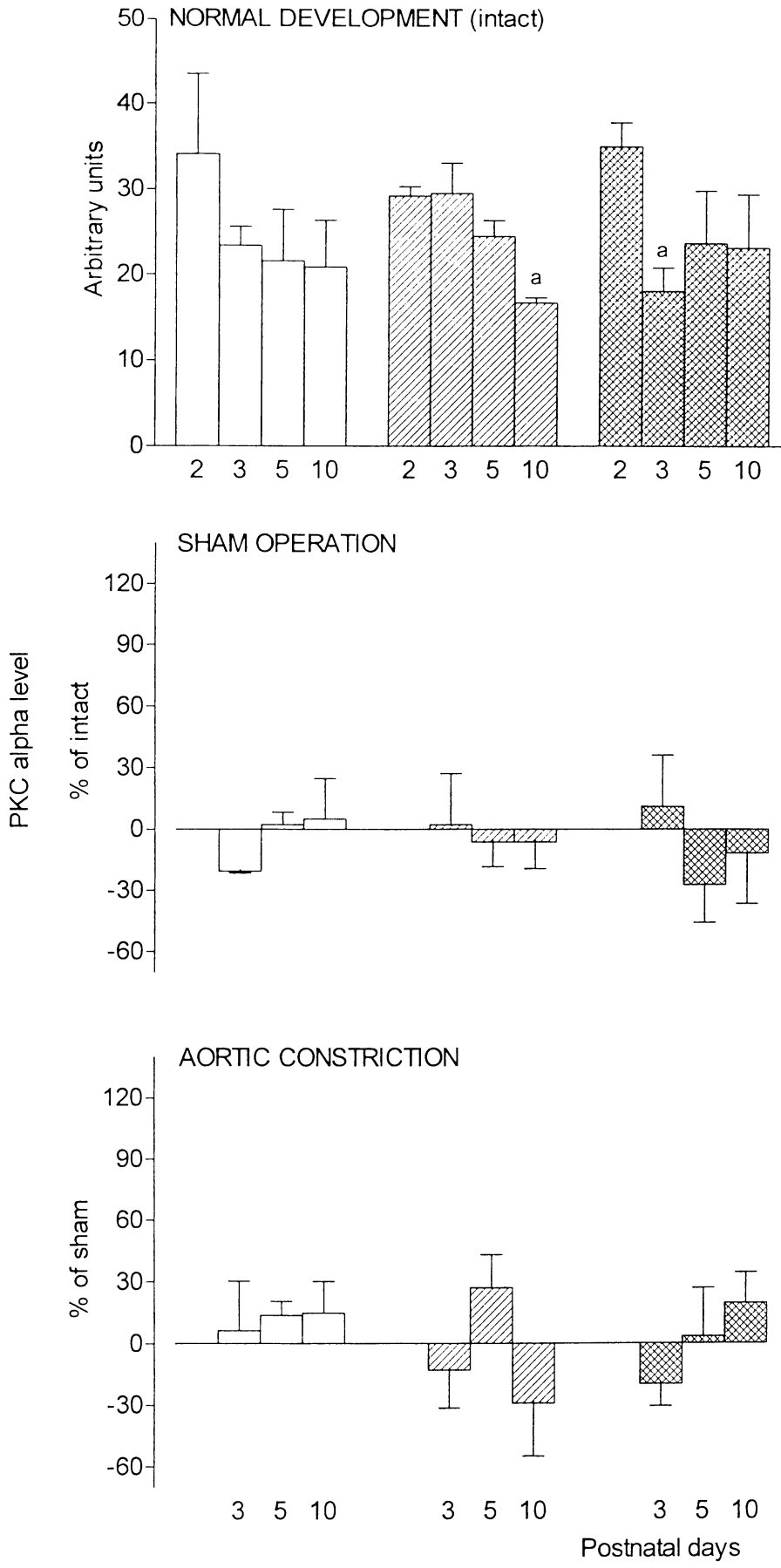
NORMAL DEVELOPMENT (intact)
Arbitrary units
50
40
30
20
10
0
a
a
2 3 5 10 2 3 5 10 2 3 5 10
SHAM OPERATION
PKC alpha level
% of intact
120
90
60
30
0
-30
-60
AORTIC CONSTRICTION
% of sham
120
90
60
30
0
-30
-60
3 5 10 3 5 10 3 5 10
Postnatal days

