Effects of Mutant and Antisense RNA of Phospholamban on SR Ca\textsuperscript{2+}-ATPase Activity and Cardiac Myocyte Contractility

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**Background**—The delayed cardiac relaxation in failing hearts has been attributed to a reduced activity of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2). Phospholamban (PLB) inhibits SERCA2 activity and is therefore a potential target to improve the cardiac performance in heart failure.

**Methods and Results**—Mutants of PLB (Adv/mPLB) or antisense RNA of PLB (Adv/asPLB) was expressed in cardiac myocytes by recombinant adenovirus, and their effects on SERCA2 activity and myocyte contractility were studied. One mPLB, K3E/R14E, pentamerized with endogenous PLB in neonatal myocytes and resulted in a 45% increase in the affinity of SERCA2 for Ca\textsuperscript{2+} and 27% faster diastolic Ca\textsuperscript{2+} decline as determined by SR \textsuperscript{45}Ca uptake assays and by indo-1–facilitated Ca\textsuperscript{2+} transient measurement, respectively. Edge-detection analysis of adult myocyte contractility showed a 74% increase in fractional shortening, accompanied by 115% increase in velocity of relengthening and 25% decrease in time to half-maximal relengthening. In parallel, infection of neonatal cardiac myocytes by Adv/asPLB decreased the endogenous PLB level by 54%, which was associated with a 35% increase in Ca\textsuperscript{2+} affinity of SERCA2 and 21% faster diastolic Ca\textsuperscript{2+} decline. However, in adult cardiac myocytes, Adv/asPLB failed to significantly alter the endogenous PLB level, the SERCA2 activity, or most of the contractile parameters.

**Conclusions**—K3E/R14E is a dominant negative mutant of PLB that disrupts the structural integrity and function of the endogenous PLB and consequently enhances SERCA2 activity and myocyte contractility. In neonatal myocytes, the decrease in steady-state abundance of PLB by asPLB also leads to increased SERCA2 activity. *(Circulation. 1999;100:974-980.)*

**Key Words:** phospholamban ■ enzymes ■ sarcoplasmic reticulum ■ RNA ■ viruses

**Congestive heart failure** is characterized by a reduced contraction and delayed relaxation of the heart. A number of studies in human failing hearts and animal models of heart failure have suggested that the reduced uptake of cytosolic Ca\textsuperscript{2+} by the sarcoplasmic reticulum (SR) is responsible for the prolonged diastolic relaxation.\textsuperscript{1–4} The removal of cytosolic Ca\textsuperscript{2+} induces cardiac relaxation and depends on the activity of SR Ca\textsuperscript{2+}-ATPase (SERCA2).\textsuperscript{5} Phospholamban (PLB), another SR protein, has been shown to be a potent inhibitor of SERCA2.\textsuperscript{6,7} The physiological functions of SERCA2 and PLB in the heart were recently demonstrated in vivo. The ablation of PLB in mice resulted in stimulated SR Ca\textsuperscript{2+} uptake and enhanced contractile performance, whereas PLB overexpression resulted in opposite effects.\textsuperscript{3,9} Furthermore, mice overexpressing cardiac SERCA2 displayed an increased contraction and accelerated relaxation of the heart.\textsuperscript{10} These studies confirmed the importance of SERCA2 and PLB in regulation of cardiac contractility and suggest that the interaction of these proteins provides a potential target for the treatment of heart failure.

