[Ca\(^{2+}\)]; homeostasis and cyclic nucleotide relaxation in aorta of phospholamban-deficient mice

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PHOSPHOLAMBAN (PLB) is a 52-amino acid protein that is localized in the sarcoplasmic reticulum (SR) membrane. PLB has an inhibitory effect on the cardiac SR Ca\(^{2+}\)-ATPase, which is relieved when PLB is phosphorylated (9, 16). PLB expression in smooth muscle has varied 12-fold (6). This evidence suggests that the level of PLB expression might play a role in regulating intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). We recently showed (17) that mouse aorta from gene-targeted mice deficient in PLB (PLB\(^{-}\)) displayed significant differences in their mechanical properties from wild-type controls. The concentration-isometric force relations of the PLB\(^{-}\) aorta for both KCl and phenylephrine (PE) stimulation were to the right of those measured for the wild type. This decreased sensitivity is consistent with a greater Ca\(^{2+}\) uptake in the SR due to a more active Ca\(^{2+}\) pump in the absence of PLB inhibition. Treatment with the SR Ca\(^{2+}\)-ATPase inhibitor cyclopiazonic acid abolished these differences, further supporting the hypothesis that these differences were related to SR Ca\(^{2+}\) handling (17). In the present study, we further tested this hypothesis by directly measuring the effects of PLB gene ablation on aortic Ca\(^{2+}\) homeostasis using the fluorescent indicator fura 2. Our results indicate that the alterations in contractility previously reported are mirrored by changes in [Ca\(^{2+}\)]\(_i\).

Because PLB plays a significant role in modulation of Ca\(^{2+}\) homeostasis and contractility in the mouse aorta, it was of considerable interest to investigate whether PLB may also be involved in the relaxation elicited by cyclic nucleotide (cNMP) pathway activation. Phosphorylation of PLB by cAMP- or cGMP-dependent protein kinase has been shown to increase Ca\(^{2+}\) uptake in SR vesicles prepared from bovine pulmonary artery (26, 27) and in cultured smooth muscle cells from rat aorta (2). Also, in rat aorta, nitric oxide-induced relaxation via the cGMP kinase pathway was associated with phosphorylation of PLB (12). These in vitro studies indicate that either cAMP or cGMP can lead to phosphorylation of PLB.

To assess the potential role of PLB in cNMP-mediated relaxation, we measured the functional consequences of PLB ablation in the aorta. We measured relaxation kinetics and agonist concentration vs. force relations after treatment with agents that stimulate the production of cAMP or cGMP. Our data support the hypothesis that PLB modulation of the SR Ca\(^{2+}\) pump is an important site for regulation of smooth muscle cytosolic Ca\(^{2+}\) and a site for coordinated regulation by cAMP and cGMP.

MATERIALS AND METHODS

Aorta preparation. The murine PLB gene was targeted in murine embryonic stem cells, and mice homozygous for the targeted PLB allele were generated (21). Genotypes of all animals were determined by PCR analysis of tail DNA biopsies. Eight- to 12-wk-old mice were anesthetized either by

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intraperitoneal injection of 1 mg pentobarbital sodium per gram body weight with 1.5 U of heparin given to prevent aortic thrombi or by CO2 gas inhalation and, then were killed by cervical dislocation. The aorta were dissected and rinsed in cold bicarbonate-buffered physiological saline solution (PSS); loose fat and connective tissue were removed. Aortas were cut into rings of 6 mm in length that had an outer diameter of 0.70 ± 0.04 mm and average wet weight of 1.24 ± 0.13 mg. The endothelium was removed mechanically by sliding the ring on a 30-gauge stainless steel needle.

For measurement. The aortic ring was threaded with two 100-μm stainless steel wires, and each wire was placed in an angle-shaped holder; each completed mounting formed a double triangle. The triangles were attached to the Harvard Apparatus Differential Capacitor Force Transducer such that isometric force in the circumferential direction was measured. Resting tension on each aorta was set to 25 mN, the tension was calculated for an in vivo aortic pressure of 100 mmHg and a cross-sectional area of 0.42 mm², and this passive tension was maintained throughout the experiment. Data were acquired using BioPac hardware and were analyzed using the companion AcqKnowledge Software.

