Mouse Phospholamban Gene Expression During Development In Vivo and In Vitro

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To establish a murine model that may allow for definition of the precise role of phospholamban in myocardial contractility through selective perturbations in the phospholamban gene, we initiated studies on the role of phospholamban in the murine heart. Intact beating hearts were perfused in the absence or presence of isoproterenol, and quantitative measurements of cardiac performance were obtained. Isoproterenol stimulation was associated with increases in the affinity of the sarcoplasmic reticulum Ca\(^{2+}\) pump for Ca\(^{2+}\) that were due to phospholamban phosphorylation. To assess the regulation of phospholamban gene expression during murine development, Northern blot and polymerase chain reaction analyses were used. Phospholamban mRNA was first detected in murine embryos on the ninth day of development (the time when the cardiac tube begins to contract). In murine embryoid bodies, which have been shown to recapitulate several aspects of cardiogenesis, phospholamban mRNA was detected on the seventh day (the time when spontaneous contractions are first observed). Only those embryoid bodies that exhibited contractions expressed phospholamban transcripts, and these were accompanied by expression of the protein, as revealed by immunofluorescence microscopy. Sequence analysis of the cDNA encoding phospholamban in embryoid bodies indicated complete homology to that in adult hearts. The deduced amino acid sequence of murine phospholamban was identical to rabbit cardiac phospholamban but different from dog cardiac and human cardiac phospholamban by one amino acid. These data suggest that phospholamban, the regulator of the Ca\(^{2+}\)-ATPase in cardiac sarcoplasmic reticulum, is present very early in murine cardiogenesis in utero and in vitro, and this may constitute an important determinant for proper development of myocardial contractility. (Circulation Research 1992;71:1021–1030)

Key Words • heart • phospholamban • development • embryoid bodies • mouse

The Ca\(^{2+}\) pump in cardiac sarcoplasmic reticulum (SR) is under regulation by the complex proteolipid phospholamban.\(^1\) Phosphorylation of phospholamban is associated with activation of the SR Ca\(^{2+}\)-ATPase,\(^2\)\(^–\)\(^10\) whereas dephosphorylation of phospholamban reverses the stimulatory effects of the protein kinases on the Ca\(^{2+}\) pump.\(^11\) Phospholamban is phosphorylated at distinct amino acids by three different protein kinases, namely, serine 10 by protein kinase C, serine 16 by cAMP-dependent protein kinase, and threonine 17 by Ca\(^{2+}\)-calmodulin–dependent protein kinase.\(^12\) Phospholamban has also been shown to be phosphorylated in vivo during isoproterenol stimulation of intact hearts.\(^13\) Phosphorylation of phospholamban and the accompanied increases in the Ca\(^{2+}\) uptake rates by SR have been postulated to be responsible for the increases in the rate of myocardial relaxation (−dP/dt) observed during isoproterenol stimulation of the heart.\(^14\)\(^,\)\(^15\) Thus, evidence from in vitro and in vivo studies suggests that phospholamban is an important regulatory phosphoprotein in the mammalian heart. However, the exact role of phospholamban in the regulation of contractility and whether phosphorylation of this protein alone is directly responsible for and sufficient to cause the alterations in myocardial relaxation observed during β-adrenergic stimulation is not presently known. Information along these lines may be obtained through the use of gene-targeting methodology, which permits introduction of specific mutations in the murine phospholamban gene, leading to biochemical and physiological alterations in the developing myocardium. Thus, it becomes important to define the regulation of phospholamban gene expression during development in the murine heart, especially since developmental studies on phospholamban have been limited. The well-characterized embryology and organogenesis of the mouse make this animal model an ideal system for studies on development.

Studies on in vivo murine embryonic development have been recently coupled to studies on in vitro development using murine embryoid bodies, which appear to provide a model system for determining the very early events in differentiation. Embryoid bodies are derived from murine embryonic stem cells, which originate directly from the inner cell mass of the mouse.

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bластоцита. Эти эмбрионные тела обладают локализованными областями сокращения, и контактирующие клетки имеют хорошо определенную саркофему, которая соответствует характеристикам миокарда. 