PLB was first identified as a major phosphorylation target in the SR membrane\textsuperscript{6} and was later found to be an inhibitor of SERCA2.\textsuperscript{7} The inhibitory effect of PLB on SERCA2 is reduced by an increase in intracellular calcium\textsuperscript{11} or by phosphorylation of PLB in response to \textbeta-\textadrenergic stimulation.\textsuperscript{7} Biochemical studies have shown that PLB exists primarily in a pentameric form that when boiled dissociates into monomers.\textsuperscript{12} Monomeric PLB is composed of 52 amino acids in 2 domains, a cytoplasmic domain (1–30) and a transmembrane domain (31–52).\textsuperscript{13} A detailed mutagenesis study by Toyofuku et al\textsuperscript{14} revealed that several amino acids in the cytoplasmic domain of PLB are important for its inhibitory function. For example, when the amino acids Glu\textsuperscript{2}, Lys\textsuperscript{3}, Arg\textsuperscript{9}, Arg\textsuperscript{14}, or Ser\textsuperscript{16} were mutated to amino acids of different charge, the PLB mutants lost their inhibitory effect on the cotransfected SERCA2 in HEK293 cells.\textsuperscript{14} However, it is
unclear whether PLB bearing these mutations can exert dominant negative effects on endogenous wild-type PLB and consequently stimulate endogenous SERCA2 in cardiac myocytes, because HEK293 cells are deficient in PLB and SERCA2. We addressed this question in cardiac myocytes. The expression of mutant PLB in cardiac myocytes can be achieved by generation of transgenic animals, as recently demonstrated by Chu et al., or by direct delivery of transgene into cultured cardiac myocytes via recombinant adeno-virus (Adv). In recent years, Adv has been used successfully for the transfer of genes into myocardium or cultured myocytes. For example, by Adv-mediated gene transfer, we and others have shown that the overexpression of SERCA2 in cultured cardiac myocytes led to acceleration of Ca\textsuperscript{2+} transients. In another study, a mutant troponin T gene was introduced into isolated adult myocytes via Adv. In the background of the endogenous wild-type troponin T, a dominant negative effect of the mutant was confirmed in contractile studies of the infected myocytes. In the present study, we generated adenoviral vectors expressing mutants or antisense RNA of PLB and studied their effects on the endogenous PLB and consequently the effects on SERCA2. The effects on myocyte contractility were also examined.

**Methods**

**Cloning and Mutagenesis of Rat PLB cDNA**

Rat PLB cDNA was synthesized by reverse transcription–polymerase chain reaction (PCR) using template polyA RNA isolated from the hearts of Sprague-Dawley rats. The sequences of the PCR primers shown below were described by Shanahan et al. and contained a BamHI (5' ) and EcoRI (3' ) linker (in lower-case letters):

5'-cggaattcTTTAAATTTCATTTATTCCCCAA-3'

The synthesized PLB cDNA was subsequently cloned in the BamHI/EcoRI sites of pBlueScript II SK (−) (Stratagene), and the identity of the gene was confirmed by sequencing. Selection of mutations was guided by the study of Toyofuku et al. and mutagenesis was carried out in PCR-based reactions. Glu2, Lys3, Arg4, and Ser6 were mutated to Ala, Glu, Gly, and Asn, respectively, and named (in single-letter symbols) E2A, K3E, R14E, and S16N. In addition, 1 mutation containing both K3E and R14E mutations (K3E/R14E) was made.

**Construction of Adv**

The cloning of transgenes into a replication-deficient human adenovirus 5 (Adv) was performed according to a protocol described by Becker et al. In brief, PLB and mutants of PLB (mPLBs) were cloned between cytomegavirus enhancer/promoter and SV40 polyadenylation signal sequence of the shuttle vector, pACCMV/PLpa, or SR(−) for short, which was kindly provided by Robert D. Girard, University of Texas Southwestern Medical Center, Dallas. Anti-sense PLB (asPLB) was generated by cloning the PLB cDNA in reversed orientation relative to the promoter. The shuttle constructs were then cotransfected with an adenovirus-derived plasmid, pJM17, into 293 cells. pJM17 was a generous gift from Frank L. Graham, McMaster University, Canada. The homologous recombination between the 2 vectors in 293 cells led to the generation of recombinant Adv containing the gene of interest. The Ads made in the present study code for the following genes: sense PLB (Adv/sPLB), antisense PLB (Adv/asPLB), and mutant PLBs (Adv/mPLB), including Adv/E2A, Adv/R14E, Adv/S16N, and Adv/K3E/R14E. In addition, Adv/SR(−) (no transgene) and Adv/LacZ (β-galactosidase) were used as experimental controls. The Ads were verified by either Southern blot analysis or PCR (data not shown) and subsequently propagated, purified by CsCl gradient centrifugation, and plaque-titered as previously described. The titer of Ads used in the following experiments was 1×10\(^6\) to 5×10\(^9\) plaque-forming units (pfu) per mL.