Ca²⁺ measurements. Aortic tissues were prepared in the manner described and were isometrically mounted on a 100-μm stainless steel wire as described above. Tissues were incubated for 2 h at room temperature in a foil-wrapped cuvette that contained MOPS-PSS, 12.5 μmol/l fura 2-AM, and 0.01% Cremaphor (vol/vol, Sigma). After incubation, the tissues were rinsed in 25°C Krebs-PSS for 15 min to remove free dye from the chamber. The stainless steel bracket and artery assembly were then attached to a Teflon mount with an inflow and outflow port and fitted into an acrylic cuvette; the final chamber volume was 2.4 ml. The cuvette was connected to a Cole-Palmer circulating pump via polyethylene tubing in inflow and outflow port and fitted into an acrylic cuvette; the artery assembly were then attached to a Teflon mount with an inflow and outflow port and fitted into an acrylic cuvette. Fluorescence was excited at 340 nm, and emission was measured at 510 nm.

As previously described (25), the fluorescence ratio was formed by dividing the intensity at the 340-nm excitation wavelength by that at 380 nm (340/380 ratio). This ratio was assigned values of 0% for resting muscle and 100% for tissue stimulated with 50 mmol/l KCl as previously reported. This protocol was chosen as a general routine over calibration of the fura 2 in absolute [Ca²⁺], because it involves the fewest assumptions.

Solutions. Krebs-PSS contained (in mmol/l) 122 NaCl, 4.73 KCl, 15 NaHCO₃, 1.19 MgCl₂, 0.02 EDTA, 1.19 KH₂PO₄, 2.50 CaCl₂, and 11.10 glucose, bubbled with 95% O₂-5% CO₂ for a final pH of 7.4 at 37°C. MOPS-PSS contained (in mmol/l) 140 NaCl, 4.70 KCl, 1.20 NaHPO₄, 20 MOPS, 0.02 EDTA, 1.2 MgSO₄, 2.50 CaCl₂, and 11.10 glucose, with a pH of 7.4 at 37°C.

Western blot analysis. Whole mouse heart and aorta homogenates were used to determine relative PLB and SR Ca²⁺-ATPase levels. Tissues were excised, placed in liquid N₂, and powdered. The powdered samples were resuspended in a homogenization buffer containing (in mM) 25 imidazole, 300 sucrose, 1 dithiothreitol, 20 sodium metabisulfite, and 0.1 phenylmethylsulfonyl fluoride, and protein concentrations were determined (Bio-Rad, Goleta, CA). Protein samples were solubilized in SDS sample buffer, and protein concentrations in the linear range for antibody detection were loaded on a 10–20% gradient SDS-polyacrylamide gel. Samples were transferred electrophoretically to a polyvinylidene difluoride membrane. After the membrane was blocked with 5% dry milk, the membrane was incubated for 1 h at room temperature with a monoclonal antibody to PLB (1:1,000 dilution) or a polyclonal antibody to SR Ca²⁺-ATPase (1:400 dilution). The antibody-antigen complex was detected after incubation of the blot with horseradish peroxidase-conjugated secondary antibody and was visualized using enhanced chemiluminescence Western blotting reagents (Amersham, Arlington Heights, IL). Blots were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Statistics. All values are expressed as means ± SE. Significance was determined using ANOVA and Bonferroni tests between wild-type and PLB⁻ mice. Concentration-response curves were fitted for each artery using Logistics Fit from Origin software, and the fitting parameters were averaged. Statistical analysis was performed using t-test for group comparisons for sequential experiments, particularly sodium nitroprusside (SNP) treatment after isoproterenol treatment. Probability of null hypothesis (P < 0.05) was considered significant; n refers to the number of mice.