Recent studies have shown that embryoid bodies recapitulate some aspects of cardiogenesis that are reminiscent of days 9–10 of embryonic mouse development. They express the appropriate developmental stage-specific cardiac and skeletal myosin heavy chain transcripts. Similar results have been also obtained for α-tropomyosin.

Thus, the developing embryoid bodies may provide an in vitro model system for studies of early events in mammalian cardiogenesis under normal or perturbed genomic expression.

This report initiates studies on phospholamban in the murine system, with the eventual goal being to determine the role of this protein in the contractile behavior of the heart by making directed mutations in the murine phospholamban gene. Although there are numerous studies on phospholamban in various mammalian species, there is very little information available on the role of this protein in the murine heart. A major limitation is the size of the murine heart (heart weight, 80–120 mg), making biochemical and functional measurements technically difficult. In this study, we demonstrate that alterations in murine cardiac function by β-adrenergic stimulation can be accurately assessed and that they may be correlated with phospholamban-induced alterations in SR Ca2+ pump properties. The amino acid sequence of phospholamban present in murine hearts is similar to that present in other mammalian species, as revealed by sequence analysis of its cDNA. Furthermore, expression of the phospholamban gene occurs in the very early stages of in utero embryonic development and of in vitro embryoid body development. The presence of phospholamban mRNA is associated with early cardiogenic events in the mouse embryo, and these transcripts are not observed in embryoid bodies that contract. These data suggest that the expression of phospholamban and regulation of the Ca2+ pump in cardiac SR may be important for proper development of myocardial contractility.

Materials and Methods

Materials

Restriction endonucleases and DNA-modifying enzymes were obtained from Bethesda Research Laboratories, Bethesda, Md. The murine cardiac cDNA library constructed in λZap II, Escherichia coli XLI-Blue, and pBluescript were from Stratagene Inc., La Jolla, Calif. [γ-32P]dATP, [α-32P]dCTP, [α-32P]dGTP, and [α-32P]dATP were purchased from New England Nuclear, Boston. Oligodeoxynucleotides used for primer extension analyses were synthesized at the University of Cincinnati. The 30-base pair oligonucleotide used for screening the murine cDNA library was a generous gift from Dr. T. Scott, Boston Biomedical Research Institute. Embryonic stem cells ES-D3 have been previously described. Dulbecco's modified Eagle's medium was from Whittaker Bioproducts, Walkersville, Md., and fetal calf serum was from Flow Laboratories, Inc., McLean, Va. Trypsin type X1 was from Sigma Chemical Co., St. Louis, Mo.

Heart Perfusion

Swiss Webster mice were anesthetized with 30 mg/kg pentobarbital i.p. and protected with 5,000 units/kg heparin sodium. The hearts were removed from the chest, and retrograde perfusion with Krebs-Henseleit buffer was performed at 37 ± 0.2°C. Contraction of the heart resumed immediately on perfusion. A catheter was inserted through a pulmonary vein and through the open mitral valve, advanced into the left ventricle, and exited through the apex to measure intraventricular pressure and dP/dt. The opening of the pulmonary vein was connected to the venous return cannula. Flow was then switched from retrograde to antegrade perfusion and adjusted to approximately 4 ml/min. The Krebs-Henseleit solution, which was oxygenated with 95% O2–5% CO2, contained (mmol/l) NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, sodium EDTA 0.5, NaHCO3 25, and glucose 5.5. The retrograde perfusion cylinder was located 70 cm above the heart. Venous inflow was monitored by an electromagnetic flow probe and adjusted via a micrometer (preload). Left atrial pressure was monitored through a side arm of the pulmonary vein cannula. In the aortic outflow catheter was a built-in “Starling resistance” to increase and decrease aortic pressure (afterload). The electrocardiograph was monitored via electrodes connected to the stainless-steel cannulas attached to the aorta and left atrium. Addition of isoproterenol to the fluid entering the heart was done with microinfusion pumps (multi-speed infusion pump, model 600, Harvard Apparatus, South Natick, Mass.). Before infusion of isoproterenol, Starling function curves were generated. All parameters were recorded simultaneously on a six-channel polygraph (model P7, Grass Instrument Co., Quincy, Mass.).

