Postnatal changes in contractile time parameters, calcium regulatory proteins, and phosphatases

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GOMBOSOVÁ, Iva, Peter Bokník, Uwe Kirchefer, Jörg Knapp, Hartmut Lüss, Frank Uličník Müllér, Thorsten Müllér, Ute Vahlensieck, Wilhelm Schmitz, Geza S. Bodor, and Joachim Neumann. Postnatal changes in contractile time parameters, calcium regulatory proteins, and phosphatases. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H2123–H2132, 1998.—Compared with isolated electrically driven neonatal ventricular preparations, the total time of contraction, the time to peak tension, and the time of relaxation were decreased to ~50% in adult ventricular preparations. The expression of sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA) was increased to 133% at the protein level and to 154% at the mRNA level in adult vs. neonatal ventricular preparations, whereas phospholamban was unchanged at both the protein and mRNA levels. Moreover, Ca²⁺ uptake was increased to 180% in adult vs. neonatal ventricular preparations. Phospholamban phosphorylation was enhanced in adult vs. neonatal ventricular preparations.

In adult ventricular preparations, phosphatase activity was reduced to 53% of neonatal preparations, the protein levels of the immunologically detectable catalytic subunits of protein phosphatase types 1 and 2A were reduced to 28 and 61% of neonatal preparations, respectively, and the mRNA levels of type 1α, 1β, 1γ, 2Aα, and 2Aβ phosphatase isoforms were decreased to 69, 68, 54, 67, and 63%, respectively. We conclude that in the adult rat heart, the shortened time parameters of contraction can be explained by an elevated expression of SERCA. In addition, an increased phosphorylation state of phospholamban due to reduced phosphatase activity may be involved.

phospholamban; sarco(endo)plasmic reticulum calcium-adenosinetriphosphatase; calsequestrin; contractility

MYOCARDIAL CONTRACTILITY changes during the postnatal development of the mammalian heart (4, 5, 9, 36, 46). A crucial step in the regulation of myocardial contractility is the dynamic alteration of intracellular Ca²⁺ levels by the sarcoplasmic reticulum (SR). Systolic contraction is brought about by an increase in free cytosolic Ca²⁺, whereas relaxation in diastole results from active removal of Ca²⁺ mainly into the SR by the cardiac sarco(endo)plasmic reticulum Ca²⁺-ATPase 2a (SERCA, Ref. 31). It can be hypothesized that altered contractility is associated with a change of Ca²⁺ homeostasis of the SR. Accordingly, a number of studies dealing with the developmental regulation of SERCA have shown that SERCA expression at mRNA and protein levels increases after birth (3, 12, 14, 33). Fittingly, the Ca²⁺ uptake of the SR is higher in adult than in fetal or neonatal mammalian hearts (sheep, Refs. 33, 47; mice, Ref. 14; rat, Ref. 55; rabbit, Refs. 40, 54). However, in principle, Ca²⁺ uptake cannot only be increased by elevation of SERCA protein levels but also by enhancing SERCA affinity for Ca²⁺, which is regulated by the phosphorylation state of phospholamban, a small intrinsic protein of the SR (for review, see Ref. 21). Only dephosphorylated phospholamban inhibits SERCA, whereas phosphorylation of phospholamban relieves this inhibition (52, 59). The β-adrenoceptor agonist isoproterenol increased the phosphorylation state of phospholamban in isolated intact ventricles and increased SERCA activity (24). Moreover, isoproterenol led to phosphorylation of phospholamban on serine-16 and threonine-17 by cAMP-dependent and Ca²⁺/calmodulin-dependent protein kinases, respectively (56). Ablation of phospholamban by injection of a specific antibody (2D12) or by gene targeting mimics the effect of isoproterenol and stimulates Ca²⁺ uptake into the SR (29, 52). These data argue that phospholamban is a prime regulator of basal myocardial contractility and is an important mediator of the β-adrenergic effects in the heart. However, little is known about the expression of phospholamban in neonatal vs. adult rat ventricular preparations. In mouse hearts, phospholamban expression increased after birth at the protein and mRNA levels (14). In rabbit hearts, after birth the phospholamban expression did not change on the mRNA level (3) but increased on the protein level (54). Although a posttranslational regulation of phospholamban expression has been suggested (54), the reason for this discrepancy is unknown. Moreover, it is conceivable that not only the expression but also the phosphorylation state of phospholamban might be subject to regulation. Some recent data support the hypothesis that protein phosphatases play an important role in the regulation of the phosphorylation state of phospholamban. We have demonstrated that phospholamban phosphorylation can be stimulated by cell membrane-permeant inhibitors of serine/threonine phosphatases (for short, phosphatases) type 1 and 2A such as okadaic acid and cantharidin (42, 44). Like β-adrenoceptor agonists, these inhibitors can exert a positive inotropic, positive lusitropic, and positive clinotropic effect (44). On the other hand, activators of phosphatases can dephosphorylate phospholamban and exert a negative inotropic effect (59). Type 1 and 2A phosphatases are the main phosphatases of the myocardium (53, 57). Both phosphatases can dephosphorylate phospholamban (30), and their activity is reduced by inhibitors like cantharidin (16). The catalytic subunits are encoded by separate genes and comprise at least type 1α, 1β, 1γ, 2Aα, and 2Aβ isoforms (for review, see Ref. 39). Bio-

