Original Contributions

Overexpression of the Sarcolemmal Calcium Pump in the Myocardium of Transgenic Rats

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Abstract—The plasma membrane calmodulin–dependent calcium ATPase (PMCA) is a calcium-extruding enzyme controlling Ca\(^{2+}\) homeostasis in nonexcitable cells. However, its function in the myocardium is unclear because of the presence of the Na\(^{+}/\)Ca\(^{2+}\) exchanger. We approached the question of the physiological function of the calcium pump using a transgenic “gain of function” model. Transgenic rat lines carrying the human PMCA 4 cDNA under control of the ventricle-specific myosin light chain-2 promoter were established, and expression in the myocardium was ascertained at the mRNA, protein, and functional levels. In vivo hemodynamic measurements in adult homozygous animals showed no differences in baseline and increased cardiac performance recruited by volume overload compared with controls. No differences between transgenic and control cardiomyocytes were found in patch clamp voltage dependence, activation/inactivation behavior of the L-type Ca\(^{2+}\) current, or fast [Ca\(^{2+}\)]\(_i\) transients (assessed by the Fura-2 method). To test whether the PMCA might be involved in processes other than beat-to-beat regulation of contraction/relaxation, we compared growth processes of neonatal transgenic and control cardiomyocytes. A 1.6- and 2.3-fold higher synthesis rate of total protein was seen in cells from transgenic animals compared with controls on incubation with 2% FCS for 24 hours and 36 hours, respectively. An effect of similar magnitude was observed using 20 \(\mu\)mol/L phenylephrine. A 1.4-fold– and 2.0-fold–higher protein synthesis peak was seen in PMCA-overexpressing cardiomyocytes after stimulation with isoproterenol for 12 hours and 24 hours, respectively. Because pivotal parts of the \(\alpha\)- and \(\beta\)-adrenergic signal transduction pathways recently have been localized to caveolae, we tested the hypothesis that the PMCA might alter the amplitude of \(\alpha\)- and \(\beta\)-adrenergic growth signals by virtue of its localization in caveolae. Biochemical as well as immunocytochemical studies suggested that the PMCA in large part was colocalized with caveolin 3 in caveolae of cardiomyocytes. These results indicate that the sarcolemmal Ca\(^{2+}\)-pump has little relevance for beat-to-beat regulation of contraction/relaxation in adult animals but likely plays a role in regulating myocardial growth, possibly through modulation of caveolar signal transduction. (Circ Res. 1998;83:877-888.)

Key Words: plasma membrane Ca\(^{2+}\)-ATPase ■ myocardium ■ transgenic rat ■ contraction ■ cardiac growth

The plasma membrane calmodulin–dependent calcium ATPase (PMCA) is a ubiquitous Ca\(^{2+}\)-transporting enzyme extruding Ca\(^{2+}\) from the cell.\(^1\)\(^-\)\(^4\) So far, 4 different PMCA isoforms in human\(^6\)\(^-\)\(^8\) and rat\(^9\)\(^-\)\(^11\)\(^,\)\(^19\) have been cloned, which are expressed in a cell type– and differentiation-specific manner.\(^11\)\(^-\)\(^17\)\(^,\)\(^19\) In the myocardium, the expression of the isoforms 1, 2, and 4 has been shown.\(^14\)\(^,\)\(^16\)\(^,\)\(^17\) Whereas much insight has been gained into the expression pattern of the various PMCA isoforms and their biochemical properties,\(^3\)\(^,\)\(^4\)\(^,\)\(^18\) studies on their physiological significance have been scant until very recently.

In nonexcitable cell types, the PMCA is the only known enzyme-mediating calcium extrusion and therefore has been postulated to have a housekeeping function.\(^19\) However, in excitable cells, which express the high capacity Na\(^{+}/\)Ca\(^{2+}\) exchanger as an additional sarcolemmal (SL) calcium transporter, the functional importance of the PMCA remains unclear.

In the myocardium, it has been assumed that the PMCA shares the burden of extruding calcium from the cell after each beat with the Na\(^{+}/\)Ca\(^{2+}\) exchanger. In particular, the high calcium affinity of the PMCA has led to the speculation that it is responsible for fine tuning of calcium in the final phase of diastole.

On the other hand, in isolated adult cardiomyocytes, the sodium/calcium exchanger has been demonstrated to have a much higher calcium transport activity than the PMCA.\(^20\) Under the assumption that these in vitro studies are represen-
tative of the situation in whole heart, the PMCA may play only a minor role in beat-to-beat regulation of contraction/relaxation.

Recently published results indicate that the PMCA plays a role in growth and differentiation processes. We reported that L6 skeletal myoblasts stably overexpressing the PMCA showed a markedly accelerated myogenic differentiation into myotubes.21 Consistent with these findings, results published by other groups also suggest an active role of the enzyme in growth regulation. Liu and coworkers22 showed a delay in G1-S phase transition in rat aortic endothelial cells overexpressing rat PMCAa. Husain et al23 demonstrated a significantly slower growth rate in PMCA-overexpressing vascular smooth muscle cells, and Brandt et al24 published results showing an inhibition of nerve growth factor–induced neurite outgrowth in pheochromocytoma cells transfected with a PMCA 1 antisense construct.

To address the unresolved issue of the function of the PMCA in the myocardium, in the present work, we established transgenic rat lines overexpressing the PMCA in the myocardium as a “gain of function” model. This allowed us to address hypotheses directly about the role of this pump in contraction/relaxation and/or cellular growth processes.

