Serine 68 Phospholemman Phosphorylation during Forskolin-Induced Swine Carotid Artery Relaxation

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Key Words

Cyclic adenosine monophosphate · FXYP protein · Phospholemman · Phosphorylation · Vascular smooth muscle

Abstract

Background: Phospholemman (PLM) is an abundant phosphoprotein in the plasma membrane of cardiac, skeletal and smooth muscle. It is a member of the FXYP family of proteins that bind to and regulate the Na,K-ATPase. Protein kinase A (PKA) is known to phosphorylate PLM on serine 68 (S68), although the functional effect of S68 PLM phosphorylation is unclear. We therefore evaluated S68 PLM phosphorylation in swine carotid arteries. **Methods:** Two anti-PLM antibodies, one to S68 phosphorylated PLM and one to unphosphorylated PLM, were made to PLM peptides in rabbits and tested with purified PLM and PKA-treated PLM. Swine carotid arteries were mounted isometrically, contracted, relaxed with forskolin and then homogenized. Proteins were separated on SDS gels and the intensity of immunoreactivity to the two PLM antibodies determined on immunoblots. **Results:** The antipeptide antibody 'C2' primarily reacted with unphosphorylated PLM, and the antipeptide anti-

body 'CP68' detected S68 PLM phosphorylation. Histamine stimulation of intact swine carotid artery induced a contraction, increased the CP68 PLM antibody signal and reduced the C2 PLM antibody signal. High extracellular [K⁺] depolarization induced a contraction without altering the C2 or CP68 PLM signal. Forskolin-induced relaxation of histamine or extracellular [K⁺] contracted arteries correlated with an increased CP68 signal. Nitroglycerin-induced relaxation was not associated with changes in the C2 or CP68 PLM signal. **Conclusions:** These data suggest that a contractile agonist increased S68 PLM phosphorylation. Agents that increase [cAMP], but not agents that increase [cGMP], increased S68 PLM phosphorylation. S68 PLM phosphorylation may be involved in cAMP-dependent regulation of smooth muscle force.

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Introduction

Phospholemman (PLM, also known as FXYP1) is a 72-amino acid sarcolemmal protein that is abundant in heart and skeletal muscle and present in other tissues such as kidney [1]. Protein kinase A (PKA) induces PLM phos-

phorylation on serine 68 (S68) [2, 3] while protein kinase C (PKC) induces phosphorylation on both serine 63 (S63) and S68 [2, 3]. Initial studies suggested a role for PLM in osmolyte flux and cell volume regulation [4, 5]. More recent findings suggest a physical and functional association of PLM with membrane ion transporters such as the Na,K-ATPase [6, 7] and the Na-Ca exchanger (NCX1) [8, 9]. These findings suggest that PLM may have functional roles similar to other members of the FXYD family of proteins [10]. For example, the γ -subunit of Na,K-ATPase (FXYD2) shares significant homology with PLM [10] that could explain the effect of PLM on Na,K-ATPase activity [11].

Very little is known about PLM in smooth muscle. Three papers describe a 16- to 17-kDa phosphoprotein in smooth muscle that may well have been PLM (PLM has an apparent molecular weight of approximately 16 kDa on SDS gels) [1]. In 1985, Boulanger-Saunier et al. [12] identified a 16-kDa phosphoprotein in a plasma membrane-enriched fraction of rat aortic smooth muscle cell membranes. This protein copurified with Na,K-ATPase and was phosphorylated by PKA in vitro and by isoproterenol in vivo. Its dephosphorylation was inhibited by 10 mM NaF. Boulanger-Saunier et al. [13] subsequently found that a phorbol ester (TPA) in vitro and arginine vasopressin in vivo induced phosphorylation of the 16-kDa protein on a second site distinct from the PKA site. In 1989, Sarevic et al. [14] found that a 17-kDa membrane phosphoprotein, likely PLM, was phosphorylated by PKA but not by protein kinase G. PLM mRNA has been reported in canine aortic, esophageal and gastric smooth muscle [1]. While these publications suggest that PLM is present and phosphorylated in intact smooth muscle, they cannot be considered definitive as they predate the cloning of PLM in 1991 and the subsequent development of reagents to study it.