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chemical data on their developmental regulation in the heart are currently lacking, however.

We hypothesized that postnatal alterations of cardiac phosphatases might occur and alter the function of SR proteins, and this could result in postnatal changes in contractility, such as shortened duration of contraction. To test this hypothesis, we have measured cardiac time parameters, SR protein expression, and phosphatase expression in neonatal vs. adult mammalian cardiac ventricular preparations.

MATERIALS AND METHODS

Animals. Sprague-Dawley CD rats from Harlan Winkelmann (Borden, Germany) were used for the present studies. Neonatal rats (within 24 h of birth) and adult rats (220–260 days old) were studied. Rats were killed by a blow to the head, hearts were rapidly removed, atria were discarded, and ventricles were immediately used for contractile studies or freeze clamped with precooled Wollenberger clamps at the temperature of liquid nitrogen and stored at −80°C. This procedure preserves the phosphorylation state of proteins (24, 25, 44). The protocols used in this study were approved by the local animal welfare review board.

Contractions experiments. Ventricular preparations (ventricular strips, ~8 mm length, <0.6 mm diameter) were used. The isolated preparations were mounted and bathed individually in glass tissue chambers for recording isometric contractions (42). The bathing solution contained (in mM) 119.8 NaCl, 5.4 KCl, 1.8 CaCl2, 1.05 MgCl2, 0.42 NaH2PO4, 22.6 NaHCO3, 0.05 Na2EDTA, 0.28 ascorbic acid, and 5.0 glucose, continuously gassed with 95% O2-5% CO2, and was maintained at 35°C and pH 7.4. Isometric force of contraction was continuously gassed with 95% O2-5% CO2, and was maintained at 35°C and pH 7.4. Isometric force of contraction was measured after each preparation was stretched to optimal length. All preparations were initially stimulated at 1 Hz with rectangular pulses of 5-ms duration (Grass stimulator SDS; Grass, Quincy, MA); the voltage was ~10–20% above threshold. Preparations were allowed to equilibrate for 30 min. In some experiments, the frequency was altered and stepwise increased from 0.2 to 1.4 Hz. Time from 10% contraction to peak contraction (TPT) and time from peak to 90% relaxation (TR) were calculated from recordings at high chart speed. Total contraction time (TCT) is the sum of TPT and TR (42, 44). Isoproterenol (10⁻⁵ to 10⁻⁶ M) was then added cumulatively, allowing 10 min for each concentration.