Materials and Methods

Construction of Transgenic Rats

We established 4 transgenic rat lines carrying the human PMCA isoform 4CI cDNA3 under the control of the ventricle-specific rat myosin light chain-2 (MLC-2) promoter25 and 2 transgenic rat lines carrying the same cDNA under the control of the α myosin heavy chain (α-MHC) promoter.26 The first expression construct was composed of the 250-bp MLC-2 promoter, a 325-bp simian virus (SV) 40 intervening sequence (IVS), the 3.6-kb hPMCA4Cl cDNA, a 600-bp SV 40 IVS, and the 240-bp SV 40 poly A signal. The second expression construct contained the 2.9-bp α-MHC promoter, a 400-bp 5’ untranslated region of the α-MHC gene, the 3.6-kb hPMCA4Cl cDNA, the 600-bp SV 40 IVS, and the 240 bp SV 40 poly A signal. The sequence of both expression constructs was confirmed by restriction endonuclease digestion and DNA sequencing. The MLC-2 promoter and the 325-bp SV 40 IVS sequence were provided kindly by Dr Kenneth Chien and Genentech Inc; the α-MHC promoter was a gift from Dr Bruce Markham (Ann Arbor, Mich). The 3.6-kb hPMCA4Cl cDNA was provided kindly by Dr Emanuel Strehler (Rochester, Minn). The expression constructs were excised from the vector, gel-purified, and used for pronuclear microinjection of fertilized oocytes from Sprague-Dawley (SD) rats to produce transgenic rats according to the procedure described by Mullins and Ganten.27 Genomic DNA (10 μg) from rat tail biopsies were subjected to EcoRI digestion and Southern blot analysis according to the standard procedures using an hPMCA4Cl cDNA–specific 1.2-kb EcoRI fragment (corresponding to nucleotides 2177 to 3403 of hPMCA4Cl3) as a probe.

Northern Blot Analysis

Expression of the human PMCA isoform 4 mRNA was detected by Northern blot analysis of total RNA from transgenic rat hearts. Twenty micrograms of total RNA was run on a 1% formaldehyde-agarose gel, and Northern blotting was performed according to standard procedures. For hybridization, a randomly primed labeled 1200-bp EcoRI fragment of the human PMCA4Cl cDNA was used. Three of the four established transgenic lines with the MLC-2 promoter–driven transgene and 1 out of 2 lines with the α-MHC promoter showed mRNA expression of the PMCA transgene. Expression was most abundant in total heart and also in isolated cardiomyocytes from adult and neonatal hearts, but lower expression was also detectable in brain, lung, and kidney. No expression of the transgene was found in atrium, skeletal muscle, liver, or noncardiomyocytes from transgenic neonatal rat hearts.

Western Blot Analysis and Analysis of Transgene Activity

Preparation of Highly Purified Plasma Membranes From Cardiac Tissue

Heart tissue (2 g) was frozen in liquid nitrogen and powdered before homogenization in 20 mL buffer containing (in mmol/L): histidine 5 (pH 7.7), KCl 750, DTT 0.2, and PMSF 0.1. The sample was centrifuged at 3000g for 15 minutes, the pellet was resuspended in 20 mL homogenization buffer, and the previous step was repeated. The pellet was resuspended in 20 mL hypotonic medium containing 10 mmol/L NaHCO3 (pH 7.4), 5 mmol/L histidine, and 0.2 mmol/L DTT, centrifuged at 3000g for 15 minutes, and the pellet was homogenized (3×30 s; 21 000 rpm) in 20 mL hypotonic medium. The homogenate was centrifuged at 12 400g for 20 minutes, the pellet was treated identically to the previous step, and the supernatants were pooled and centrifuged at 45 000g for 30 minutes. The pellet was resuspended in 1 mL sucrose-histidine buffer I (250 mmol/L sucrose, 10 mmol/L histidine [pH 7.4], 160 mmol/L KCl) and centrifuged on a sucrose gradient (18% to 35%) for 90 minutes at 100 000g. The SL fraction was harvested, and 3.2 mL of 600 mmol/L KCl was added. The SL preparation was centrifuged at 60 000 rpm for 20 minutes, and the pellet was resuspended in sucrose-histidine buffer II (250 mmol/L sucrose, 10 mmol/L histidine [pH 7.4]) and centrifuged as in the previous step. The final pellet was resuspended in 200 μL sucrose-histidine buffer II.

Western Blot Analysis

The SL preparations were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes.28 Blocking was performed overnight with 1% BSA. The membranes were incubated for 1 hour with the monoclonal anti-PMCA antibody 5F10 (1:10 000 dilution), recognizing all 4 isoforms of the PMCA expressed in rat and human tissues (Affinity BioReagents, Hamburg, Germany). After a washing step (1×PBS; pH 7.45; 1% BSA), incubation with the secondary antibody (a sheep anti-mouse antibody coupled to alkaline phosphatase; Amersham, Braunschweig, Germany) at a 1/5000 dilution was performed for 1 hour. After a further washing step in 1×PBS (pH 7.45), 1% BSA, and 0.3% Tween, immunocomplexes were visualized by chemiluminescence according to the manufacturer’s protocol (ECL-kit, Amersham). For the detection of caveolin 3 in Western blotting experiments, a monoclonal antibody (Transduction Laboratories), recognizing rat and human caveolin 3, was used as described above.

For quantification, the data were normalized to Na+/Ca2+–exchanger protein (expression of the Na+/Ca2+–exchanger protein was unchanged in the myocardium of transgenic animals as detected by Western blot analysis). Further normalization to cell number and total protein revealed similar results. The polyclonal antibody used to detect the Na+/Ca2+–exchanger protein was obtained from SWAnt.