Contraction of smooth muscle is typically associated with increased intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$), formation of a Ca^{2+} -calmodulin complex, activation of myosin light chain kinase (MLCK) and phosphorylation of myosin regulatory light chains (MRLC) on serine 19 [15]. Phosphorylation of MRLC enables crossbridge attachment to the thin filament, thereby allowing crossbridge cycling and force generation [16]. In many cases, smooth muscle relaxation proceeds via a reversal of this contraction process: reduction of myoplasmic $[Ca^{2+}]$, inactivation of MLCK and dephosphorylation of MRLC [17–19].

cAMP-mediated smooth muscle relaxation occurs via binding of agonists to specific seven-membrane-spanning receptors (e.g., β_2 -adrenergic) which activate adenylyl cy-

clase via G proteins [e.g., ref. 20]. Adenylyl cyclase can also be directly and relatively specifically activated by low concentrations of forskolin (e.g., 0.1–1 μM). It is well known that increases in $[cAMP]$ can reduce $[Ca^{2+}]_i$ and thereby inactivate MLCK and cause relaxation by MRLC dephosphorylation [see reviews in ref. 21, 22]. Specific mechanisms for reduction in $[Ca^{2+}]_i$ include (1) inhibition of Ca^{2+} mobilization from the sarcoplasmic reticulum [21], (2) hyperpolarization [23], (3) decreased Ca^{2+} influx through voltage-gated channels [24], and (4) activation of plasma membrane Ca^{2+} pumps [25]. There are also mechanisms other than reductions in $[Ca^{2+}]_i$ where increased $[cAMP]$ can cause relaxation, e.g., force suppression associated with serine 16 heat shock protein 20 phosphorylation [26].

Our general hypothesis is that cAMP-mediated phosphorylation of PLM on S68 phosphorylation causes vascular smooth muscle relaxation by increasing the activity of the Na,K-ATPase. The goals of this study were (1) to determine if PLM is present in smooth muscle, (2) to determine if cAMP-mediated relaxation is associated with S68 PLM phosphorylation, the site phosphorylated by PKA in vitro, and (3) to infer whether cAMP-mediated relaxation is associated with a decrease in intracellular Na^+ ($[Na^+]_i$). To accomplish these goals, we developed S68 phosphorylation-specific PLM antibodies. These antibodies were then tested in forskolin-induced relaxation of swine carotid artery.

Material and Methods

Tissues

Swine common carotid arteries were obtained from a slaughterhouse and transported at 0°C in physiological salt solution (PSS). PSS contained (mM): NaCl 140, KCl 4.7, 3-[N-morpholino] propane sulfonic acid 5, Na_2HPO_4 1.2, $CaCl_2$ 1.6, $MgSO_4$ 1.2, *D*-glucose 5.6, pH adjusted to 7.4 at 37°C. Zero Na^+ PSS was PSS where CholineCl was substituted for NaCl and the Na_2HPO_4 was omitted. Dissection of medial strips, mounting and determination of the optimum length for stress development at 37°C were performed as previously described [27]. The intimal surface was mechanically rubbed to remove the endothelium.

Antibodies

We developed two specific antibodies against PLM: C2 which was made to react with dephosphorylated PLM and CP68 which was made to react with PLM phosphorylated at S68, the site phosphorylated by PKA. Both were raised against C-terminal PLM peptides. To raise polyclonal antibodies against dephosphorylated PLM (C2), the 16-amino acid peptide NH_2 -CGTFRSSIRRLSTR-RR-COOH was used as the antigen and the resulting rabbit polyclonal antibody affinity purified as described [28]. To make S68 phosphorylation-specific antibodies (CP68), the 19-amino acid

peptide NH₂-DEEGTFRSSIRRL Sp (68) TRRR-COOH was made with phosphoserine (Sp) at S68. This peptide was injected into rabbits and serum collected. For affinity purification of CP68 (the phosphorylation-specific antibody), the serum was processed over an agarose immunosorbent containing the unconjugated dephosphorylated peptide to remove antibody activity that would bind the peptide irrespective of phosphorylation status. Phosphorylated PLM-specific antibodies were then affinity purified from the cleaned serum by passage over an immunosorbent containing the phosphorylated PLM peptide. ELISA showed less than 2% cross reactivity of the phosphorylated PLM-specific antibody with the dephosphorylated PLM peptide (Bethyl Labs, Montgomery, Tex., USA).