Ca²⁺ uptake. Frozen ventricles were homogenized in 250 mM sucrose, 10 µM cantharidin, and 30 mM histidine (pH 7.0). Ca²⁺ uptake in homogenates was measured by the microfiltration technique with 45Ca²⁺ (19), and rat PP2A (51), for rat calsequestrin (GenBank accession no. U33287, Aquilla TT and Rovner AS), rat cardiac inhibitory subunit of troponin (34), and human atrial natriuretic peptide (2) were employed to generate subtype-specific probes by RT-PCR (Table 1). All PCR reactions were carried out in a total volume of 50 µl containing 20 mM Tris·HCl (pH 8.3), 40 mM KCl, 6.0 mM MgCl2, 1.0 mM each dNTP (Pharmacia, Uppsala, Sweden), and 50 µl of 50 mM Tris·HCl (pH 8.3), 40 mM KCl, 6.0 mM MgCl2, 1.0 mM each dNTP (Pharmacia, Uppsala, Sweden). RNA was isolated by digestion with EcoRI. The cDNA inserts were purified from 1.5% agarose gels. Sizes were ~2,400 bp for SERCA and 1,100 bp for phospholamban. The other cDNA probes were constructed by RT-PCR. First-strand cDNA was reverse transcribed from 1 µg of total rat ventricular RNA in 10 µl of 50 mM Tris·HCl (pH 8.8), 40 mM KCl, 6.0 mM MgCl2, 1.0 mM each dNTP (Pharmacia, Uppsala, Sweden), 5.0 mM dithiothreitol, 50 µg/ml BSA, 10 units of human placental RNase inhibitor (AGS), and 30 units of TrueScript™ reverse transcriptase (AGS) at 41°C for 60 min. Primers based on the published cDNA sequences for rat protein phosphatase (PP) 1α (51), rat PP1β (51), rat PP1γ (51), rat PP2Aα (19), and rat PP2Aα (48), for rat calsequestrin (GenBank accession no. U33287, Aquilla TT and Rovner AS), rat cardiac inhibitory subunit of troponin (34), and human atrial natriuretic peptide (2) were employed to generate subtype-specific probes by RT-PCR. Each reaction was subjected to 30 cycles of denatur-

Table 1. PCR primers of PCR products

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<th>Gene</th>
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<th>3’-Primer</th>
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<tr>
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<td>108–536</td>
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<tr>
<td>ANF</td>
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<td>TCTGTGACATGCTGCTAGTT</td>
<td>111–848</td>
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PP, protein phosphatase; CSQ, calsequestrin; TnI, inhibitory subunit of troponin; ANF, atrial natriuretic factor.
capillary transfer in 2D gels (Amersham Buchler, Braunschweig, Germany) by PRISM-310 automated sequencer (Applied Biosystems) was performed. A restriction digest using the enzyme DdeI was performed on 10% polyacrylamide gels (27). Immunoblot analysis was performed after electrophoretic transfer of proteins to nitrocellulose membranes that were then incubated for 8 h with a buffer (10 mM Tris, 154 mM NaCl; TBS) containing 0.1% Tween 20 (TTBS) and 5% nonfat dry milk to block nonspecific protein binding sites on the nitrocellulose. The antibodies against the catalytic subunits for PP1α, PP2Aα, and PP2Aβ (catalogue no. 06–221, lot no. 12641 and catalogue no. 06–222, lot no. 13949; EMD Chemicals), at 2 µg/ml dilution in blocking buffer were incubated with the blot overnight. These antibodies recognized a band at the expected molecular mass of ~36 kDa that was quantified. After several rinses in TTBS, the nitrocellulose was incubated with 125I-labeled goat anti-rabbit IgG (ICN Biomedicals) diluted 1:1000 in TBS for 4 h at room temperature. After several washes with TTBS and then once with TBS, the nitrocellulose membranes were dried and the radioactive bands were visualized in a PhosphorImager and quantified as described for Northern blotting. Specificity of immunologic signals was verified by blocking experiments as described (20).

Immunoblots for mobility shift and phosphorylation. Frozen cardiac ventricles from neonatal and adult rats were homogenized at 4°C three times for 30 s each with Polytron PT-10 (Kinematica, Lucerne, Switzerland) in 300 µl of 10 mM NaHCO3. Then 100 µl of 20% SDS were added. SDS inhibited protein kinases and phosphatases and preserved thus the phosphorylation state of proteins (42). Mixtures were kept at 25°C for 30 min before centrifugation to remove debris. Thereafter, supernatants (called extracts) were kept at −20°C until further analysis. Protein concentrations were determined by the Lowry assay (27). SDS extracts were thawed, and additional SDS buffer (22) was added. Samples were incubated for 10 min at 30°C. Forty micrograms of homogenate sample protein were loaded per lane. SDS gel electrophoresis was performed on 10% polyacrylamide gels, and proteins were electroblotted to nitrocellulose membranes (Schleicher & Schuell) as described (43). This modified Laemmli system is able to separate the low- and high-molecular-weight forms of phospholamban (e.g., Refs. 24, 44).