Coupled Enzyme Assay Measuring Ca2+-Dependent ATPase Activity

The reaction mixture contained 50 nmol/L CaCl2, 50 nmol/L HEPES-Tris (pH 7.4), 160 mmol/L KCl, 2 mmol/L MgCl2, 5 mmol/L NaN3, 1 μg/mL alamethicin, 1 mmol/L ATP-Tris, 1 mmol/L phosphoenolpyruvate, 1 μM pyruvate kinase, 0.6 mmol/L NADH, 1 μM lactatedehydrogenase. The reaction was started by adding 10 μg of highly purified SL protein to 1 mL of the mixture, and the optical density of NADH/NAD was measured over 2 minutes in a spectrophotometer at 340 nm at 37°C. To stop the Ca2+-dependent ATPase activity, 2 mmol/L EGTA was added, and the measurement was continued for 2 minutes. The Ca2+-dependent ATPase activity was calculated by subtracting the 2 fitted slopes.
Measurements of L-Type Ca\(^{2+}\) Current and Fura-2 Fluorescence Ratios in Isolated Adult Cardiomyocytes

**Isolation of Adult Cardiomyocytes**

Adult cardiomyocytes from transgenic and normal SD rats (female and male; body weight, 230 to 490 g) were isolated by the method described by Stegemann et al.\(^3\) for guinea pig ventricular myocytes with slight modifications according to Linz and Meyer.\(^4\)

**Measurement of L-Type Ca\(^{2+}\) Current**

L-type Ca\(^{2+}\) current was recorded at 35±1°C using the whole-cell patch-clamp technique.\(^5\) Patch pipettes with tip resistances of 2 to 4 MΩ were pulled from borosilicate glass (Hilgenberg) and connected to a single-electrode continuous-voltage clamp amplifier (L/M EPC 7, List Medical Electronic). In all measurements, cell capacitance, series resistance, and junction potentials were compensated. Voltage-clamp protocols and data acquisition were accomplished using pClamp 6.0 software (Axon Instruments). Current signals were on-line filtered at 3 kHz and digitized at 15 kHz by a 12-bit A/D converter (Digitalis 1200, Axon Instruments).

The L-type Ca\(^{2+}\) current was determined as total peak inward current. The separation from overlapping fast Na\(^+\) current and T-type Ca\(^{2+}\) current was achieved by holding the cells at −40 mV for the time between the test pulses. K\(^+\) currents (I\(_K\), I\(_{K_{1}}\), and I\(_{Na}\)) were blocked with an external solution containing (in mmol/L): NaCl 115, tetraethylammonium chloride 20, KCl 4, BaCl\(_2\) 2, MgCl\(_2\) 1, CaCl\(_2\) 1.8, 4-aminopyridine 2, HEPES 2, and glucose 11 (pH 7.2) and an electrode-filling solution composed of (in mmol/L): CsCl 130, Mg-ATP 2, and HEPES 10; and 50 μmol/L BAPTA (pH 7.2; Molecular Probes).

To determine current-voltage relations (I/V), steady-state activation parameters (d\(n\)), and steady-state inactivation parameters (L\(r\)), gapped double-pulse protocols\(^5\) with pulse lengths of 400 ms were used (stimulation frequency, 0.1 Hz). Current amplitudes were normalized to the cell capacitance. Fitting of I/V-relations was performed according to Linz and Meyer.\(^5\)

**Fluorescence Measurement of Fast [Ca\(^{2+}\)]\(_i\) Transients**

Fast [Ca\(^{2+}\)]\(_i\) transients were elicited by Ca\(^{2+}\) influx through L-type channels in patch-clamped cardiomyocytes. In these measurements, Na\(^+/Ca\(^{2+}\)\) exchange was blocked by equimolar substitution of LiCl for NaCl.\(^3\) Lasting voltage-clamp pulses (500 ms; frequency, 0.2 Hz) were applied from a holding potential of −40 to 20 mV in an external solution containing (in mmol/L): LiCl 135, KCl 4, MgCl\(_2\) 1, CaCl\(_2\) 1.8, 4-aminopyridine 2, HEPES 2, and glucose 11 (pH 7.2). The electrode-filling solution was composed of (in mmol/L): CsCl 130, Mg-ATP 2, HEPES 10, Fura-2/Na\(^{+}\) 50, and 50 μmol/L BAPTA (pH 7.2; Molecular Probes).

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**Fluorescence Measurement of Slow [Ca\(^{2+}\)]\(_i\) Transients**

Slow [Ca\(^{2+}\)]\(_i\) transients were elicited by external sodium depletion. After a 25-s baseline interval, standard Tyrode’s buffer (see above) was replaced by sodium-free buffer (equimolar substitution of LiCl for NaCl). Fluorescence image acquisition lasted for a total of 17 minutes. Hypercontracting and noncontracting cells were not evaluated. In some experiments, sarcoplasmic reticulum (SR) block was achieved with 30 minutes of preincubation with ryanodine-thapsigargin (10 and 5 μmol/L, respectively). Blocker substances were present throughout the experiment.

**Hemodynamic Measurements**

For hemodynamic measurements, rats were anesthetized with ether. Polyethylene cannulas were inserted into the trachea for artificial ventilation and into the right carotid artery, right jugular vein, and femoral vein. Pressure was measured through a short segment of a fluid-filled PE 50 tubing connected to a microtip manometer (Millar) via a 3-way stopcock, with 0 adjusted to midchest level. The carotid cannula was advanced briefly into the left ventricle and then was withdrawn into the aortic arch while pressures were recorded. The jugular vein cannula was advanced to the right atrium. Left ventricular systolic and end-diastolic pressures, the maximum rate of rise in left ventricular systolic pressure, dP/dt\(_{max}\), and mean right atrial pressure (RAP) were measured in heart-lung preparations (MAP), heart rate, and mean right atrial pressure (RAP) were measured under light ether anesthesia and spontaneous respiration.

During positive pressure ventilation, and after midsternal thoracotomy, a calibrated flowmeter (2.0 mm; Statham, Inc) was placed around the ascending aorta for continuous measurement of aortic blood flow. Mean aortic blood flow was obtained electronically and was taken as cardiac index (CI), as described by Pfeffer et al.\(^6\) Systemic vascular resistance index was calculated as (MAP−RAP)/CI and was expressed as mm Hg/mL per min/kg body weight.

After baseline measurements, warmed (39°C to 40°C) Tyrode’s solution was infused into the femoral vein at a rate of 40 mL/kg per minute for 45 seconds or until maximal flow was achieved.\(^6\) This infusion produces a rise in cardiac output to peak values, followed by a plateau, despite further elevation of RAP. Maximum cardiac performance was defined as peak values of cardiac output and stroke volume during this Tyrode’s infusion.