Calcium Uptake

At the peak of the inotropic response to isoproterenol, mouse hearts were frozen and powdered under liquid nitrogen. Control hearts were perfused and treated under identical conditions but in the absence of isoproterenol. Frozen cardiac powder from control and isoproterenol-treated hearts was homogenized in 50 mM KF (pH 7.0), 10 mM NaF, 1 mM EDTA, 0.3 M sucrose, 0.3 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. Ca2+ uptake in homogenates (0.1 mg/ml) from hearts that had been perfused with or without isoproterenol was measured by a modification of the Millipore filtration technique. The reaction contained 40 mM imidazole (pH 7.0), 100 mM KCl, 5 mM MgCl2, 5 mM NaCl, 5 mM potassium oxalate, 500 μM EGTA, and various concentrations of CaCl2 to yield 0.01–10 μM free Ca2+. Homogenates were incubated at 30°C for 2 minutes in the above buffer, and the reaction was initiated by the addition of ATP (final concentration, 5 mM). Ca2+ uptake rates were linear up to 6 minutes at 30°C.

Cloning and Isolation of cDNA Clones

A cardiac cDNA library (Stratagene) made from 12-week-old BALB/c female mice, constructed in λZap II, was screened by plaque hybridization. The 32P-labeled oligonucleotide 3'-TACCTATTTCCAGTTATGGAGTGGCCAGAGA-5', which is complementary to the first 30 nucleotides from the translation initiation codon of dog cardiac phospholamban, was used to screen 1 x 107 plaques. Ten putative positive plaques were carried through three rounds of plaque purification, and the purified plaque that gave the strongest hybridization signal was chosen for sequence analysis.
The pBluescript plasmid containing the cDNA was excised from the bacteriophage DNA according to the manufacturer's protocol and converted to the plasmid vector pBluescript (Stratagene), and it was designated as pMPLB.

**DNA Sequence Analysis**

DNA sequence analysis was performed by the dideoxynucleotide chain terminating method\(^\text{29}\) with a Sequenase II kit (United States Biochemical Corp., Cleveland, Ohio) on both double- and single-stranded DNA templates according to manufacturer's recommendation.

**RNA Isolation and Northern Blot Analysis**

Total cellular RNA was isolated from various tissues of mice (FVB/N strain) at different stages of development and from embryoid bodies using RNAzol (Cinna/Biotech, Friendswood, Tex.). Total RNA (10 μg) was electrophoresed on a 1% agarose gel containing 1 M formaldehyde. The gel was blotted onto charged nylon, and the RNA was covalently bound to the membrane by illumination with ultraviolet light. The blot was probed with full-length cDNA fragments, which were \(^3^P\)-la- beled by the random primer extension method using Random Primer Plus (New England Nuclear).

**Analysis of RNAs From Mouse Embryos or Embryoid Bodies by Polymerase Chain Reaction**

Total RNA was isolated from mouse (FVB/N strain) embryos, embryonic stem cells, or embryoid bodies by using RNAzol, as described above. For subsequent amplifications, the RNA was denatured at 80°C for 5 minutes. The tubes were placed on ice, and the RNA present was used to direct the synthesis of cDNA without further purification.