Nitrocellulose sheets were incubated with the monoclonal antibody A1 raised against phospholamban (BIOMOL). Under our assay conditions, the affinity of the antibody did not depend on the phosphorylation state of phospholamban (see also Ref. 11 and Fig. 7B). Proteins binding antibodies were visualized using 125I-labeled anti-mouse IgG (ICN Biomedicals). Radioactive bands were visualized by a PhosphorImager as described for Northern blotting.

Preparation of membrane vesicles. Membrane vesicles were prepared as described previously (1) with minor modifications. Frozen neonatal rat ventricles were homogenized in 10 ml of medium containing (in mM) 4.0 EDTA, 1.0 NaH2PO4, and 0.1% (vol/vol) β-mercaptoethanol. The tissue was homogenized three times for 30 s each with a Polytron PT-10 (Kinematica). The sample was sedimented for 20 min at 14,000 g. The supernatant was sedimented at 45,000 g for 30 min, and the resulting pellet was resuspended in 10 ml of homogenization medium containing 0.6 M NaCl. This material was sedimented at 45,000 g for 30 min. The final pellet containing the membrane vesicles was resuspended in 200 µl of 50 mM Tris-HCl (pH 7.0), 0.1 mM EDTA, and 0.1% β-mercaptoethanol. The membrane vesicles (10 µg protein/assay) were immediately phosphorylated in a medium (final volume 50 µl) containing (in mM) 40 histidine HCl (pH 6.8), 10 MgCl2, 15 NaF, 1 EGTA, and 0.75 ATP at 37°C. The reaction was initiated by the addition of ATP. After 2 h, 5 µl of a solution of BSA (10 mg/ml) and 3 ml of 15% TCA containing 50 mM H3PO4 and 0.5 mM ATP were added. After centrifugation at 4°C, the precipitates were processed as described.
Ref. 18. The electrophoretic separation was performed on 10% polyacrylamide gels according to Laemmli (22). Phospholamban was identified by immunoblotting as described above.

PP assay. Assays for PP activity were performed exactly as described previously (42). Phosphatase activity was measured at 30°C using [32P]phosphorylase as a substrate. The 50 µl incubation mixture contained (in mM) 20.0 Tris (pH 7.0), 5.0 caffeine, 0.1 EDTA, and 0.1% (vol/vol) β-mercaptoethanol. The reaction was terminated after 10 min by addition of TCA. Samples were centrifuged, and radioactivity in the supernatants was determined by scintillation counting.

The reaction was terminated after 10 min by addition of TCA. Samples were centrifuged, and radioactivity in the supernatants was determined by scintillation counting.

Statistics. Data shown are means ± SE. Statistical analysis was performed using Student’s t-test or by two-way ANOVA followed by Bonferroni’s t-test as appropriate. A P value <0.05 was considered significant.

RESULTS

Contractile response. TCT, TPT, and TR were about twofold longer in neonatal compared with adult ventricular preparations (259.0 ± 8.86 vs. 126.1 ± 4.77 ms (TCT), 106.0 ± 4.85 vs. 52.8 ± 2.52 ms (TPT), 153.0 ± 4.90 vs. 73.3 ± 2.50 ms (TR); n = 5–9, 1 Hz, P < 0.05). Similar results were obtained at lower (0.2 Hz) and higher (1.4 Hz) rates of stimulation (data not shown). As expected, isoproterenol cumulatively applied (0.001–10 µM) shortened time parameters in adult and neonatal preparations in a concentration-dependent manner. The effect was maximal at 10 µM isoproterenol (data not shown). In neonatal rat ventricular preparations, 10 µM isoproterenol shortened TPT by 39% (from 106 ± 4.85 to 64 ± 1.7 ms, n = 5–7, P < 0.05) and TR by 40.7% (from 153 ± 4.90 to 91 ± 10.14 ms, n = 5–7, P < 0.05). In adult ventricular preparations, 10 µM isoproterenol shortened TPT by 17.9% (from 53 ± 2.52 to 43 ± 1.44 ms, n = 9, P < 0.05) and TR by 28.8% (from 73 ± 2.50 to 52 ± 2.37 ms, n = 9, P < 0.05). It is noteworthy that after maximum β-adrenergic stimulation, the duration of contraction in neonatal preparations was 21% longer than in adult preparations.