RESULTS

In our previous report (17), we characterized the time course of isometric force in response to 50 mmol/l KCl or 10 μmol/l PE for wild-type and PLB⁻ aorta (17). Figure 1 shows typical responses from a pair of aorta in the
current study that exhibited similar behavior for comparison with \([Ca^{2+}]_i\) data. Briefly, there were little differences in the contraction kinetics for a 50 mmol/l KCl stimulation. For contractures to 10 µmol/l PE, the PLB\(^{-}\) aorta demonstrated a more rapid rise in force. Relaxation from either stimulus was faster in PLB\(^{-}\)aorta but did not obtain statistical significance. The steady-state force in response to 10 µmol/l PE is lower in the PLB\(^{-}\)aorta (relative to the maximum KCl contraction) than in the wild type, indicative of the decrease in sensitivity of the PE concentration-force relation in PLB\(^{-}\)aorta. To further test our hypothesis that these differences were related to increased SR Ca\(^{2+}\) uptake in the PLB\(^{-}\)aorta, we measured \([Ca^{2+}]_i\) in these aorta using the ratiometric dye fura 2.

Figure 2 shows the 340/380 ratio of fura 2 in response to stimulation by 50 mmol/l KCl or 10 µmol/l PE for wild type (A) and PLB\(^{-}\) (B) aorta. The signal reached a maximum for both wild-type and PLB\(^{-}\)aorta within 10 s after increasing stimulus. Most notable are the more rapid decline in the Ca\(^{2+}\) indicator signal in the PLB\(^{-}\)aorta upon washout of the bath contents with fresh PSS. The height of the peak during PE stimulation was approximately two times its steady-state value in the PLB\(^{-}\).

Figure 3 presents graphically the averaged \([Ca^{2+}]_i\) measurements for the type of experiment shown in Fig. 2. The risetimes of the fura 2 ratio for either PE or KCl stimulation did not differ. After washout of the stimu-
lus, the return to baseline of the fura 2 ratio for the PLB\(^{-}\) aorta was two times as fast than that of the wild-type aorta. The steady-state fura 2 ratio after stimulation with KCl or PE did not differ in the wild-type aorta. However, in the PLB\(^{-}\) aorta, the steady-state ratio for PE was significantly \((P < 0.05)\) less than that for the reference KCl contracture.

Fig. 2. Ratio of fluorescence at 340 to 380 nm of the Ca\(^{2+}\) indicator fura 2 loaded in wild-type and PLB\(^{-}\) aorta in response to KCl and PE stimulation. A: original record of the 340-to-380 nm fluorescence ratio (baseline = 0, maximum KCl = 100) from wild-type mouse aorta loaded with fura 2-AM. Note that there is little difference in the steady-state values whether the tissue is stimulated with KCl or PE. Also note the shallow slope of signal decline after "wash," which indicates that the cuvette contents were replaced with fresh physiological saline solution (PSS). B: response of PLB\(^{-}\)aorta to the same conditions as in A. Note the following 3 distinct features: 1) there is a very steep drop in the fura 2 signal upon replacement of the bath contents with fresh PSS (wash); 2) upon stimulation with 10 µmol/l PE, there is a biphasic response (a steep increase in fura 2 signal, followed by an immediate decline of the signal); 3) the final steady-state level after PE stimulation is much lower than that seen with KCl stimulation in the wild type.

Fig. 3. Summary of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) kinetic data for wild-type and PLB\(^{-}\) aorta for the experiment shown in Fig. 1. Ratio of the 340/380 fluorescence for peak PE (PE\(_{peak}\)) to KCl is normalized to the steady-state KCl ratio (KCl\(_{ss}\)) and presented as percent. Rise and relaxation half-times are given in seconds. Bars represent averages for 6 preparations ± SE. *Significant difference between that measured for wild-type and PLB\(^{-}\) aorta at the \(P < 0.05\) confidence level.
aorta, whereas the peak and steady-state ratios were similar for the wild-type aorta.