**Culture of Neonatal Cardiomyocytes and Protein Synthesis Measurements**

**Primary Cell Culture of Neonatal Rat Cardiomyocytes**

Cardiomyocytes from 48-hour-old transgenic and normal SD rats were prepared according to Simpson and Savion\(^7\) with minor modifications. The hearts were cut into small pieces and digested in CBFFHH (calcium and bicarbonate free Hank’s with HEPES) containing (in mmol/L): NaCl 137, KCl 5.36, MgSO\(_4\) ½ H\(_2\)O 0.81, glucose 5.55, KH\(_2\)PO\(_4\) 0.44, Na\(_2\)HPO\(_4\) 0.34, HEPES 20.06; pH 7.4 containing 31 μg/L penicillin G, 10 μg/mL DNase, and 1.5 mg/mL trypsin. FCS was added to the resulting cell suspension to inactivate trypsin and DNase, and the cells were pelleted by 10 minutes of centrifugation at 700g and resuspended in MEM/5 (containing 31

imaging system (Hamamatsu Photonics KK) described in detail by Gollnick et al.\(^8\) A dual excitation interference filter wheel (340 and 380 nm) and a 150-W Xenon arc lamp were used for Fura-2 excitation (0.4-s double flashes) at 5- or 10-s intervals. Digital intensity images (256×256 pixel; 8-bit resolution; no image averaging) of emission (425 to 575 nm) were acquired for each excitation wavelength using a Hamamatsu C2400-77H ICCD camera. Myocytes were placed in an open perfusion chamber (0.2 mL) and adhered to the laminin-coated glass bottom. Fura-2 fluorescence was measured in an inverted epifluorescence microscope (Zeiss Axiovert 100 TV with Zeiss Fluar 40× objective, Carl Zeiss) under permanent perfusion of buffers at 37±0.5°C. For evaluation of intensity changes with custom-written software (4 to 5 evaluable cells/experiment), digital measuring windows were placed within the cell borders. Background fluorescence was measured separately for each wavelength and subtracted from each intensity value before the calculation of 340:380 nm ratio images. Relative ratio changes were not converted to absolute changes of [Ca\(^{2+}\)]\(_i\). Slow [Ca\(^{2+}\)]\(_i\) transients were elicited by external sodium depletion. After a 25-s baseline interval, standard Tyrode’s buffer (see above) was replaced by sodium-free buffer (equimolar substitution of LiCl for NaCl). Fluorescence image acquisition lasted for a total of 17 minutes. Hypercontracting and noncontracting cells were not evaluated. In some experiments, sarcoplasmic reticulum (SR) block was achieved with 30 minutes of preincubation with ryanodine-thapsigargin (10 and 5 μmol/L, respectively). Blocker substances were present throughout the experiment.

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mu/mL penicillin G, 50 mu/mL streptomycin, 30.74 mu/mL BrdU, 2 mu/mL vitamin B12, and 5% FCS). Most noncardiomyocytes were removed by preplating for 1 hour. After preplating, the supernatant containing >90% cardiomyocytes was removed, and cells were counted in a Fuchs-Rosenthal chamber and plated in MEM/5 on 6-well plates at a density of 0.7 million cells/well (low-density culture). After 24 hours, the MEM/5 was removed, and the cells were grown for 48 more hours in the serum-free medium MEM/TI (additionally containing 31 mu/mL penicillin G, 50 mu/mL streptomycin, 30.74 mu/mL BrdU, 2 mu/mL vitamin B12, 1 mu/mL transferrin, and 1 mu/mL porcine Zn-insulin). Growth experiments were started on day 3.

Growth Experiments, Measurement of Cellular Protein Synthesis and Apoptosis Analysis
Primary cell cultures of 48-hour-old transgenic and normal SD rats were prepared as described above. On day 3, the MEM/TI was replaced by MEM/TI containing either a growth stimulus, such as 2% FCS, 20 mu/mL phenylephrine, and 1 mu/mL isoproterenol, or no stimulus. The cells then were incubated for several different time periods (6 to 12 to 18 to 24 to 36 hours). Four hours before the end of the incubation period, medium was changed into MEM/TI with/without growth stimulus and ['H-Leucine at an activity of 2.5 Ci/muL. After incubation, the cells were washed 2 times with PBS, lysed with 1% SDS, and harvested. A small amount of the lysate was used to determine DNA concentration as a measure of number of cells, using the Hoechst 33258 dye. An equal volume of 10% TCA was added to the lysate. Proteins were precipitated for 30 minutes at room temperature, pelleted by centrifugation (10 minutes, 14 000 g). The CPM:DNA concentration ratio was calculated for each sample, and changes in protein synthesis by the growth stimuli were depicted as percentage of the CPM:DNA concentration ratio of each sample, and changes in protein synthesis by the growth stimuli were depicted as percentage of the CPM:DNA concentration ratio of each sample, and changes in protein synthesis by the growth stimuli were depicted as percentage of the CPM:DNA concentration ratio of the nonstimulated control cells. Terminal deoxynucleotidyl transferase-mediated end-labeling of fragmented nuclei (TUNEL assay) was performed on neonatal cardiomyocytes according to the manufacturer’s protocol (Boehringer Mannheim Biochemicals).

Preparation of Caveolar Membranes
To purify caveolae, we used the protocol described by Song et al.29 Purified plasma membranes from 5-day-old SD rats were suspended in MES buffered saline (MBS), disrupted by sonication and placed at the bottom of a discontinuous 45% to 35% sucrose gradient. After centrifugation at 260 000 g for 20 hours, 1-mL fractions were taken starting from the top, and an equal amount of protein of each fraction was used in Western blot analysis detecting caveolin 3 (monoclonal anti–caveolin 3 antibody, Transduction Laboratories) and the PMCA (Ab 5F10, Affinity Bioreagents).