SR Ca2+ uptake. To determine whether developmental changes in SR function occur, Ca2+ uptake measurements were performed. SR Ca2+ uptake in adult preparations amounted to 0.30 ± 0.09 Ca2+ mg protein −1·min −1, whereas Ca2+ uptake was 0.06 ± 0.01 Ca2+ mg protein −1·min −1 in neonatal preparations (n = 5, P < 0.05 neonatal vs. adult). Ca2+ uptake was stimulated to 0.83 ± 0.12 and 0.26 ± 0.06 Ca2+ mg protein −1·min −1 in adult and neonatal preparations, respectively, in the presence of anti-phospholamban antibody 2D12 (n = 5, P < 0.05 neonatal vs. adult).

Quantification of SERCA, phospholamban, calsequestrin, the inhibitory subunit of troponin, and atrial natriuretic peptide mRNA levels. Total RNA was isolated from neonatal and adult rat ventricles. In Northern blots from both adult and neonatal ventricles, the following transcripts were detectable: one transcript of 4.4 kb for SERCA (Fig. 1A), two major transcripts of 3.3 and 3.3 kb for phospholamban (Fig. 1B), one transcript of 3.3 kb for calsequestrin (CSQ) mRNA (Fig. 1C), and two major transcripts of 1.1 and 0.7 kb for inhibitory subunit of troponin (TnI) mRNA (Fig. 1D), and a major transcript detected at 0.7 kb corresponds to ANF mRNA (Fig. 1E).

![Image](http://ajpheart.physiology.org/Downloadedfrom)
of 2.9 kb for calsequestrin (Fig. 1C), one transcript of 0.7 kb for the inhibitory subunit of troponin (Fig. 1D), and one transcript of 0.7 kb for the atrial natriuretic peptide (Fig. 1E). Comparing adult and neonatal preparations, we found that the content of Ca\(^{2+}\)-ATPase mRNA level was lower in the neonatal rat ventricle than in the adult (0.28 ± 0.02 vs. 0.58 ± 0.03 PhosphorImager units, n = 3, P < 0.05). In contrast, phospholamban mRNA expression level was unchanged (4.6 ± 0.30 vs. 4.64 ± 0.27 PhosphorImager units, n = 3). Calsequestrin mRNA level was lower in the neonatal rat ventricle (1.03 ± 0.09 vs. 2.30 ± 0.14 PhosphorImager units, n = 3, P < 0.05); the mRNA for the inhibitory subunit of troponin was likewise less abundant in neonatal compared with adult ventricles (1.89 ± 0.36 vs. 3.49 ± 0.11 PhosphorImager units, n = 3, P < 0.05). In contrast, atrial natriuretic peptide mRNA level was markedly higher in neonatal than in adult ventricular preparations (6.80 ± 0.40 vs. 1.05 ± 0.07 PhosphorImager units, n = 3, P < 0.05).

Quantification of SERCA, phospholamban, calsequestrin, the inhibitory subunit of troponin, and α-actin protein levels. mRNA levels have often been used to predict changes at the protein level. However, there is evidence that protein and mRNA levels, for instance, of phospholamban or SERCA, do not always correlate (23, 37). Thus it was important to determine whether the observed changes at mRNA levels in the developing hearts reflected changes at protein levels. Quantitative immunoblotting was used to determine the expression of SERCA, phospholamban, calsequestrin, the inhibitory subunit of troponin, and α-actin. Initial experiments showed that the detection method was linear between 20 and 60 µg protein of ventricular homogenate (e.g., for the inhibitory subunit of troponin, Fig. 2). Thus 40 µg protein were used for quantitative immunoblotting. SERCA protein expression in adult ventricular preparations was 3.3-fold of values in neo-

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Fig. 2. Immunoblot of TnI in adult ventricular preparations from rats. Increasing amounts of ventricular homogenate (20, 40, 60, and 80 µg) were electrophoresed on SDS gel. After transfer of protein to nitrocellulose membranes, blots were cut and probed with antibodies specific for TnI as described in MATERIALS AND METHODS.

Fig. 3. Immunoblot of SERCA, CSQ, α-actin, and TnI in neonatal and adult ventricular preparations from rats. Forty micrograms of ventricular homogenate were electrophoresed on SDS gel. After transfer of protein to nitrocellulose membranes, blots were cut and probed with antibodies specific for SERCA, CSQ, α-actin, and TnI as described in MATERIALS AND METHODS. Definitions are as in Fig. 1.