**Adenoviral Infection of Cardiac Myocytes or H9c2 or Solo8 Cells**

Neonatal rat cardiac myocytes were prepared as previously described. Approximately 6×10\(^7\) cells per 6-cm dish, 2×10\(^7\) cells per 10-cm dish, or 2×10\(^6\) cells per 3.5-cm coverslip were plated and used for Western blot analysis, SR \(^{45}\)Ca uptake assays, or Ca\textsuperscript{2+} transient measurement, respectively. The preparation of adult ventricular myocytes used the collagenase-dissociation method as described by Hilal-Dandan et al. for rat and by Yasuda and Lew for rabbit. Approximately 4×10\(^7\) adult rat or rabbit myocytes in 3-cm dishes were infected by Adv for SR \(^{45}\)Ca uptake assays or edge-detection assays. A rat embryonic heart–derived cell line (H9c2) or rat skeletal myoblast-derived cell line (Solo8) was used to study adenovirus-expressed PLB because these cells are deficient in endogenous PLB (Figure 1B). For all experiments, the cells were infected with Ads at a multiplicity of infection of 100 for 1 hour followed by 3 days of incubation.

**Preparation of Polyclonal Antibody Against PLB for Western Blot Analysis**

To raise a polyclonal PLB antibody, a chicken was repeatedly immunized with a rat PLB peptide (NH\(_2\)-KVQYLLTRSABRSTIEC) linked to keyhole limpet hemocyanin. The peptide represents amino acids 3 to 19 of the cytoplasmic domain of PLB. After 3 rounds of booster immunization (at 15 days, 42 days, and 54 days), total IgY was purified from the egg yolk by use of the EGGstract IgY Purification System (Promega). The specificity of this antibody was confirmed by comparison to a commercial monoclonal antibody (Affinity BioReagent) in Western blots, because it specifically recognized both pentamer and monomeric PLB (data not shown and Figure 1C). Furthermore, the chicken PLB antibody recognized the K3E/R14E PLB mutant, whereas the monoclonal antibody failed to detect the mutant PLB (Figure 1C).

**SR \(^{45}\)Ca\textsuperscript{2+} Uptake Assays**

Oxalate-facilitated SR \(^{45}\)Ca\textsuperscript{2+} uptake assays were based on a modified protocol of Pagni and Solaro. Ad-affected myocytes (≈2×10\(^6\)) were scraped in PBS, pelleted by centrifugation, and resuspended in 25 mmol/L imidazole (pH 7.0). Cells were homogenized by 10 passages through a 27-gauge syringe. Aliquots (75 μL) of lysates were transferred into tubes containing 750 μL of uptake buffer (in mmol/L: KCl 100, potassium oxalate 10, imidazole 40, sodium azide 10, MgCl\(_2\) 4.5, sodium ATP 2.5, creatine phosphate 3.0, and creatine phosphokinase 2.0 U/mL, pH 7.0). The uptake reaction was initiated by addition of 10,000 Ci/mL \(^{45}\)Ca (Amersham) and a given amount of free Ca\textsuperscript{2+} (pCa 5.5 to 8.5), which was calculated on the basis of the amount of added EGTA. After 10 minutes of incubation, 300 μL of the reaction mixture was transferred onto a 0.45-μm nitrocellulose membrane in a Millipore filtration apparatus, and radioactivity on the membrane was measured by liquid scintillation spectroscopy. The uptake activity at each pCa was expressed as the percentage of uptake at the maximal Ca\textsuperscript{2+} concentration (pCa 5.5). EC\(_0\) was defined as the Ca\textsuperscript{2+} concentration at which the uptake was half-maximal.