Immunostainings and Confocal Microscopy
Primary cell culture of neonatal cardiomyocytes was performed as described above. Cells were cultured on slides for 48 hours in medium containing 5% FCS and for another 24 hours in serum-free medium. They were fixed for 30 minutes with 2% paraformaldehyde at room temperature and then rinsed twice in PBS. Samples were blocked and permeabilized in PBS, containing 1% BSA, 0.5% Triton X-100, and 0.1% Tween 20 for 30 minutes at 20°C and then were incubated at 4°C overnight in a PBS/0.1% BSA solution containing the primary antibodies. For double-staining, the monoclonal antibody IA3 (a generous gift from Drs A. Filoteo and J.T. Penniston, Mayo Clinic and Foundation, Rochester, Minn), generated against the human PMCA isoform 4, and the polyclonal rabbit anti–caveolin 3 antibody (0.25 mg/mL, Transduction Laboratories) were used. Working dilutions of these antibodies were 1:500. After incubation, the samples were washed 5 times in PBS/0.1% Tween 20, then in Texas Red-conjugated goat anti-rabbit IgG (1.4 mg/mL) and the Cy2-conjugated goat anti-mouse IgG antibodies (1.3 mg/mL, Jackson ImmunoResearch Laboratories) were applied in PBS (1:500 and 1:250, respectively) for 1 hour at 4°C. After final washing steps, the samples were mounted in Vectashield mounting medium (Vector) and analyzed with a BioRad MRC 1024 confocal laser scanning system using the LaserSharp software. Statistical analysis was performed using Student t test, and values are expressed as SEM unless stated otherwise.

Results
Generation of Transgenic Rats Overexpressing the PMCA in the Myocardium
Several independent transgenic rat lines carrying the cDNA for the human PMCA isoform 4C1 under the control of a cardiac specific promoter were established as described in Materials and Methods. To assess the developmental-dependent expression of the transgene in the heart under the control of the MLC-2 promoter, we analyzed 3-, 14-, 30-, and 90-day-old homozygous transgenic (1142+/+) and SD wild-type rats were hybridized to a 32P-labeled probe of 1.2 kb corresponding to human PMCA isoform 4 (n=4). The specific signal at 3.6 kb was detected only in transgenic rats, whereas negative SD−/− controls displayed no signal for the transgene mRNA. The filter was rehybridized to a GAPDH cDNA probe as a loading standard. The intensity of the PMCA and the GAPDH signal was quantified, and relative PMCA mRNA levels were calculated. B, Western blot analysis of transgene expression. Highly purified plasma membrane protein preparations of cardiac tissue from neonatal and 12-week-old transgenic line 1142 rats and SD controls (10 mu g and 20 mu g in each left and right lane, respectively) were electrophoresed, transferred, probed with the antibody 5F10 against the PMCA, and reprobed with an antibody against the Na+/Ca2+ exchanger. The 5F10 antibody recognizes the endogenous rat and the overexpressed human PMCA. Quantification of the Western blot signals revealed a 2.5±0.3-fold increase in PMCA protein level in cardiac tissue from transgenic neonatal and adult rats, respectively. At least 2 Western blots were carried out for each time point, showing similar results for the quantification of the signals. For each Western blot, plasma membrane protein samples were prepared from 10 adult and 10 neonatal transgenic hearts and from the same number of control hearts. Two different protein concentrations were loaded for each parameter, and the signals were quantified.
3- and 14-day-old animals. The level of mRNA encoding the transgene decreased only slightly in adult animals.

**Quantitative Immunoblotting and Measurement of ATPase Activity in Transgenics and Controls**

To determine the levels of transgene overexpression, highly purified SL protein preparations from the myocardium of transgenics and controls were subjected to Western blot analysis. Quantitative immunoblotting revealed a 2.5-fold PMCA overexpression in transgenic neonatal cardiomyocytes and 1.6-fold overexpression of the calcium pump in adult cardiomyocytes compared with controls (Figure 1B).

To assess the function of the over expressed PMCA, Ca
2++-dependent ATPase activity was measured in highly purified plasma membrane preparations of the myocardium. The Ca
2+-dependent ATPase activity in the hearts of adult homozygous rats (line 1142) was 12.6 μmol ADP/mg protein/h compared with a level of 7.1 U in controls. These results fit well with our protein data (see above) and suggest that the transgene was entirely functional.

**Expression of Endogenous Calcium-Transporting Systems in the Heart of hPMCA4C1-Overexpressing Rats**

In cardiac tissue from PMCA-transgenic rats, no compensatory up- or down-regulation of the Na+/Ca
2+ exchanger, SERCA2a, and endogenous rat PMCA 1 mRNA was detected (Figure 2). The transgenics showed the normal decrease in expression of the Na+/Ca
2+ exchanger and increase in expression of the SERCA2a and the endogenous PMCA1 during development from neonatal to adult rats.

Furthermore, endogenous MLC-2 expression was not altered in transgenic animals (data not shown). This was excluded, because transgene expression was driven by the MLC-2 promoter; hence, down-regulation of the endogenous promoter activity might occur. This data showed that phenotypic differences between transgenics and controls would be due to transgene overexpression rather than compensatory changes in other Ca
2+ transporters.

**In Vivo Hemodynamic Experiments**

One hypothesis was that the PMCA might play a role in the cardiac contraction/relaxation cycle. Physiological measurements assessing the contraction/relaxation parameters such as left ventricular systolic pressure, mean aortic pressure, dP/dtmax, left ventricular end-diastolic pressure, RAP, and heart rate are summarized in Tables 1 and 2. Adult transgenic rats showed no significant differences in baseline or peak cardiac performance compared with SD wild-type rats.