Fig. 4. Autoradiograms of Northern blots (A–C) and quantification (D) of mRNA levels of catalytic subunits of protein phosphatases (PP) 1α (A), PP1β (B), and PP1γ (C) in ventricular preparations isolated from neonatal (N) and adult (AD) rats. Total RNA was isolated, blotted onto nylon membranes, and then hybridized with specific 32P-labeled DNA probes as described in MATERIALS AND METHODS. Radioactivity bound to membranes was assessed and expressed in pixel units as described in MATERIALS AND METHODS. Representative Northern blots detected major transcripts at 1.8 kb corresponding to PP1α (A), at 3.2 kb corresponding to PP1β (B), and at 2.6 and 1.6 kb corresponding to PP1γ (C). In D, each bar represents mean ± SE of 3–5 experiments. * Significant differences vs. adult preparations.
nental preparations (27.4 ± 0.82 vs. 8.36 ± 1.23 PhosphorImager units, n = 5, P < 0.05, Fig. 3). The expression of phospholamban and α-actin was unchanged (8.55 ± 0.41 vs. 8.67 ± 0.32 PhosphorImager units for phospholamban and 1.30 ± 0.17 vs. 1.34 ± 0.16 PhosphorImager units for α-actin, n = 4 or 5, Figs. 3 and 7A). Protein levels of calsequestrin and the inhibitory subunit of troponin were lower in neonatal ventricular preparations (27.4 ± 0.82 vs. 8.36 ± 1.23 PhosphorImager units, n = 5, P < 0.05, Fig. 3). The expression of phospholamban and α-actin was unchanged (8.55 ± 0.41 vs. 8.67 ± 0.32 PhosphorImager units for phospholamban and 1.30 ± 0.17 vs. 1.34 ± 0.16 PhosphorImager units for α-actin, n = 4 or 5, Figs. 3 and 7A). Protein levels of calsequestrin and the inhibitory subunit of troponin were lower in neonatal ventricular preparations and amounted to 63% (4.03 ± 0.30 vs. 6.35 ± 0.32 PhosphorImager units, n = 5, P < 0.05, Fig. 3) and 23% (8.55 ± 0.73 vs. 36.73 ± 2.91 PhosphorImager units, n = 5, P < 0.05, Fig. 3) of those in adult preparations.

Phosphatase activity. We compared phosphatase activity in ventricular homogenates from neonatal and adult rat ventricles. Phosphatase activity was higher in neonatal rat ventricle preparations compared with adult (1.020 ± 59 nmol·mg protein⁻¹·min⁻¹ in neonatal vs. 540 ± 33 nmol·mg protein⁻¹·min⁻¹ in adult, n = 9 or 10, P < 0.05).

Phosphatase expression at mRNA and protein levels. To understand the underlying mechanism of enhanced phosphatase activity, the mRNA and protein expression of the catalytic subunits of PP1 and PP2A were studied in neonatal and adult rat ventricular preparations. In neonatal and adult ventricular preparations, Northern blots showed transcripts for PP1α at 1.8 kb (Fig. 4A); for PP1β at 3.2 kb (Fig. 4B); for PP1γ at 6.8 and 2.6 kb (Fig. 4C); for PP2Aα at 1.1, 2.0, and 2.7 kb (Fig. 5A); and for PP2Aβ at 2.0 kb (Fig. 5B). Summarizing the data of all experiments, we found that the mRNA in neonatal preparations for PP1α amounted to 145%, PP1β amounted to 148%, PP1γ amounted to 185%, PP2Aα amounted to 150%, and PP2Aβ amounted to 158% of adult ventricular mRNA (Figs. 4D and 5C). Data of protein expression correlated with mRNA expression. Quantitative immunoblotting with the antibodies for PP1α and for PP2A was performed. Protein levels of PP1 in neonatal ventricular preparations were elevated to 354%, and protein expression of PP2A amounted to 165% of adult ventricular values (Fig. 6, A and B).

Mobility shift and phosphorylation of phospholamban. Phosphorylation of phospholamban leads to a higher apparent molecular mass of pentameric phospholamban in SDS-PAGE as described before (56). Interestingly, we observed that the apparent molecular mass of phospholamban was higher in adult rat ventricles than in neonatal ventricles (Fig. 7A), suggesting a higher state of phosphorylation of phospholamban in adult cardiac preparations. This interpretation is supported by the fact that phosphorylation of phospholamban from neonatal ventricular preparations by exogenous cAMP-dependent protein kinase decreased the mobility of phospholamban under the same conditions (Fig. 7B).