If our hypothesis of PLB modulation were to be valid, the decreased sensitivity of isometric force to agonist in the PLB<sup>−</sup> aorta must reflect [Ca<sup>2+</sup>]<sub>i</sub>. We thus tested whether the differences in force were associated with similar changes in [Ca<sup>2+</sup>]<sub>i</sub>. Figure 4 presents the average [Ca<sup>2+</sup>]<sub>i</sub> data for the steady-state response to 10 µmol/l PE normalized to the steady-state response to a maximal concentration of KCl (50 mmol/l). This steady-state PE-to-KCl ratio is nearly two times as large in wild-type than in PLB<sup>−</sup> aorta. Figure 4 also shows that a similarly constructed PE-to-KCl ratio of the developed isometric forces was also approximately two times as large in the wild-type than in the PLB<sup>−</sup> aorta. This is a reflection of the decreased sensitivity of force to PE stimulation in the PLB<sup>−</sup> aorta demonstrated in our previous study (17). Thus the decreased sensitivity of isometric force in the PLB<sup>−</sup> aorta was associated with a similar decrease in the [Ca<sup>2+</sup>]<sub>i</sub> response.

cNMP. Numerous mechanisms, including those involving PLB, have been proposed for cNMP-mediated vasodilation (11, 22). The PLB<sup>−</sup> mouse provides a unique animal model for estimating the contribution of PLB to these pathways. We investigated the role of PLB in the cAMP-signaling pathway using isoproterenol and forskolin. In contrast to rat aorta, which relaxes completely to 1 µmol/l isoproterenol (23), mouse aorta showed only a slight decrease in force at this concentration. Even at 30 µmol/l, ~70% of its original force was maintained. No differences were noted between the PLB<sup>−</sup> and wild-type aortas in this response. In contrast, forskolin, which directly stimulates adenylyl cyclase, completely relaxed mouse aorta (Fig. 5A) with an ED<sub>50</sub> of ~0.25 µmol/l for both tissue types. Again, little difference between PLB<sup>−</sup> and wild-type aorta was observed; these data are summarized in Table 1. We also examined whether there were any differences in the time course of relaxation after treatment with 1 µM forskolin, which is sufficient to elicit a complete relax-

![Fig. 5. Time courses of relaxation of a 10 µM PE contracture after treatment with 1 µM of either forskolin (A) or sodium nitroprusside (B). There were no statistically significant differences between PLB<sup>−</sup> and wild-type aorta for either time course of relaxation. Points are mean values ± SE for 4 wild-type and 5 PLB<sup>−</sup> mouse aortas in A and 4 each in B.](http://ajpheart.physiology.org/)

Fig. 4. Comparison of the averaged isometric force and [Ca<sup>2+</sup>]<sub>i</sub> data for wild-type and PLB<sup>−</sup> aorta. Responses to 10 µM PE were normalized to the maximum KCl (KCl<sub>max</sub>) response. Note that the relative differences in [Ca<sup>2+</sup>]<sub>i</sub> parallel those of force for both wild-type and PLB<sup>−</sup> aorta.

![Fig. 4. Comparison of the averaged isometric force and [Ca<sup>2+</sup>]<sub>i</sub> data for wild-type and PLB<sup>−</sup> aorta. Responses to 10 µM PE were normalized to the maximum KCl (KCl<sub>max</sub>) response. Note that the relative differences in [Ca<sup>2+</sup>]<sub>i</sub> parallel those of force for both wild-type and PLB<sup>−</sup> aorta.](http://ajpheart.physiology.org/)
Table 1. Concentration vs. force relations for PLB− and wild-type aorta contracted with 10 μM PE and relaxed with forskolin, SNP, or SNP after exposure to Iso

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>ED₅₀</th>
<th>Force, mN/mm²†</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin, μmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>5</td>
<td>0.267 ± 0.04</td>
<td>14.44 ± 1.69</td>
<td>0.91 ± 0.0</td>
</tr>
<tr>
<td>PLB</td>
<td>4</td>
<td>0.245 ± 0.01</td>
<td>12.94 ± 2.31</td>
<td>0.85 ± 0.0</td>
</tr>
<tr>
<td>SNP, nmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>12</td>
<td>45.6 ± 16.7</td>
<td>13.50 ± 0.84</td>
<td>0.88 ± 0.0</td>
</tr>
<tr>
<td>PLB−</td>
<td>10</td>
<td>16.0 ± 4.6</td>
<td>11.40 ± 1.01</td>
<td>0.86 ± 0.0</td>
</tr>
<tr>
<td>SNP (post-Iso), nmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>7</td>
<td>5.38 ± 1.81*</td>
<td>14.74 ± 0.90*</td>
<td>1.13 ± 0.1</td>
</tr>
<tr>
<td>PLB−</td>
<td>7</td>
<td>1.19 ± 0.25</td>
<td>11.71 ± 1.01</td>
<td>1.34 ± 0.1</td>
</tr>
</tbody>
</table>