**Electrophysiological Studies and Calcium Measurements**

Electrophysiological studies and Fura-2 measurements on isolated adult cardiomyocytes were performed to determine if the increase in PMCA expression resulted in corresponding alterations of electrophysiological parameters and/or the calcium transient. As shown in Figure 3, there are no differences in voltagedependent, activation, and inactivation behavior of L-type Ca
2+ current between transgenic cells and control adult cardiomyocytes. Furthermore, transgenic and control cardiomyocytes displayed an identical time course in fast [Ca
2+]i transients induced by calcium influx through L-type channels in the presence of thapsigargin and ryanodine (SR block; Figure 4A). The slow decline of [Ca
2+]i transients after Na+ depletion was faster slightly but not significantly in cardiomyocytes isolated from PMCA-transgenic animals (Figure 4B), attesting to the activity of the transgene but suggesting that a decrease in [Ca
2+]i, in the myofilament compartment was not a major effect of transgene overexpression. When the SR was blocked by thapsigargin and ryanodine, a marginally different time constant (τ) of [Ca
2+]i, decline was seen: τ=180.5 s (n=14) in controls versus τ=214.7 s (n=17) in transgenic cardiomyocytes (data not shown). The τ values of the monoexponential fit functions are given in the legend to Figure 4B. They are given without SD.
because mean values of an exponential function constant were fitted. The Fura-2 fluorescence ratios at rest were slightly different: ratio 340:380 nm was $0.89 \pm 0.17$ (n=14 cells; 4 different animals) in controls versus $1.14 \pm 0.36$ (n=17 cells; 4 different animals) in transgenic animals. Although both alterations could be indicative of compensatory mechanisms, they are so small that their biological relevance is probably minor.

### Cardiomyocyte Growth

Our second main hypothesis, derived from our\textsuperscript{16,21} and others\textsuperscript{22–24} results, was that the PMCA is involved in long-term cellular processes such as growth and differentiation rather than in beat-to-beat regulation of contraction/relaxation. As a model system for cardiac hypertrophy, we used primary low-density cultures of neonatal cardiomyocytes as a well-established model.\textsuperscript{37} Transgenic compared with wild-type cardiomyocytes re-

### TABLE 2. Baseline Cardiac Performance and Peak Cardiac Performance During Acute Volume Loading

<table>
<thead>
<tr>
<th></th>
<th>Baseline Performance</th>
<th>Peak Performance</th>
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<tbody>
<tr>
<td></td>
<td>SD −/+</td>
<td>SD −/+</td>
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<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>99±15</td>
<td>94±14</td>
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<td>Heart rate, min(^{-1})</td>
<td>356±32</td>
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<td>Cardiac output, mL/min</td>
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<td>Cardiac index, mL/min per kg</td>
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<tr>
<td>Stroke volume, mL</td>
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<td>0.27±0.08</td>
</tr>
<tr>
<td>Stroke volume index, mL/kg</td>
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<td>0.84±0.13</td>
</tr>
<tr>
<td>Total peripheral resistance, mm Hg/mL per min</td>
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<td>1.09±0.34</td>
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<tr>
<td>Total peripheral resistance index, mm Hg/mL per min/kg</td>
<td>0.32±0.10</td>
<td>0.33±0.07</td>
</tr>
</tbody>
</table>

SD −/− indicates Sprague-Dawley control rats; 1142 +/+, PMCA-transgenic rats.

Figure 3. Measurements of L-type calcium current. Characterization of L-type Ca\(^{2+}\) current in patch-clamped adult cardiomyocytes from control (■; n=33 cells; 4 animals) and transgenic animals (∆; n=45 cells; 4 animals). A, Averaged current-voltage relations of L-type Ca\(^{2+}\) current. Current amplitudes were normalized to single cell capacitance. B, Voltage dependence of time to peak of L-type Ca\(^{2+}\) current. C, Voltage dependence of $d_{\text{Ca}}$ and $f_{\text{Ca}}$. Error bars=SD.
revealed a 1.6- and 2.3-fold higher protein synthesis rate on stimulation with 2% FCS for 24 hours and 36 hours, respectively (Figure 5A). After incubation with 20 μmol/L phenylephrine, PMCA-overexpressing cells displayed a 2.0-fold higher level in protein synthesis (Figure 5B). Transgenic cardiomyocytes displayed a 1.4- and 2.0-fold higher peak in growth response on incubation with 1 μmol/L isoproterenol for 12 hours and 24 hours, respectively (Figure 5C).

Differences in growth were not due to a difference in the viability of transgenic and wild-type cardiomyocytes, as there were no significant differences in the number of apoptotic cells in both cultures (Figure 6). Apoptotic cells were detected by the TUNEL assay.

**Figure 4.** Measurement of [Ca\(^{2+}\)]\(_i\) in single cardiomyocytes. A, Fast [Ca\(^{2+}\)]\(_i\) transients induced by voltage clamp pulses (duration, 500 ms) from -40 to 20 mV, recorded after inhibition of Na\(^+\)/Ca\(^{2+}\) exchange and SR. Top, Command voltage. Bottom, Averaged [Ca\(^{2+}\)]\(_i\) transients recorded in cells from control (solid line; n=13 cells; 4 different animals) and transgenic animals (dotted line; n=17 cells; 4 different animals). Ratio values were normalized to the mean of the first 500 ms (0) and the maximum values (1). B, Slow decline of [Ca\(^{2+}\)]\(_i\) transients after Na\(^+\) deprivation by equimolar substitution of LiCl for NaCl in the external solution. Mean values of single cell measurements in control (n=14 cells; 4 different animals) and transgenic cardiomyocytes (transgenic; n=17 cells; 4 different animals). To compare the recordings on different cells, the peak of [Ca\(^{2+}\)] transient was set to t=0 s. Data were normalized to the maximum ratio values (1). Error bars=SD. The first 300 s of the averaged data were fitted by monoexponential functions with time constants of τ=71.4 s (control) and τ=68.8 s (transgenic).

**Figure 5.** Cardiomyocyte growth. Primary low density neonatal cardiomyocyte cultures from SD wild-type and transgenic rats were incubated with different stimuli (A, 2% FCS; B, 20 μmol/L phenylephrine; C, 1 μmol/L isoproterenol) for various periods. As a control parameter SD and transgenic cardiomyocytes were cultured in MEM/TI without additional factors. Protein synthesis rate was assessed by H\(^3\) leucin incorporation. Counts were standardized to DNA concentration, and the relative protein synthesis rate in stimulated cells was standardized to the data obtained from nonstimulated cells. For each parameter, 6 independent experiments (each in double) were performed. On average, 20 neonatal animals from at least 2 litters (for transgensics and controls each) were sacrificed for 1 experiment. Data are presented as SEM. *P≤0.05.
analogy to several other proteins localized to caveolae, there seem to be areas of the cell membrane where the PMCA is localized outside caveolae (Figure 8; also see Discussion).