Optimal conditions for first-strand cDNA synthesis of mouse embryonic mRNA (1 μg) required the use of 100 pmol primer 1 (5'-CCAGACTGAGACGATATAAAGTG-3'), which was allowed to anneal to the nucleic acid at 60°C for 15 minutes. Subsequently, polymerase chain reaction (PCR) buffer consisting of 18.6 mM Tris-HCl (pH 8.3), 93 mM CaCl2, 2.5 mM MgCl2, 0.002% gelatin, 3.7 mM each dGTP, dCTP, dTTP, and dATP, and 7 units/μl Superscript H (Bethesda Research Laboratories) was added to a final volume of 10 μl. cDNA synthesis was allowed to proceed at 42°C for 45 minutes. The reaction was stopped by incubation at 94°C for 1 minute and brought to 11.72 mM Tris-HCl (pH 8.3), 58.6 mM CaCl2, 2.64 mM MgCl2 (1.5 mM free Mg\(^2+\)), 0.0012% gelatin, 1.14 mM dATP, 1 unit Taq polymerase, and 50 pmol primer 2 (5'-GCTAAGCTCCCAT-AAGACTT-3') in a final volume of 50 μl. A 50-μl mineral oil overlay was added, and tubes were placed in a thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, Conn,) for an initial denaturation cycle of 94°C for 3 minutes (before the amplification process). The mixture was then cycled 30 times as follows: 94°C for 1 minute, 58°C for 2 minutes, and then 72°C for 2 minutes plus a 3-second extension per cycle. The mixture was then incubated at 72°C for 10 minutes to allow for the extension of all unfinished products. Control reactions using cloned phospholamban cDNA and adult mouse heart total RNA as a template were treated exactly as described above. A 10-μl volume of the amplified DNA was electrophoresed on a 1% agarose gel and stained with ethidium bromide. The gel was then photographed and subsequently blotted onto a charged nylon membrane. The filter was hybridized with a \(^3^P\)-labeled full-length cDNA fragment in 5× standard saline citrate, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate, and 0.1 mg/ml denatured salmon sperm DNA at 55° or 65°C for 12 hours. Probe concentration was 3 ng/ml (10° cpn/ng). The filter was washed in 0.1× standard saline citrate and 0.1% sodium dodecyl sulfate at 55° or 65°C and processed for autoradiography.

Optimal conditions for first-strand cDNA synthesis of RNA from stem cells or embryoid bodies consisted of using 10 μg RNA plus 1 μg oligo dT (15 base) primer, which was allowed to anneal to the nucleic acid at 37°C for 15 minutes. Subsequently, reverse transcriptase buffer (Bethesda Research Laboratories) consisting of 50 mM Tris-HCl (pH 8.3), 3 mM MgCl2, 75 mM KCl, 10 mM dithiothreitol, 2.5 mM each dGTP, dCTP, dTTP, and dATP, and 200 units Maloney murine leukemia virus reverse transcriptase was added to a final volume of 10 μl. cDNA synthesis was allowed to proceed at 37°C for 60 minutes. Subsequently, 8 μl of 0.3 M NaOH including 3 mM EDTA was added, and incubation was continued at 60°C for 60 minutes; 1.8 μl of 3 M NaAc (pH 5.2) was added, and the nucleic acid was precipitated by the addition of 40 μl ethanol (100%) at −20°C for 60 minutes. The reactions were centrifuged in a microfuge at 4°C for 5 minutes, and the pellets were resuspended in 20 μl H₂O. Ten-microliter aliquots were used as a template for PCR amplification. Reaction buffer (PCR buffer) consisting of 50 mM Tris-HCl (pH 8.3), 10 mM KCl, 1.5 mM MgCl₂, 0.002% gelatin, 0.2 mM each dGTP, dCTP, dTTP, and dATP, 125 ng each of primer 1 and primer 2, and 1 unit Taq polymerase (Perkin-Elmer Cetus) was added to a final volume of 50 μl. The reactions were overlaid with 50 μl mineral oil, and amplification of cDNA was carried out in a Perkin-Elmer Cetus thermocycler. The cycle sequence consisted of an initial denaturation at 94°C for 1 minute, and this was followed by 35 cycles of 1 minute at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C. A final 10-minute extension at 72°C was done. Seven-microliter aliquots of each sample were electrophoresed using 1.2% agarose gels. The samples were then blotted onto nylon-charged membranes, which were subsequently hybridized with a \(^3^P\)-labeled full-length cDNA fragment, as indicated above.

For sequence analysis, the PCR product, identified on agarose gels, was gel-purified, cloned into pBluescript SK †, and transformed into XL-1 Blue Cells (Stratagene). Single-stranded DNA sequence analysis was performed as described above.