Parameters were averaged and are presented as means ± SE. PLB−, phospholamban deficient; PE, phenylephrine; SNP, sodium nitroprusside; Iso, isoproterenol. Concentration-force data for individual aorta were each fit with the power logistic relation F = Fmax × [1 - (ED₅₀ + [c/ED₅₀]²)], where F is the developed force, Fmax is the maximum force, c is the concentration of vasodilator, ED₅₀ is the concentration of vasodilator at one-half maximum relaxation, and h is the Hill coefficient. *Indicates P < 0.05 for the difference between wild-type and PLB− aorta. †P > 0.05 for the difference in average force for all data between wild-type and PLB− aorta.

The table shows the concentration vs. force relations for PLB− and wild-type aorta contracted with 10 μM PE and relaxed with forskolin, SNP, or SNP after exposure to Iso. The data is presented as the number of observations (n), ED₅₀, force in mN/mm², and Hill coefficient. The table highlights the concentration-force data for individual aorta, each fitted with a power logistic relation. The data shows significant differences between the two tissue types and indicates the relative PLB-to-SERCA ratio in the mouse heart.

We have shown that the time course of the decline in Ca²⁺ upon removal of agonist is substantially faster in the PLB− aorta than in the wild type (Fig. 3). In our previous studies, the differences between PLB− and wild-type aorta were abolished by cyclopiazonic acid, localizing the site of the difference in contractility to the PLB-to-SERCA ratio. To better understand these results, it is important to know the relative PLB-to-SERCA ratio, since this determines the level of maximum potential inhibition. This is technically difficult, even when tissue mass is not limiting, as is the case for the mouse aorta. To achieve reasonable precision, our strategy was to determine the PLB-to-SERCA ratio in the mouse aorta relative to that in the mouse heart, for which absolute protein levels have been exhaustively measured to establish this ratio (10, 15). Figure 6 shows Western blots of PLB and SERCA from mouse heart and aorta. Approximately 20 times more aortic homogenate protein needed to be loaded on the gels to achieve similar levels of immunoreactivity as that in heart. To obtain the relative PLB-to-SERCA ratio in the aorta, we first obtained the slope in the linear region of the immunoreactivity vs. protein relation for both heart and aorta from the same gel or gels run simultaneously. PLB immunoreactivity slopes for aorta were divided by those of the heart to form the PLB aorta-to-PLB heart ratio. A similar procedure was carried out for SERCA, run on the same gels. Dividing these ratios yields a value for the ratio of PLB to SERCA in the aorta relative to the heart. With the use of the immunoreactivity of the pentameric form of PLB, this ratio was 0.87. With the use of boiled protein samples, to assure all PLB was present in the monomeric form, a ratio of 1.25 was obtained. Thus, for the mouse, the ratio of PLB to SERCA in the aorta is similar to that in the heart.

**DISCUSSION**

The ratio of PLB to SERCA in the mouse heart is reported to be in the range from 0.5 to 1.0 (10, 15). On the basis of a comparison of heart and aortic proteins using Western blot analysis, the aorta has a similar PLB-to-SERCA ratio. This is significantly higher than that reported for slow skeletal muscle (32) and supports our hypothesis that PLB can be a major factor in modulation of vascular contractility.

**Ca²⁺ handling.** The Ca²⁺ handling ability was compared in aorta from PLB− mice vs. the wild type. PLB is known to have an inhibitory effect on the SR Ca²⁺-ATPase, and this inhibition is relieved upon phosphorylation (9). Thus complete removal of PLB from the regulatory system should result in a dephosphorylated Ca²⁺-ATPase, which should enhance the ability of the SR to remove Ca²⁺ from the cytosol. This hypothesis has previously been validated for cardiac myocytes (34). In the present studies, our data suggest that a similar mechanism involving PLB is also valid in smooth muscle.