PLM Standards

Purified recombinant dephosphorylated PLM was prepared as described [29]. A pig cardiac sarcolemmal vesicle preparation [30] was loaded on most blots since it contained dephosphorylated and phosphorylated PLM that was detected by both anti-PLM antibodies.

PLM in vitro Phosphorylation

Forty micrograms of purified recombinant dephosphorylated PLM (trifluoroethanol removed by N₂ evaporation) [29] were incubated in 200 μ l of a buffer containing 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, 0.1% Triton X-100, 25 mM NaF and 0.1 mM ATP with γ -³²P-ATP. Samples were removed both before and after addition of 1 μ l (1.1 μ g) of the catalytic subunit of PKA (specific activity 6.3 μ mol/min/mg, a generous gift of Tom Lincoln, PhD). The reaction was stopped by addition of 1% SDS. The stoichiometry of PLM phosphorylation was calculated from the ³²P activity of the phosphorylated PLM (dpm/mol PLM) divided by the ³²P-specific activity of the ATP employed (dpm/mol γ -phosphate in ATP).

Measurement of PLM Phosphorylation

Swine carotid arteries were pharmacologically treated and frozen in an acetone-dry ice slurry (20 g/20 ml) at -78°C [27]. The frozen tissue was allowed to slowly thaw to room temperature in the slurry (2 h), the tissues were air dried, weighed and homogenized in a buffer containing 1% SDS, 10% glycerol and 20 mM dithiothreitol (20 mg wet weight/ml buffer). Twenty microliters of homogenates (concentration normalized to tissue weight) were then loaded identically onto two 12% SDS electrophoresis gels, blotted to nitrocellulose and then incubated with the anti-PLM antibodies (1:10,000 for C2 and 1:1,000 for CP68). After washing, incubation with secondary antibodies (1:5,000 of goat anti-rabbit) and detection with enhanced chemiluminescence, the blots were imaged and digitized with UnScanIt (Silk Scientific Inc.).

Two internal standards were loaded on every gel to allow comparison of immunoblotting intensity on different blots. One standard, 'KF', was a pooled homogenate of swine carotid tissues that had been activated with 109 mM extracellular [K⁺] ([K⁺]_o) for 10 min followed by the addition of 10 μ M forskolin for 30 min. The second standard, 'K', was a pooled homogenate of swine carotid tissues that had been activated with 109 mM [K⁺]_o for 40 min. The intensity of the PLM antibody immunoreactivity was determined for the experimental samples and these standards on every blot. We found that the ratio of the intensity of experimental sample CP68 immunoblotting to the intensity of KF immunoblotting

from the same blot was the most reproducible for CP68 (similar results were seen with other normalizations); therefore, we report the CP68/KF ratio as an index of S68-PLM phosphorylation (fig. 3b, d). We found that the C2 blots were most reproducible when the intensity of sample C2 immunoblotting was normalized to the intensity of the C2 immunoblotting from the unstimulated control swine carotid artery tissue from the same blot; therefore, we report the C2/control ratio as an index of unphosphorylated PLM (fig. 3a, c).

Statistics

The significance of the correlation between the intensity of CP68 immunoblotting and contractile force shown in figure 4 was tested using analysis of covariance (ANCOVA). The analysis showed no significant difference among the slopes for different experimental conditions, so the final model allowed intercepts but not slopes to vary. Significance was defined as $p < 0.05$.