**Embryonic Stem Cell and Embryoid Body Cultures**

The embryonic stem cell line ES-D3 was derived from the inner cell mass of a 129/Sv+J blastocyst.\(^\text{18}\) The embryonic stem cell line was maintained undifferentiated by culturing in the presence of murine primary embryonic fibroblast feeder layers prepared from 14–16-day BALB/c fetuses. Before use as a feeder layer, the embryonic fibroblasts were treated with 10 μg/ml mitomycin C for 1.5 hours. Cells were propagated in high-glucose Dulbecco’s modified Eagle’s medium containing 0.1 mM β-mercaptoethanol and 15% heat-inactivated...
fetal calf serum. The embryonic stem cells were transferred to new feeder layers every 3 days. Cells were grown at 37°C in 10% CO₂. Embryoid bodies were obtained by treating the embryonic stem cells and embryonic fibroblast feeder layer with 0.05% trypsin type X1 and placing them in 100-mm plastic tissue culture dishes for 1 hour to allow fibroblasts to attach to the plate. The unattached embryonic stem cells were transferred to 100-mm plastic bacterial Petri dishes containing 10 ml Dulbecco’s modified Eagle’s medium, 15% heat-inactivated fetal calf serum, and 0.1 mM β-mercaptoethanol. Forty-eight hours later, the embryoid bodies were transferred to another 100-mm plastic bacterial Petri dish containing Dulbecco’s modified Eagle’s medium as described above. The media were changed every other day.

**Preparation and Purification of Phospholamban Antibody**

Antibodies to purified phospholamban were raised in rabbits, and sera were applied to a phospholamban Affi-Gel 10 column to obtain an affinity-purified phospholamban antibody, as previously described.²⁶

**Immunofluorescence Labeling**

Embryoid bodies. Six to 10 embryoid bodies were collected using a fine-tipped, fire-polished Pasteur pipette and placed in 5–10 μl culture medium. The small droplet containing the embryoid bodies was placed into a nearly frozen drop of O.T.C. compound (Tissue Tek) adhered to a brass specimen holder cooled on dry ice. Frozen sections, 4–6 μm in thickness, were obtained using a cryostat microtome and were mounted on cooled glass slides coated with 0.5% gelatin. For immunolabeling, the sections were treated for 45 minutes with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) to block the nonspecific binding sites, incubated for 1 hour in the affinity-purified phospholamban polyclonal antibody, given three 20-minute washes with PBS/BSA and incubated for 1 hour with donkey-anti-rabbit–TRITC conjugate (Accurate Chemical & Scientific Corp., Westbury, N.Y.). The excess secondary label was removed using three 20-minute washes in PBS/BSA, and the sections were mounted using Citifluor (Connaught Laboratories). Fluorescence micrographs were obtained using a Nikon Labophot microscope.

Embryonic stem cells. The staining procedures were identical to those for the frozen sections except that embryonic stem cells in tissue culture dishes were fixed with PBS-buffered 4% paraformaldehyde for 5 minutes, solubilized with 0.1% Triton X-100 for 15 minutes, and washed with three changes of PBS before immunostaining.

**Results**

**Isoproterenol Stimulation of Murine Hearts and Increases in Ca²⁺ Transport by Sarcoplasmic Reticulum**

Murine hearts were perfused in the presence of increasing concentrations (from 10⁻⁹ to 10⁻⁶ M) of isoproterenol, and the increases in contractility (as measured by +dP/dt of left ventricular pressure) and relaxation (as measured by −dP/dt of left ventricular pressure) were recorded (Figure 1). These changes in contractility and relaxation were maximally developed within 1 minute of isoproterenol administration, and they were maximal at 10⁻⁷ M isoproterenol. Higher concentrations of isoproterenol appeared to be toxic, causing arrhythmias and decreases in contractility. The ED₅₀ for the isoproterenol response was obtained at 3.3±0.6, 2.7±0.3, and 4.9±0.6 M isoproterenol for +dP/dt, −dP/dt, and heart rate, respectively.
partially during relaxation and contraction. Ca\(^{2+}\) uptake rates by the homogenates of the isoproterenol-stimulated hearts were higher than those by the control hearts, and this stimulation was mostly pronounced at low [Ca\(^{2+}\)] (Figure 2). Analysis of these data indicates that the EC\(_{50}\) of the SR Ca\(^{2+}\) pump for calcium was 0.14±0.01 \(\mu\)M (n=6) for isoproterenol-stimulated hearts and 0.24±0.01 \(\mu\)M (n=6) for control hearts. This finding suggests that the increases in the velocity of SR Ca\(^{2+}\) uptake were associated with an increase in the sensitivity of the transport system for calcium, which is known to be regulated by phospholamban. Thus, the role of phospholamban in regulating myocardial contractility in the mouse appears to be similar to that observed in other mammalian species,\(^{14,15}\) and the mouse may serve as an appropriate animal model for delineating the function of phospholamban in the intact heart and its role during cardiogenesis.