**DISCUSSION**

There are conflicting reports whether duration of contraction declines or increases after birth. The time parameters are longer in adult feline and canine hearts than in neonatal hearts (36, 46). The underlying biochemical reason for this change is speculative, postnatal developmental regulations of, for example, phospholamban and SERCA expression in feline or canine hearts have not yet been reported. In mouse heart, the
expression of both phospholamban and SERCA increased in parallel during maturation (14). However, contractile measurements are apparently lacking in neonatal vs. adult mouse ventricular preparations. Time parameters of contraction were shortened in ventricular preparations from fetal vs. adult mice (3). In rabbit, mRNA data indicated that phospholamban is unchanged and SERCA is increased on maturation but protein data are apparently lacking (3). In rats, reduced developed tension, prolonged TPT, and prolonged TR have been observed in neonatal vs. adult ventricular preparations (9). These contractile measurements in rats are in agreement with our present findings. The purpose of the present work was to further our understanding on the biochemical basis for these contractile differences between adult and neonatal hearts using an integrative approach in one animal species (rat).

We detected an enhanced expression (on mRNA and protein levels) of the cardiac form of the inhibitory subunit of troponin in adult rat ventricular preparations compared with neonatal ventricular preparations in accordance with previous work in the rat heart (49, 34). Our results indicate an increase in the inhibitory subunit of troponin in relation to α-actin. β-Adrenergic stimulation phosphorylates the inhibitory subunit of troponin in isolated cardiac preparations, and this phosphorylation is thought to contribute to the relaxant effect of β-adrenergic stimulation. Thus an enhanced level of unphosphorylated inhibitory subunit of troponin in adult ventricles is expected to lead to prolonged relaxation. Paradoxically, shortened relaxation is actually observed in adult preparations. It could be speculated that the effect of enhanced levels of the inhibitory subunit of troponin might be functionally antagonized, at least in part, by an enhanced phosphorylation state of the inhibitory subunit of troponin in adult preparations due to reduced activity of protein phosphatases (see below).

The main Ca2+-binding protein of the heart is situated in the junctional and corbular SR and has been termed calsequestrin (32). Increased calsequestrin levels in adult vs. fetal hearts have been observed in rabbit (3) and sheep (33). Postnatal increases have been noted in rabbit heart (3). Data for calsequestrin in rat cardiac development have previously not been available. In the present study, we noted a higher expression of calsequestrin in adult vs. neonatal rat ventricular preparations at mRNA and protein levels.

Unphosphorylated phospholamban reduces the ability of SERCA to pump Ca2+ from the cytosol into the SR (21). This inhibition can be relieved by phosphorylation by cAMP-dependent protein kinase.

cAMP-elevating agents, like the β-adrenoceptor agonist isoproterenol, increase phospholamban phosphorylation in the heart and enhance Ca2+ uptake (13, 24). Moreover, the Ca2+ uptake of the cardiac SR can be stimulated by incubation with an anti-phospholamban antibody (2D12) that impedes the interaction of phospholamban with SERCA (52).

Conflicting data have been reported on the postnatal regulation of phospholamban expression. After birth, a parallel increase in phospholamban expression on protein and mRNA levels was noted in mouse heart (14). In sheep, an increase in phospholamban protein level was noted in adult vs. fetal hearts (33). In rabbits, no change in phospholamban mRNA was detectable in fetal, neonatal, or adult hearts (3). Thus it appears that species differences in postnatal phospholamban expression exist and rat cardiac differences have not been studied before. Here, we report that phospholamban levels are unchanged during rat cardiac development on both protein and mRNA levels.