We have shown that the time course of the decline in Ca²⁺ upon removal of agonist is substantially faster in the PLB− aorta than in the wild type (Fig. 3). In our previous studies, the differences between PLB− and wild-type aorta were abolished by cyclopiazonic acid, localizing the site of the difference in contractility to the PLB-to-SERCA ratio. This is significantly higher than that reported for slow skeletal muscle (32) and supports our hypothesis that PLB can be a major factor in modulation of vascular contractility.

**[Ca²⁺] AND cGMP RELAXATION IN PLB− MICE**
SR (17). Together these data support the hypothesis that, in the absence of PLB, the SR Ca\(^{2+}\)-ATPase would have a higher affinity for Ca\(^{2+}\) and thus would be able to more rapidly remove Ca\(^{2+}\) from the cytosol. An enhanced Ca\(^{2+}\) uptake in the absence of PLB could also be anticipated to decrease the steady-state [Ca\(^{2+}\)] levels. This appears to be the case with 10 \(\mu\)mol/l PE stimulation, because the steady-state fura 2 signal in the PLB\(^{-}\) aorta was only \(\sim 60\%\) of the signal compared with the wild type.

In cardiac myocytes, the lack of PLB leads to an increase in SR Ca\(^{2+}\) loading and/or Ca\(^{2+}\) release (1, 31). This may be similar in PLB\(^{-}\) aorta in which the peak [Ca\(^{2+}\)], response to 10 \(\mu\)mol/l PE was larger than that in the wild type (106 vs. 84%, relative to the KCl reference), although this trend was not statistically significant (Figs. 2 and 3). We previously reported that the peak increase in force with PE stimulation in a Ca\(^{2+}\)-free medium was also greater in the PLB\(^{-}\) aorta (17). This would also be consistent with a greater SR Ca\(^{2+}\) loading and/or Ca\(^{2+}\) release.

Alternatively, when using gene-targeted or transgenic animals, one always must be cognizant of potential compensatory changes. In hearts from PLB\(^{-}\) and wild-type mice, a variety of Ca\(^{2+}\)-handling, metabolic, and contractile proteins were compared (1). The only significant change was a 25% decrease in the ryanodine receptor. These types of experiments are difficult in the mouse aorta; however, it was not statistically different from that of the wild-type aorta. These types of experiments are difficult in the wild type (106 vs. 84%, relative to the KCl reference), although this trend was not statistically significant (Figs. 2 and 3). We previously reported that the peak increase in force with PE stimulation in a Ca\(^{2+}\)-free medium was also greater in the PLB\(^{-}\) aorta (17). This would also be consistent with a greater SR Ca\(^{2+}\) loading and/or Ca\(^{2+}\) release.

In these cNMP relaxation pathways. It is possible that, because functional removal of the SR with cyclopiazonic acid eliminated differences between wild-type and PLB\(^{-}\) aorta (17), any compensation would likely be limited to the SR. Thus the changes in Ca\(^{2+}\) handling seen here in the absence of PLB would only be greater if compensation is occurring.

Although [Ca\(^{2+}\)] and force production do not always correlate in smooth muscle (33), our data indicate a good correlation between force production and cytosolic Ca\(^{2+}\) levels. Both parameters in PLB\(^{-}\) aorta are \(\sim 60\%\) of the response observed in the wild type (Fig. 4). The changes in force are thus likely due to changes in Ca\(^{2+}\) and not due to changes in Ca\(^{2+}\)-sensitivity of the contractile apparatus. In our previous study (17), relaxation of isometric force upon washout of agonist was slightly faster in PLB\(^{-}\) aorta; however, it was not statistically different from that of the wild-type aorta. The half-time for this mechanical relaxation was \(\sim 1\) min. This is similar to the half-time for [Ca\(^{2+}\)] to return to the prestimulus baseline (Fig. 3) for the wild-type aorta but considerably longer than that for the PLB\(^{-}\) aorta (30 s). This supports our previous suggestion that the reduction in [Ca\(^{2+}\)] was unlikely to be the limiting step for relaxation of force. Presumably, dephosphorylation of the regulatory myosin light chains is also involved (4).

cNMP. Prominent pathways for relaxation in smooth muscle include cAMP- and cGMP-mediated responses, which have been shown to have several important effects (22, 29, 33), including activation of cNMP-dependent protein kinases, regulation of cNMP-gated cation and anion channels (8, 19, 36), regulation of cAMP phosphodiesterase (28, 35), regulation of myosin phosphorylation levels, reduction in rate of inositol trisphosphate synthesis (14, 30), and phosphorylation of PLB (9).