Discussion

The present results demonstrate that: (1) overexpression of the SL calcium pump (PMCA) in the myocardium of transgenic rats did not lead to measurable alterations in basal and stimulated contractile activity of the adult heart or other hemodynamic parameters; (2) transgenic and control adult cardiomyocytes showed no differences in L-type calcium current and fast calcium transients; (3) PMCA-overexpressing neonatal cardiomyocytes showed a greatly altered amplitude of growth in response to phenylephrine and isoproterenol (and FCS as an aggregate stimulus); and (4) at least partial localization of the PMCA in caveolae of cardiomyocytes was demonstrated on the basis of differential density- and confocal immunofluorescence experiments, compatible with the hypothesis that the pump might be responsible for modulating the amplitude of caveolar signal transduction.

It has been discussed whether the PMCA is a calcium pump simply maintaining low cytosolic calcium concentrations and preventing cells from Ca\(^{2+}\) overload or whether it has additional significant physiological roles. In the present work, we have investigated the following 2 hypotheses using transgenic overexpression in the rat heart muscle as a “gain of function” model.

First, we hypothesized that the PMCA might play a role in the cardiac contraction/relaxation cycle. We reasoned that an increased PMCA expression might influence the diastolic Ca\(^{2+}\)-decline and hence cardiac relaxation. The second hypothesis—based on previous data—tentatively assigned the pump a potential role in regulation of long-term cellular processes in muscle cells, e.g., growth and differentiation.

The data presented here support an affirmation of the second hypothesis, whereas the first is made unlikely. With regard to the role of the PMCA and other Ca\(^{2+}\)-transporting enzymes such as the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase and the Na\(^+/\)Ca\(^{2+}\) exchanger in the cardiac contraction/relaxation cycle, most studies have investigated calcium transients and concomitant contractions of isolated cardiac myocytes. On the basis of these data, it has been calculated that in the rat heart, at each beat, only 1% to 2% of [Ca\(^{2+}\)]\(_i\) decline during relaxation is mediated by the PMCA and the mitochondrial Ca\(^{2+}\)-uniporter together, whereas Na\(^+/\)Ca\(^{2+}\) exchange across the plasma membrane accounts for ~25% of calcium removal; the sarcoplasmic reticulum pump is responsible for pumping the bulk flow of calcium (~75%) out of the myofilament compartment.

These data have been disputed on the grounds that the use of isolated cells in these experiments may not be representative of the situation in the intact myocardium. Furthermore, the sequential use of inhibitors of the various calcium transporters may have interfered with other cellular functions, and no specific inhibitor of the PMCA is available.

In our transgenic “gain of function” model, overexpression and activity of the transgene were ascertained, and compensatory changes in the expression level of major endogenous

**Figure 6.** Percentage of apoptotic cardiomyocytes detected by the TUNEL assay. After 12, 24, and 36 hours under basic culture conditions in MEM/11 (described in Materials and Methods), neonatal cardiomyocytes isolated from PMCA-transgenic animals did not show a different percentage of apoptotic cells compared with wild-type controls. Cultures incubated with isoproterenol, FCS, and phenylephrine in comparison to the growth experiments (Figure 4) showed the same result for transgenics and controls (data not shown).

**Localization of the PMCA in Caveolae**

To begin to unravel the mechanisms for the altered growth in transgenic cardiomyocytes, we tested the hypothesis that the PMCA is localized to caveolae. In differential density centrifugation, the PMCA could be detected in the same membrane fraction as caveolin 3 (Figure 7). Importantly, this fraction contained only ~3% of total membrane protein but close to 100% of the PMCA as well as the bulk of caveolin 3 protein. These results represent strong biochemical evidence for the localization of the PMCA in caveolae.

In addition, confocal microscopy studies demonstrated that the overexpressed hPMCA4CI (as well as the total PMCA-fraction; Figure 8g through 8i) and caveolin 3 were colocalized in cultured cardiac myocytes derived from transgenic animals (Figure 8). Small color dots represent caveolae, whereas larger aggregates likely are Golgi complexes; shutting of caveolae between the plasma membrane and the Golgi complex has been shown repeatedly. Interestingly, in

**Figure 7.** Preparation of caveolar membranes on a density gradient. Displayed are equal amounts of protein, taken from the 12 1-mL fractions of the density gradient column (fraction No. 1 is at the top). The table shows the amount of protein in each fraction as a percentage of total protein on the gradient. In fraction 5, in which the caveolin 3-positive caveolar membranes are enriched, the signal for the PMCA could be detected, whereas the other fractions displayed only a faint or no signal for the PMCA. SL indicates SL protein was loaded as a control and displayed the signal for caveolin 3 and the PMCA.
Figure 8. Confocal microscopy studies showing colocalization of hPMCA4CI and caveolin 3. a through c, Double-immunolabeling of hPMCA4CI (Cy2, green) and caveolin 3 (Texas Red, red) in isolated neonatal cardiomyocytes from PMCA-transgenic rats. In (a), green indicates the localization of hPMCA4CI. Red staining in (b) shows distribution of caveolin 3. Colocalization of both stainings is indicated in yellow in (c). Note that there is some PMCA staining outside the areas of caveolin staining, indicating that the PMCA may not be localized exclusively to caveolae, a phenomenon often observed within the group of caveolar proteins. Larger patches represent the Golgi complex, a compartment with intense caveolar trafficking. d through f, Panels show stainings in cardiomyocytes isolated from neonatal wild-type control rats. Stainings are as in (a) through (c). Some background staining by the hPMCA4CI antibody is seen because of faint cross-reactivity with rat PMCA. g through i, Confocal microscopy using the PMCA antibody 5F10, which is neither species- nor isoform-specific. As in panels (a) through (f), where the Texas Red staining shows the distribution of PMCA, the Cy2 (green) staining displays the caveolin 3 localization. Extensive, but not exclusive colocalization of PMCA and caveolin 3 is seen. A computerized image analysis revealed that ~56% of the PMCA pixels are colocalized with the caveolin 3 pixels. j through k, Negative controls demonstrating the absence of nonspecific binding of the secondary antibodies, j. The polyclonal rabbit anti–caveolin 3 and the Cy2-conjugated goat anti-mouse IgG antibodies were used for immunostaining. k, The broadly reactive monoclonal (and hence mouse) antibody 5F10 and the Texas Red–conjugated goat anti-rabbit IgG were utilized. l, Both secondary antibodies were used in parallel. Furthermore, the isoform specificity of the used primary antibodies has been shown extensively by us and others.
genes involved in cardiac calcium handling because of the PMCA overexpression were excluded. Thus, we believe that differences in phenotype between transgenics and controls were due to overexpression of the transgene rather than to compensatory changes in other Ca\textsuperscript{2+} transporters.