**Intracellular \(^{45}\)Ca Transient Measurement**

The Indo 1–facilitated Ca\textsuperscript{2+} transient measurement after adenoviral infection was as described previously. In brief, myocytes were incubated with 3 μmol/L indo 1-AM for 30 minutes at 37°C. The cells were then rinsed and placed in Tyrode’s solution containing 2 mmol/L CaCl\(_2\). Cells were stimulated to contract (0.3 Hz) by use of platinum electrodes. Indo 1 measurements were made with a data collection rate of 20 Hz at room temperature. For data analysis, representative Ca\textsuperscript{2+} transients were imported into a spreadsheet program and aligned and averaged with the initial upstroke of each transient as a reference point. Indo 1 ratios for each transient were also normalized with the basal (diastolic=0%) and maximum (sys-
tolic (100%) indo 1 ratios as references. The experiment was carried out by investigators unaware of sample identity.

Measurement of Myocyte Contractility (Edge Detection) in Adult Myocytes

The edge detection of cultured adult rabbit myocytes was performed according to a protocol described by Yasuda and Lew.26 For analysis, myocytes were transferred into a 0.5-mL glass chamber and examined under a microscope. Only rod-shaped cells with clear striations were studied. Myocytes were continuously perfused with Tyrode’s solution containing 2.5 mmol/L Ca$^{2+}$ at flow rate of 0.1 mL/min (23°C). Myocytes were field-stimulated to contract at 0.2 Hz (5-ms pulse duration). The pulse polarity was reversed every 10 stimulations. The cell length of the contracting myocyte was recorded at 60-Hz sampling frequency with a Panasonic GP-CD60 solid-state camera connected to a video edge-detection system (Crescent Electronics). The stimulus signal and myocyte lengths were converted from analog to digital and stored on a personal computer (Windaq software, Data Instruments). Myocyte shortening was calculated as the percent change in myocyte length from resting to minimal myocyte length. In addition, the time from minimal length to half-maximal relengthening (RT50) and the maximal velocity of relengthening ($\frac{dL}{dt}$) were calculated from the recordings. Data from 3 consecutive beats were averaged for each myocyte. Three animals were analyzed.

Statistical Analysis

Data are expressed as mean±SD except where indicated. One-way ANOVA was used to examine the statistical difference between control, Adv/K3E/R14E, and Adv/asPLB.

Results

Western Blot Analysis of PLB in Adenovirus-Infected Cells

Figure 1A shows a Western blot analysis of PLB in neonatal myocytes infected with Adv expressing indicated genes. SR(−) indicates control Adv vector; sPLB, PLB cDNA cloned in sense orientation; asPLB, PLB cDNA cloned in antisense orientation; and E2A, R14E, S16N, and K3E/R14E, mutants of PLB. PLB$\beta$, and PLB$\alpha$ indicate monomeric and pentameric PLB, respectively. (PLB/mPLB)$_5$ indicates putative heteropentamer consisting of both PLB and mPLB. Equal loading of protein is assessed by α-actin. Proteins were resolved on a 4% to 20% Tris-glycylamide gel (Novex) and blotted with mouse monoclonal antibody against PLB (Affinity BioReagents). B, Western blot analysis of PLB from a skeletal muscle myoblast cell line (Sol8) infected with Adv expressing sPLB or K3E/R14E alone or both. Equal amounts of protein were resolved on an 18% Tris-tricine gel and blotted with mouse monoclonal antibody against PLB (Affinity BioReagents). C, Western blot analysis of PLB from an embryonic cardiac cell line (H9c2) infected with Adv expressing LacZ, sPLB, K3E/R14E, or both sPLB and K3E/R14E. Proteins were resolved on a 4% to 20% Tris-glycylamide gel (Novex) and blotted with a chicken polyclonal antibody against PLB. mPLB$\alpha$ indicates pentameric mutant phospholamban.
decrease in PLB protein content. In addition, Adv/K3E/R14E infection of myocytes led to formation of a distinct pattern of pentamer PLB. Multiple PLB bands appeared in addition to PLB5. This was accompanied by a reduced abundance of PLB3 compared with the control. The nature of the banding pattern of PLB was further explored in PLB-deficient Sol8 cells. Sol8 cells were infected with either Adv/sPLB or Adv/K3E/R14E alone or both. The Western blot showed that the monoclonal PLB antibody detected PLB in cells infected by Adv/sPLB but failed to detect K3E/R14E (Figure 1B). Infection of Sol8 cells with both Advs resulted in formation of multiple bands of PLB. Moreover, PLB3 decreased in abundance simultaneously with the appearance of the upper bands.