Results

As detailed in the methods, two antibodies were made to C-terminal PLM peptides. To evaluate the specificity of these antibodies, purified recombinant PLM [29] (shown to be dephosphorylated by mass spectrometry) was phosphorylated with the catalytic subunit of PKA in the presence of γ -³²P-ATP. Different amounts of phosphorylated and dephosphorylated PLM (exposed to γ -³²P-ATP without PKA) were loaded on SDS gels, blotted, analyzed for ³²P activity and immunoblotted with both antibodies. Increasing ³²P activity was observed with an increasing amount of PKA-treated PLM (fig. 1, top panel). Calculated stoichiometry revealed that approximately 50% of PLM was phosphorylated by PKA treatment (a time course suggested we had reached steady-state phosphorylation). Dephosphorylated PLM was not detected by the CP68 antibody (fig. 1, second panel). The intensity of the CP68 antibody signal was proportional to the amount of PKA-treated PLM loaded on the immunoblot (fig. 1, third panel). The intensity of the C2 antibody signal was proportional to the amount of dephosphorylated PLM loaded on the immunoblot (fig. 1, fourth panel). The intensity of the C2 antibody signal was less intense on immunoblots loaded with PKA-treated PLM (fig. 1, bottom panel) compared with that observed with dephosphorylated PLM.

Swine carotid medial smooth muscle rings were first equilibrated and then contracted with 10 μ M histamine or 40 mM [K⁺]_o for 10 min. Some were then relaxed with 0.1, 0.3, 1, 3 or 10 μ M forskolin, and others were left in the contraction solution. After an additional 30 min, tissues were frozen and homogenized. Proteins were separated on SDS gels and the intensity of immunoreactiv-

Fig. 1. Specificity of the anti-PLM antibodies. Purified recombinant PLM was either phosphorylated with or without PKA in the presence of γ - ^{32}P -ATP and then run on SDS gel electrophoresis. The top panel shows an autoradiogram of PLM treated with PKA (there was no ^{32}P activity in PLM exposed to γ - ^{32}P -ATP without PKA). The second panel shows an immunoblot of untreated PLM with the CP68 antibody. The third panel shows an immunoblot of PLM treated with PKA with the CP68 antibody. The fourth panel shows an immunoblot of untreated PLM with the C2 antibody. The fifth panel shows an immunoblot of PLM treated with PKA with the C2 antibody.

ity to the two PLM antibodies determined on immunoblots. As detailed in 'Material and Methods', the intensity of both the C2 and CP68 antibodies were normalized to internal standards. For clarity, the term 'C2 signal' will refer to the intensity of the C2 antibody immunoreactivity normalized to the intensity of the C2 antibody immunoreactivity from the untreated control tissue homogenate loaded on the same blot. Similarly, the term 'CP68 signal' will refer to the intensity of the CP68 antibody immunoreactivity normalized to the intensity of the CP68 antibody immunoreactivity from the KF pooled homogenate loaded on the same blot.

Fig. 2. Changes in the CP68 (top panel) and C2 (bottom panel) PLM signals in swine carotid artery tissue homogenates. Lane 1 is the heart sarcolemmal PLM standard that is detected by all antibodies. Lane 2 is from unstimulated swine carotid tissues, i.e. the control (which is used to normalize the C2 signals in figure 3 as detailed in 'Material and Methods'). Lane 3 is from 10 μM histamine-stimulated tissues. Lanes 4–7 are from tissues first stimulated with 10 μM histamine followed by addition of 0.3, 1, 3 or 10 μM forskolin to induce relaxation. Lanes 8 and 9 are the K and KF standards (Std) (see 'Material and Methods').

This normalization procedure allows comparison of changes in the C2 and CP68 antibody intensity on different immunoblots. Figure 2 shows a representative immunoblot of tissues stimulated with histamine, and figure 3 shows aggregate data. PLM was present in swine arterial smooth muscle, and its phosphorylation status depended on the treatment.

Unstimulated (control) tissues had low levels of CP68 signal and force (fig. 2, lane 2; fig. 3, filled symbols). Histamine (10 μM) induced a large decrease in the C2 signal, a small increase in the CP68 signal and a maximal contraction (fig. 2, lane 3; fig. 3, open squares). Adding forskolin to histamine-stimulated tissues was associated with no change in the C2 signal, dose-dependent increases in the CP68 signal (fig. 2, lanes 4–7) and dose-dependent reduction in force (fig. 3).