Functional alterations that may result from increased PMCA expression first were investigated by in vivo hemodynamic measurements in adult transgenic rats and by determining calcium transients in isolated adult cardiomyocytes. Hemodynamic parameters reflecting the contractility of the adult myocardium and pertinent fast calcium transients, as well as a variety of other pivotal components of electromechanical coupling, were unchanged.

In this context, it is interesting to note that overexpression of the SERCA2 (≈20%) in transgenic mice indeed could induce a significantly faster decline of the calcium transient and resulted in enhanced cardiac contractility.\textsuperscript{46} Furthermore, transgenic mice overexpressing wild-type or a mutant form of phospholamban, the regulator of the cardiac SERCA, to approximately the same extent in the myocardium as in our animals showed alterations in contractile parameters predicted by in vitro and single cell experiments.\textsuperscript{49,50} Overexpressing the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in the myocardium of transgenic mice did not significantly alter the resting calcium concentration, the magnitude of triggered Ca\textsuperscript{2+}-transients, or the Ca\textsuperscript{2+} current density but did accelerate the rate of removal of Ca\textsuperscript{2+} from the cytosol, when SR uptake was impaired by caffeine.\textsuperscript{51}

Because in our animals, overexpression of the PMCA was at least as high or higher than the overexpression of these other proteins, which did lead to alterations in contractility, we conclude that the PMCA has little, if any, role in the beat-to-beat regulation of the contraction/relaxation cycle.

The second hypothesis tested in the present work tentatively assigned the PMCA a role in growth regulation. This concept was based on our previous results, showing a dramatic effect of PMCA overexpression on the differentiation of skeletal muscle cells\textsuperscript{21} and on results reported by others demonstrating an influence of PMCA overexpression on cellular growth.\textsuperscript{22,23,52,53} Furthermore, inhibition of the PMCA using the antisense approach showed an influence of the pump on differentiation of pheochromocytoma cells.\textsuperscript{34}

Therefore, we focused our analysis on the growth of neonatal cardiomyocytes in culture, which is an established model for the analysis of hypertrophic growth.\textsuperscript{57} There was a significant difference in the response of transgenic and wild-type cardiac muscle cells to a variety of stimuli. PMCA overexpressing cells on average showed a 2-fold higher protein synthesis rate compared with controls.

These differences were not due to a difference in the viability of transgenic cardiomyocytes, as the percentage of apoptotic cells was similar. Whereas neonatal PMCA overexpressing cardiomyocytes showed an increased growth, the adult myocardium of transgenic rats did not display signs of hypertrophy. This could be because of compensatory mechanisms (frequently seen in transgenic animals) preventing hypertrophy in the adult heart at least under normal conditions. The effect of PMCA overexpression under pathological conditions awaits further testing.

Dissecting the mechanisms responsible for the alteration in growth was not the immediate goal of the present work, but as a step toward creating testable hypotheses, we investigated whether the PMCA is localized in caveolae of rat cardiomyocytes. Previously, there had been preliminary indications that the PMCA is localized in caveolae: Fujimoto\textsuperscript{54} published results based on immunogold labeling of the PMCA, showing the presence of the calcium pump in caveolae of various mouse tissues, but only tentatively in the myocardium. Schnitzer et al\textsuperscript{55} reported the presence of the PMCA in purified caveolar fractions of rat lung endothelial cells. For the first time, our results present biochemical evidence for localization of the PMCA in caveolae of the myocardium and show colocalization of the PMCA and caveolin 3 based on double-immunostaining. Caveolin 3, but not caveolin 1 or 2, is expressed in the myocardium.\textsuperscript{40} It is of interest that our immunofluorescence studies show that there may be compartments of the cell where the PMCA is not colocalized with caveolin, a phenomenon well known for other proteins.\textsuperscript{40} One possible interpretation is that there may be areas of the cell membrane ("cholesterol-sphingolipid rafts") that fulfill specialized functions in signal transduction in the absence of caveolin.\textsuperscript{56} This possibility currently is under investigation in our laboratory.

Caveolae (which are abundant in striated muscle) have been implicated in signal transduction.\textsuperscript{40,57} Several proteins recently have been localized to caveolae. Among these are important signaling molecules such as Gs\textsubscript{a}, nitric oxide synthase, ras, src-tyrosine kinase, and channels such as the IP3-sensitive Ca\textsuperscript{2+} channel.\textsuperscript{40} In view of our results and these recent findings about caveolae, we hypothesize that the calcium pump modulates the amplitude of signaling through molecules targeted to caveolae either by direct interaction and/or a modification of subcellular Ca\textsuperscript{2+} pools.\textsuperscript{58-60} It is not yet technically possible to measure calcium directly in 50- to 100-nm structures. Therefore, indirect methods will have to be used to address the latter question.

In conclusion, the data presented in this paper strongly suggest that future concepts about the function of the PMCA in the myocardium (and possibly in other tissues) should include a role for PMCA in long-term cellular processes such as growth and caveolar signal transduction. The assumption of participation of the pump in the contraction/relaxation cycle, as well as the hypothesis that the PMCA represents a mere “evolutionary remnant” in the myocardium, are both made unlikely by our data.

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