There is general agreement in the literature that SERCA expression is increased during development. For example, there is an increase in SERCA at the protein level in fetal, neonatal, and adult sheep hearts (47). SERCA mRNA increased gradually from fetal to neonatal and to adult rabbit hearts (3). Recently, a postnatal increase in SERCA on protein and mRNA level was reported in the mouse heart (14), whereas data in rat heart are apparently lacking. The new
information here is that SERCA was increased (on mRNA and protein level) postnatally. This increase was accomplished by an increased Ca\textsuperscript{2+} uptake. An increase in Ca\textsuperscript{2+} uptake has been noted before in sheep heart (47), mouse heart (14), or rat heart (41, 55). We extend on previous work by showing that ablation of phospholamban inhibition of SERCA function by use of the anti-phospholamban antibody 2D12 could stimulate Ca\textsuperscript{2+} uptake in both neonatal and adult preparations. This antibody offers the possibility to assess the maximal SERCA-mediated Ca\textsuperscript{2+} uptake. This maximum Ca\textsuperscript{2+} uptake was greatly increased postnatally. This is expected if phospholamban is functionally active in both neonatal and adult ventricular preparations. Finally, the higher maximum Ca\textsuperscript{2+} uptake data are consistent with elevated SERCA protein levels in adult in comparison with neonatal rat ventricular preparations.

It can be asked whether the change in SERCA and calsequestrin relative to phospholamban expression is mainly because of histological changes of the SR after birth. Anatomic changes in the SR are well known. For instance, the t-tubule system develops only after birth (15, 45). It can be suggested that the relative amount of the SR in the cells increases, and this could account for all biochemical alterations that were observed in the present study. However, SERCA is mainly detectable in the free SR, whereas calsequestrin is present in the corbular and junctional SR (17). Hence, the present data at least indicate that the amounts of two proteins (SERCA and calsequestrin) located in different parts of the SR increase postnatally related to phospholamban (mainly located in the free SR). A caveat is in order that an optimal constant denominator for postnatal changes in SR proteins is not readily available. Even muscle lipids change postnatally (7).

One should bear in mind that altered expression of other proteins that affect the Ca\textsuperscript{2+} homeostasis in the heart might also affect duration of contraction. For instance, there is evidence that the expression of the ryanodine receptor (Ca\textsuperscript{2+}-release channel, feet, pedes) or the L-type calcium channel is changed postnatally in the heart (58). For instance, the current through L-type Ca\textsuperscript{2+} channels and the channel density (by radioligand binding) is increased postnatally (58, 28). Interestingly, currents through L-type Ca\textsuperscript{2+} channels were differently increased by phosphatase inhibitors in adult vs. neonatal rabbit cardiac cells. Hence, the authors (28) suggested that the phosphatase activity (mainly type 1) might decline postnatally. However, no biochemical measurements of phosphatases or their activity were performed.

Type 1 and 2A phosphatases comprise >90% of phosphatase activity in the heart and dephosphorylate, for instance, phospholamban (53). We were able to detect the catalytic subunits of 1\alpha, 1\beta, 1\gamma, 2A\alpha, and 2A\beta in the neonatal and adult rat hearts by Northern blotting. Interestingly, the mRNA and the protein expression of phosphatase type 1 and type 2A declined postnatally. Fittingly, phosphatase activity was diminished in preparations from adult vs. neonatal rabbit cardiac cells. Hence, the authors (28) suggested that the phosphatase activity (mainly type 1) might decline postnatally. However, no biochemical measurements of phosphatases or their activity were performed.
lar preparations indicates enhanced phosphorylation of phospholamban in adult preparations. We suggest that this may in part result from reduced phosphatase activity in adult compared with neonatal ventricles.

In summary, SERCA but not phospholamban expression changed postnatally, and this increase is accompanied by enhanced Ca\(^{2+}\) uptake. The repressor function of phospholamban on SERCA is diminished in adult myocardium by a reduced phosphorylation state of phospholamban. This could result from the observed decreased activity and expression of type 1 and 2A phosphatase isoforms in adult hearts. This is the first biochemical report on developmental regulation of phosphatases in the heart.

The excellent technical assistance of R. Plitzko is gratefully acknowledged. We thank Dr. K. R. Boheler for kindly providing the rat SERCA\(a\) cDNA and rat phospholamban cDNA. We thank Dr. K. R. Boheler for kindly providing the rat SERCA\(a\) cDNA and rat phospholamban cDNA. We thank Dr. K. R. Boheler for kindly providing the rat SERCA\(a\) cDNA and rat phospholamban cDNA. We thank Dr. K. R. Boheler for kindly providing the rat SERCA\(a\) cDNA and rat phospholamban cDNA. We thank Dr. K. R. Boheler for kindly providing the rat SERCA\(a\) cDNA and rat phospholamban cDNA. We thank Dr. K. R. Boheler for kindly providing the rat SERCA\(a\) cDNA and rat phospholamban cDNA.

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