Because K3E/R14E was not detected by the antibody and the formation of multiple bands of PLB only suggested but did not prove the expression of K3E/R14E and its interaction with sPLB, it is imperative to demonstrate the expression of K3E/R14E directly. A chicken polyclonal PLB antibody was raised against a peptide corresponding to the cytoplasmic domain of PLB. In addition, an H9c2 cardiac cell line was used because of its high infectivity by Adv. As shown in Figure 1C, this antibody recognized both wild-type and K3E/R14E PLB. Furthermore, K3E/R14E, both its pentamer and monomer, appeared to have a slower migration relative to sPLB and resulted in the formation of additional bands in the presence of sPLB (Figure 1C). These data suggest that Adv-expressed K3E/R14E formed heterotetramers with transgenic sPLB (in Sol8 or H9c2 cells) or endogenous PLB (in myocytes). In addition, the banding pattern suggests the formation of heterotetramers with different compositions of sPLB and K3E/R14E.

The effects of asPLB and K3E/R14E were also characterized in adult rat myocytes under the same conditions. It was found that Adv/K3E/R14E infection resulted in a similar pattern in electrophoresis, whereas Adv/asPLB infection did not alter the PLB protein level significantly (data not shown).

**Effects of mPLB and asPLB on SERCA2 and Intracellular Ca\(^{2+}\) Transients**

The effects of mPLB and asPLB on SERCA2 were examined by determination of the SR \(^{45}\)Ca uptake activity. Adv/E2A, Adv/R14E, and Adv/S16N did not affect SR \(^{45}\)Ca uptake compared with Adv/SR(−) and were therefore not included in the later studies. The initial rate of Ca\(^{2+}\) uptake by SR measured at certain [Ca\(^{2+}\)] \(_{o}\) reflects the activity of SERCA2. As shown in Figure 2, Adv/K3E/R14E and Adv/asPLB infection of neonatal rat myocytes decreased the [Ca\(^{2+}\)] \(_{o}\) needed by SERCA2 for the same activity compared with Adv/SR(−) and therefore stimulated SERCA2 activity. The EC\(_{50}\) of [Ca\(^{2+}\)] \(_{o}\) at which the uptake activity was half-maximal were, in \(\mu\)mol/L, 0.20±0.02 for Adv/SR(−), 0.11±0.01 for Adv/K3E/R14E, and 0.13±0.01 for Adv/asPLB, as summarized in Table 1. The effects of K3E/R14E and asPLB on SERCA2 were also examined in adult rat myocytes. Adv/K3E/R14E lowered the EC\(_{50}\) significantly (by 36%), whereas the change due to Adv/asPLB infection did not reach statistical significance (Table 1).