Isolation and Characterization of Murine Phospholamban cDNA

To determine the amino acid sequence of murine cardiac phospholamban and the resemblance to its homologues in other species, we isolated and sequenced a cDNA clone. A total of 1\(\times\)10\(^7\) plaques were screened from an amplified murine cardiac muscle cDNA library in bacteriophage \(\lambda\)Zap II. The library was probed with a synthetic DNA oligomer, based on the phospholamban cDNA sequence from dog cardiac muscle.\(^{24}\) Ten phospholamban-positive clones were identified. The cDNA insert from one clone, which was approximately 900 nucleotide residues (nt), was rescued from \(\lambda\)Zap II as a plasmid and sequence analysis was performed using double-stranded as well as single-stranded DNA. The partial restriction map of the phospholamban cDNA and the sequencing strategy are shown in Figure 3A. The complete nucleotide sequence of the phospholamban cDNA is shown in Figure 3B. An open reading frame of 156 nt (nt1–nt156, Figure 3B) encoded a peptide of 52 amino acid residues. This peptide shares 100% homology with rabbit cardiac and slow-twitch skeletal muscle phospholamban,\(^{29}\) and it differs from dog cardiac muscle and pig antrum phospholamban at position 2\(^{24,30}\) and from human cardiac muscle phospholamban at position 27\(^31\) (Figure 4). Thus, the amino acid sequence of mouse cardiac phospholamban is very similar to those from other previously reported mammalian species.

**Phospholamban Transcript Amplification During Early Stages of In Utero Embryogenesis**

To determine whether phospholamban mRNA is expressed in different mouse tissues and whether different mRNA species exist, we performed Northern blot analysis of mRNA isolated from heart, fast-twitch skeletal muscle, brain, liver, kidney, and lung at 15 days after conception (p.c.) and at 2, 21, and 50 days after birth. Two major forms of phospholamban mRNA, corresponding to 0.7 and 2.8 kb were detected in the heart, and they were present throughout the developmental stages examined (Figure 5). There was no detection of phospholamban transcripts in any of the other tissues in either the embryonic, neonatal, or adult stages (data not shown).

To determine the time when phospholamban transcripts are first detected in the mouse during development, we used a method for amplification of the phospholamban mRNA that would allow detection of low levels of expression. A set of oligonucleotide primers was developed (Figure 3); these primers were designed such that an intron was present between them and were based on the known genomic sequences of rabbit\(^3\) and chicken\(^22\) phospholamban. The location and direction of the primers is shown in Figure 3. The sequences are as follows: primer 1, 5'–CCAGACGGAGCATATT-TTG-3'; and primer 2, 5'–GCTAAGCTCCATAG-GCTTCC-3'. This set of primers was then used to amplify the RNA from embryos at days 8–14 p.c. Amplification of the phospholamban transcript from adult mouse heart yields the expected product of 700 base pairs (Figure 6, lane 6), and this product is also observed on amplification of the phospholamban cDNA. This product was also observed in embryos that were at least 9 days old, but it was not present in embryos that were 8 days old. To determine that the lack of observing the presence of phospholamban mRNA on day 8 was not due to the mRNA samples used for amplification, the \(\beta\)-myosin heavy chain transcript, which was previously shown to be first detected at day 5 p.c.,\(^{20}\) was also amplified\(^{20}\) using the same mRNA samples. Amplification of the \(\beta\)-myosin heavy chain transcript revealed the presence of a 205–base pair product in 8-day-old embryos, indicating that the mRNA samples were not degraded. Furthermore, to ensure that the PCR products obtained were not due to amplification of genomic DNA, which may have contaminated the total RNA samples, 1 \(\mu\)g intact mouse genomic DNA was amplified in parallel with the RNA samples. However, there was no PCR product detected.
using this genomic DNA as a template. This finding is expected, since the phospholamban gene contains a large intron (6.5–10.5 kb) between the two PCR oligomers and the methodology used here would not allow for amplification of sequences greater than ~2 kb.