Relaxation in response to cNMP has been suggested to involve a number of sites in smooth muscle and is known to involve both a lowering of [Ca\(^{2+}\)] and also a decrease in Ca\(^{2+}\)-sensitivity (33). Our expectation would be that, if PLB played a major role in cAMP- or cGMP-mediated pathways, then concentration vs. relaxation curves for these two aorta would be different. Specifically, the PLB\(^{-}\) curve would lie to the left of the wild type, especially at low doses of agonist. Mouse aorta was quite sensitive to both SNP and forskolin, which could elicit complete relaxation of a PE (10 \(\mu\)mol/l) contraction. Importantly, complete relaxation could also be obtained in aorta lacking PLB. There were little differences between aorta types in the sensitivity or the time course of the relaxation. A slight (2-fold) increase in sensitivity to SNP was observed in the PLB\(^{-}\) aorta, but this was not statistically significant (Table 1). Even if this were significant, these data indicate that PLB does not play a major role in either cAMP- or cGMP-mediated relaxation in mouse aorta. This is an important finding, since PLB has often been suggested to be a major effector in cNMP-mediated vascular relaxation based largely on correlations between relaxation and PLB phosphorylation (11, 12). This gene-targeting approach is a powerful tool for separation of causality from correlation.

There is considerable evidence indicating that the cAMP or cGMP pathways can interact in smooth muscle (2, 20, 24). Our data indicate that there is potentiation of the cGMP pathway by cAMP. Relaxation to SNP was significantly potentiated after prior exposure to isoproterenol (Table 1). Moreover, a significant difference in the ED\(_{50}\) between the PLB\(^{-}\) and wild-type aorta was also seen. Our data suggest that cNMP relaxation may not be primarily modulated by PLB inhibition of the SR but that fine tuning of the steady-state Ca\(^{2+}\) levels may occur through interaction of these pathways. The large differences observed between wild-type and PLB\(^{-}\) aorta would suggest that PLB is not highly phosphorylated in the resting state, at least in terms of its inhibition (17). Thus one would have anticipated a greater role for PLB in these cNMP relaxation pathways. It is possible that, in other vascular tissues, phosphorylation of PLB by cNMP pathways may be a more significant factor. Because PLB is also phosphorylated by a Ca\(^{2+}\)- and calmodulin-dependent kinase, this may be a more physiologically relevant pathway in smooth muscle, although it is not in the heart (13).

In conclusion, our two-part study demonstrates a significant role for PLB in modulation of smooth muscle contractility. The site of altered contractility in the PLB\(^{-}\) aorta was localized to the SR, suggesting alterations in Ca\(^{2+}\) handling mediated by PLB (17). This study confirms this previous hypothesis by direct measurement of [Ca\(^{2+}\)]. The data presented in this paper...
are consistent with a deinhibited SR Ca\(^{2+}\)-ATPase in the PLB\(^{-}\) aorta that can more rapidly lower [Ca\(^{2+}\)]\(i\) than one regulated by PLB. In addition, our data support the hypothesis that a deinhibited pump would produce a lower steady-state [Ca\(^{2+}\)]\(i\) during stimulation than in the wild type. Somewhat surprisingly, PLB did appear to modulate not a major effector in cAMP- or cGMP-mediated relaxation. However, PLB did appear to modulate effects due to interactions between these two nucleotides. The wide range of PLB content in smooth muscles in conjunction with our studies suggests that regulation of PLB expression may play a long-term role in the modulation of force and [Ca\(^{2+}\)]\(i\).

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REFERENCE


31. Santana, L. F., E. G. Kranias, and W. J. Lederer. Calcium sparks and excitation-contraction coupling in phospholamban-