Forty millimoles $[\text{K}^+]_o$ depolarization induced a maximal contraction without significant changes in the C2 or CP68 signal (fig. 3, open circles). Adding forskolin to high $[\text{K}^+]_o$ -depolarized tissues was associated with a reduced C2 signal, dose-dependent increases in the CP68 signal and dose-dependent reduction in force. When relaxed with forskolin, the histamine-stimulated tissues exhibited a greater CP68 signal and lower force than that observed in the 40 mM $[\text{K}^+]_o$ -depolarized tissues.

Fig. 3. Biochemical events occurring in forskolin relaxed swine carotid artery activated by histamine (a–c) or high [K⁺]_o depolarization (d–f). Immunoblot intensity was normalized as described in ‘Material and Methods’ and is presented as mean ± 1 SEM with n = 4–5. Tissues were activated with 10 μM histamine (□) or 40 mM [K⁺]_o (○) and then relaxed by addition of various [forskolin] as described on the abscissa. Unstimulated control tissues are shown as ■ or ●. Unphosphorylated PLM was estimated as the C2 signal (a, d), putative S68 PLM phosphorylation was estimated as the CP68 signal (b, e) and force as percent of a prior 109 mM [K⁺]_o contraction (c, f). Some error bars are obscured by the symbols.

The relation between the CP68/KF ratio and active force is shown in figure 4. There was a highly significant negative correlation between the CP68 signal and force ($r^2 = 0.5$; $p < 0.0001$, ANCOVA). There was no significant difference in the slopes or intercept of the regression lines for tissues stimulated with histamine or high [K⁺]_o, suggesting a simple inverse relation between the degree of forskolin-induced relaxation and the degree of S68 PLM phosphorylation.

We evaluated whether agents that increase [cGMP] also induce PLM phosphorylation. Histamine (10 μM)

Fig. 4. Dependence of contractile force on the CP68 signal (putative S68 PLM phosphorylation). Data are replotted from figure 3. ■ = Histamine-stimulated tissues; ○ = high [K⁺]_o.

induced a small increase in the CP68 signal and a maximal contraction. Addition of 100 μM nitroglycerin (to increase [cGMP]) induced a relaxation without changing the CP68 signal from the values observed with histamine alone. Addition of 1 μM forskolin (to increase [cAMP]) further increased the CP68 signal.

If S68 PLM phosphorylation were to cause relaxation by increasing the activity of the Na,K-ATPase, then forskolin should reduce [Na⁺]_i. [Na⁺]_i is difficult to measure in intact smooth muscle, but can be inferred by the response to removal of extracellular [Na⁺] ([Na⁺]_o) [31, 32]. If L-type Ca²⁺ channels are blocked, contraction induced by removal of [Na⁺]_o should result from the increase in [Ca²⁺]_i that occurs from Ca²⁺ influx through reversal of the Na-Ca exchanger. If forskolin were to reduce [Na⁺]_i, then there would be less Na⁺ for the Na-Ca exchanger, less increase in [Ca²⁺]_i and less contraction. We evaluated the effect of forskolin pretreatment on swine carotid artery contraction induced by a zero Na⁺ saline in the presence of 10 μM diltiazem (fig. 6). Pretreatment with 1 μM forskolin for 15 min significantly reduced the zero Na⁺ contraction compared with a control that was not treated with forskolin.

Fig. 5. Biochemical events occurring with forskolin (FSK) or nitroglycerin (NTG) induced swine carotid artery relaxation. Immunoblot intensity was normalized as described in 'Material and Methods' and is presented as mean $\pm$ 1 SEM with $n = 4-5$. Tissues were unstimulated (control), contracted with 10 μ M histamine for 60 min, contracted with 10 μ M histamine for 10 min and relaxed by addition of 100 μ M nitroglycerin for 50 min, or contracted with 10 μ M histamine for 10 min and relaxed by addition of 1 μ M forskolin for 50 min. Putative S68 PLM phosphorylation was estimated as the CP68 signal (**a**) and force as percent of a prior 109 mM $[K^+]_o$ contraction (**b**).