Thus, in embryonic development we were able to detect the phospholamban mRNA at a stage (day 9 p.c., Figure 6) that is relatively early in cardiovascular development and corresponds to the first signs of myocardial contractility.

Expression of Phospholamban mRNA in Mouse Stem Cells and Embryoid Bodies

Mouse embryonic stem cells can remain morphologically undifferentiated when grown on fibroblast feeder layers, but in the absence of feeder cells, embryonic stem cells spontaneously differentiate into embryoid bodies, which may provide an in vitro differentiation system for studying early cardiogenic processes. To determine the in vitro expression pattern of phospholamban, embryonic stem cells were removed from the
fibroblast layer and allowed to differentiate for 1–12 days. Under the culture conditions used in these experiments, foci exhibiting spontaneous contractions were first detected by day 8 of suspension culture. Embryonic stem cells and embryoid bodies at different stages of development were analyzed by PCR for the presence of phospholamban mRNA. The presence of the phospholamban transcript did not immediately appear to be apparent at any stage of embryoid body development. However, blotting onto a nylon membrane and hybridization to 32P-labeled cDNA revealed low levels of this transcript present by day 7 of embryoid body development, and by day 8, a sizeable increase in the phospholamban expression was observed (Figure 7). Undifferentiated embryonic stem cells did not express the phospholamban transcript.

In this in vitro cardiogenesis system, typically =40% of the embryoid bodies would beat by day 12 in culture suspension. To determine whether phospholamban transcripts were expressed in both beating and nonbeating embryoid bodies, individual beating and nonbeating embryoid bodies were identified by microscopic inspection and collected in two separate pools (20 embryoid bodies each), and the RNA transcripts were subjected to amplification by PCR, as described above. The phospholamban transcript was only present in embryoid bodies that were beating; it was not expressed in nonbeating embryoid bodies (Figure 7). Furthermore, the product obtained by PCR amplification of the phospholamban transcript in 9-day-old beating embryoid bodies was sequenced in parallel with the PCR product obtained from adult mouse hearts. Comparison of the obtained nucleotide sequences (n=3) revealed complete identity between the products obtained with beating embryoid bodies and with adult hearts. These findings suggest that the amino acid sequence of phospholamban, which is present in the early stages of development, is similar to that in the adult animal.

**Expression of Phospholamban in Mouse Embryoid Bodies**

An affinity-purified polyclonal antibody, raised against the purified canine cardiac phospholamban, was also used to determine whether phospholamban was differentially expressed in beating and nonbeating embryoid bodies. Figure 8 shows the results of phase-contrast microscopy and detection of phospholamban using immunofluorescence techniques in embryonic stem cells, beating embryoid bodies, and nonbeating embryoid bodies. Phospholamban was only detected in beating embryoid bodies, in agreement with the findings on phospholamban mRNA expression (Figure 7).