![Figure 2. SR Ca\(^{2+}\) uptake assay in homogenates of neonatal rat cardiac myocytes that were infected with Advs expressing indicated genes. SR(−) indicates control Adv without insert; K3E/R14E, mutant of PLB; and asPLB, PLB cDNA cloned in antisense orientation. Initial rate of SR Ca\(^{2+}\) uptake was determined at free Ca\(^{2+}\) concentrations ranging from pCa 5.5 to pCa 8.5. Uptake activity at each pCa was expressed as percentage of uptake at maximal Ca\(^{2+}\) concentration (pCa 5.5). Values are mean±SD of 5 samples from 3 independent experiments.](image)

To further examine the effects of K3E/R14E and asPLB on SERCA2, intracellular Ca\(^{2+}\) transients in neonatal myocytes were measured by use of the indo 1 fluorescence indicator. Indo 1 ratiometric data obtained from each condition were normalized to the respective maximum and minimum of each contractile Ca\(^{2+}\) transient and then aligned and averaged. As shown in Figure 3, the decay curves of K3E/R14E and asPLB are displaced to the left of the LacZ control. Furthermore, for most of the diastolic time points, K3E/R14E was significantly different from LacZ control, whereas at several diastolic time points, asPLB was also significantly different from LacZ. The half-times for decay, RT\(_{50}\), for LacZ, K3E/R14E, and asPLB were determined to be 0.28 seconds (or 100%), 0.20 seconds (or 73%), and 0.22 seconds (or 79%), respectively. The values for K3E/R14E (73%) and PLB (79%) are significantly different (\(P<0.05\)) from the values obtained from the LacZ-expressing virus. In a complementary approach, plasmid transfection rather than adenoviral infection was used for gene delivery. It was found that K3E/R14E- and asPLB-transfected myocytes, as monitored by cotransfected green fluorescence protein, exhibited 43% (\(P<0.05\)) and 9% (\(P>0.1\)) decreases in RT\(_{50}\), respectively, relative to vector-transfected cells. Thus, introducing K3E/R14E and asPLB into the cardiac myocytes by either the adenovirus or cotransfection technique reduced the duration of the diastolic Ca\(^{2+}\) transients.

**Effect of mPLB (K3E/R14E) and asPLB on Myocyte Contractility**

To determine whether the enhanced SERCA2 activity and accelerated Ca\(^{2+}\) transients lead to changes in contractile behavior of myocytes, we analyzed myocyte contractility by edge detection. Adult rabbit myocytes were infected with
Adv/LacZ, Adv/K3E/R14E, or Adv/asPLB. Three days later, we noticed a significant difference in the number of spontaneously contracting cells between the different groups (Adv/LacZ<<Adv/asPLB<Adv/K3E/R14E). Compared with the LacZ control, K3E/R14E increased fractional shortening by 74%, which was accompanied by a 25% decrease in RT50 and a 115% increase in +dL/dt (Table 2). When the myocyte contractility was examined after Adv/asPLB infection, it was found that the fractional shortening of the myocytes increased significantly (by 57%), whereas the changes in RT50 and +dL/dt were not significant (Table 2).

**Discussion**

Decreased SERCA2 activity, which leads to prolonged Ca2+-transients, is suggested to be the underlying mechanism of the delayed relaxation in failing hearts.1,2 In an attempt to improve the contractile phenotype in heart failure, it is of interest to identify mechanisms that can increase SERCA2 activity. Because it has been shown that the interaction of SERCA2 and its inhibitory protein PLB is conserved in failing hearts,28 we investigated 2 different approaches that specifically target PLB. The first approach is to disrupt the inhibitory effect of PLB on SERCA2 by a dominant negative mutant of PLB, and the second one is directed against the de novo synthesis of PLB by use of an antisense RNA strategy.