Fig. 2

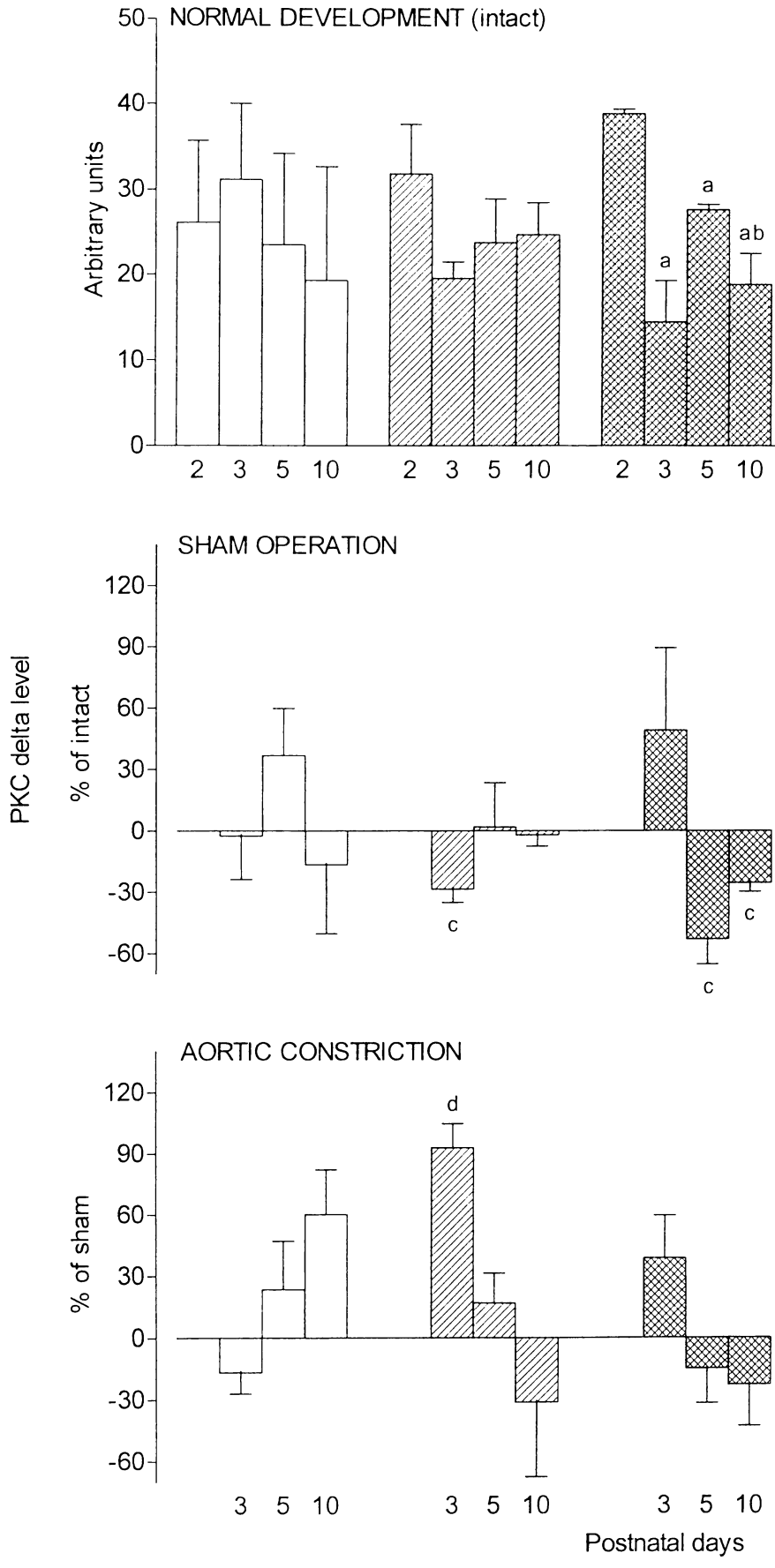
NORMAL DEVELOPMENT (intact)
Arbitrary units
50
40
30
20
10
0
2 3 5 10 2 3 5 10 2 3 5 10
a
a
ab
SHAM OPERATION
PKC delta level
% of intact
120
90
60
30
0
-30
-60
c
c
c
AORTIC CONSTRICTION
% of sham
120
90
60
30
0
-30
-60
d
3 5 10 3 5 10 3 5 10
Postnatal days