**Discussion**

Evidence from in vitro and in vivo studies suggests that phospholamban, the regulator of the Ca2+-ATPase in cardiac SR, is an important determinant of myocardial contractility in the mammalian heart. The results...
contractions are first observed.\textsuperscript{35} In mouse development, blood circulation can first be detected in the visceral yolk sac at approximately somite stage 8 (days 8–8.5 p.c.), and fetal–placental circulation is beginning to be established at approximately somite stage 13 (day 9 p.c.). By this stage, there is a single endocardial tube, consisting of a common atrium and ventricle, that is capable of maintaining some circulation, and rhythmic contractions can be observed. By somite stage 21 (day 9.5 p.c.), formation of the cardiac loop and septation is under way, and the fetal arteriovenous circulatory system is rapidly developing. Thus, cardiac muscle represents one of the first functional organs during mouse development, and it is interesting that phospholamban is present at the very early stages of cardiogenesis. Detection of phospholamban transcripts in the early stages of embryonic development was possible through their amplification by the use of polymerase chain reaction. At later stages of development, when the phospholamban transcripts were abundant, total cardiac RNA was analyzed using Northern blots, and two phospholamban mRNA species were detected with sizes of 0.7 and 2.8 kilobase pairs, which were due to different polyadenylation signals (data not shown). Multiple phospholamban mRNA species have also been previously shown to be present in dog heart (3.6, 2.8, and 1.0 kb),\textsuperscript{24} rabbit heart and rabbit slow skeletal muscle (predominant 3.4 kb),\textsuperscript{29} and pig antrum (2.8 and 0.9 kb),\textsuperscript{30} but the significance of these multiple phospholamban mRNA species is not presently known.

We have previously suggested that embryoid bodies derived from murine embryonic stem cells may provide a suitable system for studies on embryogenesis, including early events in cardiogenesis.\textsuperscript{18–21,30} In this study, we have extended our observations to include phospholamban gene expression in this in vitro model system for embryogenesis. Phospholamban appeared to be first detected on day 7 of embryoid body development. Since light microscopic examination of these embryoid bodies reveals that approximately half of them possess distinct regions of rhythmic contractions, we separated the embryoid bodies into pools of beaters and nonbeaters and analyzed them via the PCR for the presence of phospholamban mRNA. Phospholamban mRNA was only present in the pool of beating embryoid bodies, and it was not detected in the nonbeating embryoid bodies. These results are similar to those recently reported on \(\alpha\)-cardiac myosin heavy chain transcripts, which were also observed to be only expressed in beating embryoid bodies.\textsuperscript{30} The presence of phospholamban mRNA in beating embryoid bodies was shown to be accompanied by the presence of the protein as well, as revealed by immunofluorescence microscopy.

In previous studies it was suggested that a unique form of phospholamban may be present early in development in sheep\textsuperscript{37} and chicken\textsuperscript{38} hearts. This form either lacked the dissociation pattern to its lower \(M_1\) or was not phosphorylated by the SR-associated \(Ca^{2+}\)-calmodulin–dependent protein kinase.\textsuperscript{37,38} Since embryoid bodies may represent very early stages of embryonic heart development, we investigated whether an isoform of phospholamban may be present in this system. Thus, the PCR products from 9-day-old beating embryoid bodies were analyzed in parallel with the products from adult murine hearts. However, there were no differ-
ences detected between their nucleotide sequences, suggesting that the phospholamban present in the very early stages of in vitro development is similar to that in the adult mammalian heart.

Our findings on the presence of phospholamban in the very early stages of mouse embryonic development and in contracting mouse embryoid bodies lead to interesting speculations on the role of phospholamban in cardiogenesis. With the ability to target the mouse phospholamban gene and modify or even ablate its expression, we are now in a position to design specific animal models that will elucidate the function of phospholamban in development and differentiation. Furthermore, these animal models may be used for biochemical measurements at the SR level and physiological measurements at the isolated heart level to elucidate the role of phospholamban in myocardial contractility. To this end, we describe here initial studies on the regulatory role of phospholamban in the affinity of the SR Ca\(^{2+}\) pump for Ca\(^{2+}\) and in the contractile responses of murine hearts. Although the size of the murine heart is very small, we were able to accurately record its contractile parameters and the alterations in contractility and relaxation by β-adrenergic stimulation. Thus, studies with mutated mouse myocardia are now feasible. Accurate assessment of the alterations in the affinity of the SR Ca\(^{2+}\) pump for Ca\(^{2+}\) and the alterations in the relaxation parameters will be especially helpful in subsequent analyses of the transgenic mice generated by the phospholamban gene-targeting studies, since these parameters have been shown to be under phospholamban regulation.

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