Our results demonstrate, for the first time, that a mutant of PLB interacts with wild-type PLB in a dominant inhibitory fashion. The effects of this mutant, K3E/R14E, on SERCA2 activity were examined in multiple assays. First, in SR Ca2+ uptake assays, K3E/R14E expressed by Adv (Adv/K3E/R14E) caused 45% and 36% decreases in EC50 of [Ca2+]i in neonatal and adult myocytes, respectively (Figure 2 and Table 1). The decrease in EC50 indicated an increase in affinity of SERCA2 for Ca2+ and therefore a stimulation of SERCA2 activity at a given submaximal concentration of Ca2+. The smaller effect of Adv/K3E/R14E in adult myocytes might be due to the higher abundance of endogenous PLB in adult myocytes than in neonatal myocytes and suggests a competition between endogenous and mutant PLB. In addition, changes in SR Ca2+ uptake activity were further, and more directly, determined by indo 1–facilitated Ca2+ transient measurement. It was found that the diastolic Ca2+ decline was accelerated in K3E/R14E and asPLB-expressed myocytes. Taken together, these data confirmed that K3E/R14E and asPLB stimulated the SERCA2 activity, which indeed resulted in faster Ca2+ transients in myocytes.

To determine whether the increased SR Ca2+ uptake activity is accompanied by changes in contractile phenotype, the contractile characteristics of Adv/K3E/R14E-infected adult myocytes were analyzed by edge detection. The in-

### TABLE 1. Relative Change in EC50 of SERCA2 in Adv/K3E/R14E- and Adv/asPLB-Infected Cardiac Myocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>K3E/R14E</th>
<th>asPLB</th>
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<tbody>
<tr>
<td>Neonatal cardiac myocytes (n=3)</td>
<td>100±10</td>
<td>55±5 (P&lt;0.05)</td>
</tr>
<tr>
<td>Adult cardiac myocytes (n=3)</td>
<td>100±12</td>
<td>64±6 (P&lt;0.05)</td>
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SR Ca2+ uptake assays were carried out in neonatal or adult rat cardiac myocytes that were infected by Adv expressing the indicated genes. SR(−) indicates control Adv without insert; K3E/R14E, mutant of phospholamban (PLB); and asPLB, PLB cDNA cloned in antisense orientation. The data from 3 independent experiments were plotted, and the Ca2+ concentration at which uptake activity was half maximal (EC50) was determined. The mean EC50 of asPLB and K3E/R14E is expressed as a percentage of the EC50 of SR(−). The data represent mean ± SD of 3 independent experiments (n=3). One-way ANOVA was used to examine the statistical difference between SR(−), K3E/R14E, and asPLB. P values indicate comparison with the SR(−) control group.