Fig. 3

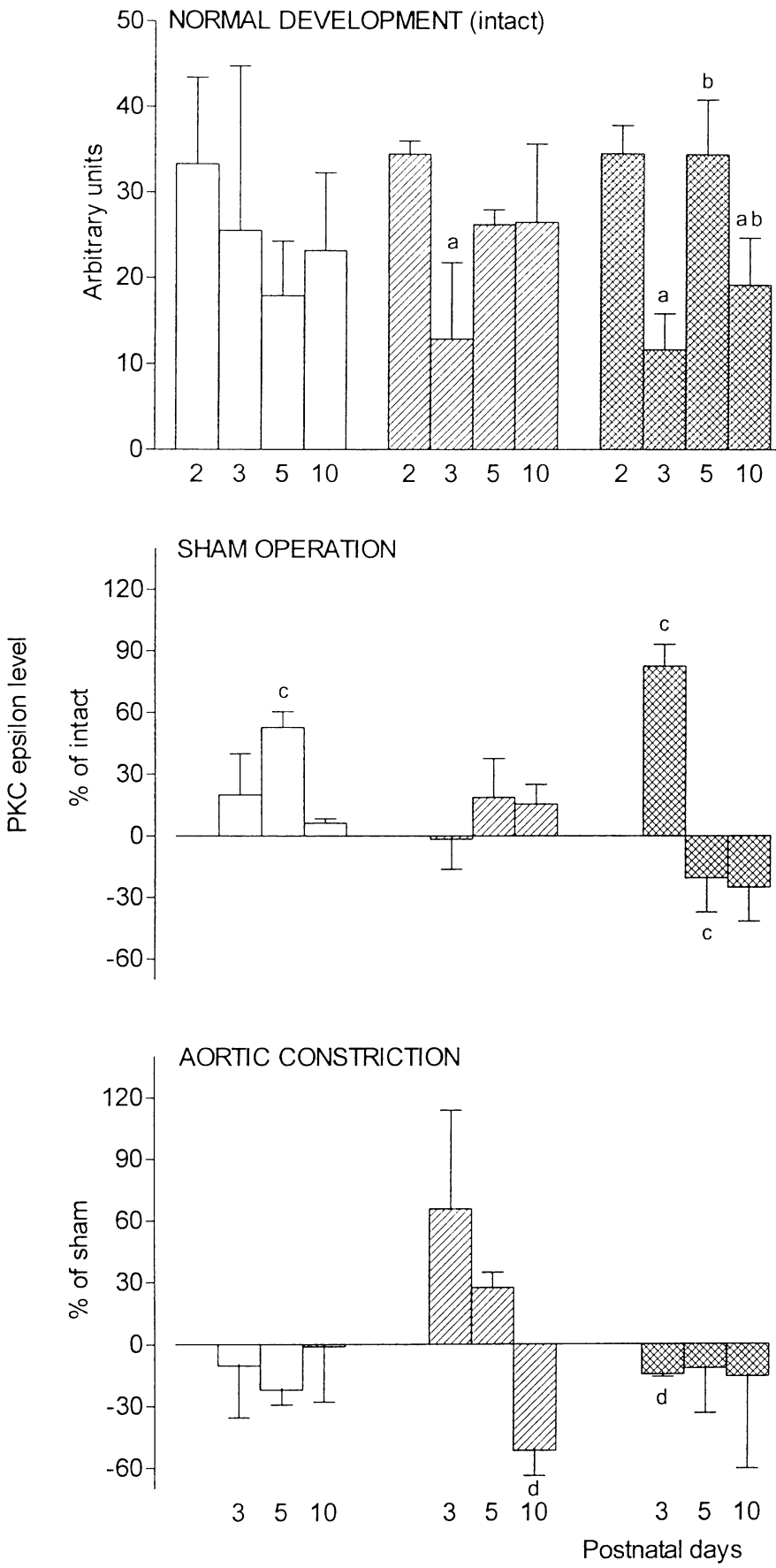
NORMAL DEVELOPMENT (intact)
Arbitrary units
50
40
30
20
10
0
a
a
b
a b
2 3 5 10 2 3 5 10 2 3 5 10
SHAM OPERATION
PKC epsilon level
% of intact
120
90
60
30
0
-30
-60
c
c
c
AORTIC CONSTRICTION
% of sham
120
90
60
30
0
-30
-60
d
d
3 5 10 3 5 10 3 5 10
Postnatal days

Fig. 4