### TABLE 2. Effects of K3E/R14E and asPLB on Myocyte Contractility

<table>
<thead>
<tr>
<th>Group</th>
<th>LacZ</th>
<th>K3E/R14E</th>
<th>asPLB</th>
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<tr>
<td>+dL/dt, μm/s</td>
<td>11.7±1.9</td>
<td>25.1±1.6 (P&lt;0.05)</td>
<td>18.4±2.0 (P&lt;0.05)</td>
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<tr>
<td>RT50, ms</td>
<td>539.0±27.0</td>
<td>402.0±19.0 (P&lt;0.05)</td>
<td>483.0±29.0 (P&lt;0.1)</td>
</tr>
<tr>
<td>Shortening, %</td>
<td>6.2±0.5</td>
<td>10.8±0.5 (P&lt;0.05)</td>
<td>8.0±0.6 (P&lt;0.1)</td>
</tr>
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Adult rabbit cardiac myocytes were infected with Adv expressing β-galactosidase (LacZ) as control, mPLB (K3E/R14E), or asPLB. Myocyte shortening was analyzed by edge detection. Shortening is expressed as % change from resting myocyte length to minimal myocyte length. Data are expressed as mean ± SEM of 3 animals, and n indicates the total number of myocytes analyzed. Statistical analysis between groups was performed by ANOVA. P values indicate comparison with the LacZ control group.
crease of the maximal +dL/dt was paralleled by an abbreviated RT_{50}. These data show that the increased SERCA2 activity translates into an accelerated relaxation of the myocytes. In addition, Adv/K3E/R14E-infected myocytes displayed an enhanced fractional shortening, which suggests an increase in SR loads of Ca^{2+} due to the enhanced SERCA2 activity. This is also in accord with the findings in PLB-deficient and SERCA2 transgenic mice.8,10 Furthermore, it is noteworthy that Adv/K3E/R14E infection increased the number of spontaneously contracting myocytes, a phenomenon most likely associated with the increased amount of oscillating Ca^{2+} due to the elevated SR loading of Ca^{2+}.30 Taken together, these data show that K3E/R14E affects endogenous wild-type PLB in a way that significantly reduces its inhibition of SERCA2. Although our experiments do not unveil the underlying mechanism that disrupts the SERCA2-PLB interaction, a study by Toyofuku et al14 indicates that certain mutations change the net charge of PLB and therefore result in a loss of inhibitory function. It has been further shown by Kimura et al31 and Autry and Jones32 that PLB interacts with and inhibits SERCA2 predominantly as a monomer that exists in equilibrium with the noninhibitory pentamer. On the basis of this hypothesis, the heteropentamer of K3E/R14E and wild-type PLB might be more stable than the homopentamer of wild-type PLB. Therefore, the dissociation of the heteropentamer into monomers, which ultimately results in inhibition of SERCA2, is disfavored. Although these assumptions are speculative, we were able to demonstrate that K3E/R14E interacts with endogenous PLB and forms such a complex, accompanied by a decrease in homopentamer formation (Figure 1). Alternatively, the monomeric K3E/R14E may act as a noninhibitory competitor for endogenous wild-type PLB simply by blocking SERCA2-PLB interaction sites. However, in contrast to the hypothesis that favors monomeric PLB as the active SERCA2 inhibitor, Chu et al15 recently demonstrated that the pentamer formation is necessary for optimal inhibition, which suggests that the presence of K3E/R14E in heteropentamer impairs the inhibitory function of PLB in the present study.

In a second experimental approach, we used a full-length asPLB to reduce endogenous PLB levels. We hypothesized that a reduction of PLB levels would lead to increased SERCA2 activity, similar to the findings in the PLB-deficient mice.8 The injection of myocytes with Adv/asPLB led to a decrease in PLB protein content in neonatal myocytes, presumably due to the increased degradation of PLB mRNA. The decrease in PLB was confirmed in SR Ca^{2+} transient measurement (Figures 2 and 3). However, in adult rat myocytes, the steady-state level of PLB was not affected by the expression of asPLB. This was partially consistent with the results of edge detection, in which RT_{50} and fractional shortening were not significantly affected, although the velocity of shortening was increased by 57% (P=0.05). The effect of asPLB in adult myocytes needs to be further examined in the future when a more effective antisense oligonucleotide of PLB becomes available. In separate studies published recently,33 we could show that increasing PLB levels in adult rabbit myocytes by Adv vector expressing sense PLB resulted in a significant prolongation of RT_{50}. Nevertheless, the discrepancy in the effects between neonatal and adult cardiac myocytes might be related to the different abundances of PLB in myocytes at different developmental stages. It has been shown that PLB is nearly twice as abundant in adult as in neonatal myocardium.29 Because an antisense approach depends completely on the inhibition of the de novo synthesis of a protein and the half-life of preexisting proteins, it is conceivable that a more effective antisense oligonucleotide or a longer incubation period in adult cardiac myocytes might generate significant effects. Nonetheless, the data obtained in neonatal cardiac myocytes confirmed in principle the feasibility of this approach.

In summary, the present study has identified a dominant negative mutant of PLB, K3E/R14E, that disrupts the function of the wild-type PLB, resulting in enhanced contractility of cardiac myocytes. Furthermore, we could demonstrate that antisense PLB led to decreased PLB protein content and increased SERCA2 activity in neonatal cardiac myocytes. This study further underlines the experimental value of adenovirus-mediated gene transfer into isolated cardiac myocytes and offers potential strategies to improve contractile performance in the diseased heart.

Acknowledgments

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