Discussion

We found PLM immunoreactivity in smooth muscle (fig. 2). These data suggest that the 16/17-kDa phosphoprotein identified in smooth muscle plasma membrane preparations [12–14] was likely PLM. We found that the CP68 PLM signal correlated with cAMP-, but not cGMP-mediated relaxation (fig. 3, 5). These data suggest a potential role for S68 PLM phosphorylation in cAMP-, but not cGMP-mediated relaxation. The CP68 PLM signal was increased by histamine stimulation but not high $[K^+]_o$ depolarization (fig. 3), suggesting that histamine stimulation, but not high $[K^+]_o$ depolarization, also increases S68

Fig. 6. Forskolin-dependent contractile response to removal of extracellular $[Na^+]$. Four sets of tissues were incubated in 10 μ M diltiazem for 15 min prior to removal of $[Na^+]_o$ by changing to a Na^+ -free PSS which had choline $^+$ substituted for Na^+ . Forskolin (1 μ M) was either not added (control), added 1 min prior to removal of $[Na^+]_o$ or added 15 min prior to removal of $[Na^+]_o$. The resulting peak contraction is shown as mean $\pm$ 1 SEM and analyzed by an ANOVA with Student-Newman-Keuls testing with * indicating a significant difference compared with no forskolin. Forskolin pretreatment for 15 min significantly reduced the zero Na^+ contraction.

PLM phosphorylation, either via histamine-induced activation of PKC or PKA. Pretreatment with 1 μ M forskolin for 15 min significantly reduced swine carotid contraction induced by zero $[Na^+]_o$ (fig. 6). This result is consistent with the hypothesis that forskolin reduced $[Na^+]_i$.

PLM Antibody Specificity

Our results suggest that the CP68 antibody detects S68 PLM phosphorylation. PKA treatment of recombinant PLM increased the CP68 signal (fig. 1). Since dephosphorylated PLM did not produce a CP68 signal, we suggest that the CP68 antibody is specific to phosphorylated PLM. However, we cannot state that the CP68 antibody is absolutely specific to S68 because we could not make PLM that was only phosphorylated on S63 (there is no kinase that specifically phosphorylates PLM on S63).

The specificity of the C2 antibody could not be clearly delineated either, since PKA treatment induced only approximately 50% phosphorylation. Nevertheless, the C2 signal decreased by approximately 50% with PKA treatment, suggesting that the C2 antibody is at least partially specific to dephosphorylated PLM over phosphorylated PLM. Similar results were seen in intact swine carotid: forskolin treatment increased the CP68 signal and decreased the C2 signal (fig. 2). We found it important to use large dilutions (1:10,000) of the C2 antibody to detect

the fall in the C2 signal when PLM was phosphorylated. These results confirm and extend those of Silverman et al. [33] who evaluated the C2 and CP68 immunoreactivity in forskolin and phorbol ester-treated rat cardiac myocytes. Both studies agree that PKA activation leads to an increase in the CP68 signal and a decrease in the C2 signal.

Histamine alone induced a large decrease in the C2 signal with only a small increase in the CP68 signal (fig. 3). This suggests that histamine alone may have caused phosphorylation of PLM on sites other than S68. These sites may include S63 phosphorylated by PKC [2, 3] or other sites phosphorylated by other kinases. Supporting this contention was the finding that addition of forskolin to histamine increased in the CP68 signal without further decreases in the C2 signal. Perhaps forskolin-induced S68 PLM phosphorylation (detected by CP68) on PLM already phosphorylated at other sites (possibly S63) so that there was no change in the C2 signal. The results with high K^+ -depolarized tissues support this contention; there was no change in the C2 signal with high K^+ alone, consistent with a lack of PKC activation with high K^+ . After high K^+ activation, forskolin decreased the C2 signal and increased the CP68 signal, consistent with the C2 antibody detecting the decline in unphosphorylated PLM and CP68 detecting the S68 phosphorylation. These data suggest that forskolin can induce S68 PLM phosphorylation regardless of PLM phosphorylation at other sites.

Physiology of PLM Phosphorylation in Smooth Muscle

Addition of forskolin to high $[K^+]_o$ increased S68 PLM phosphorylation, but at any given [forskolin], there was less S68 PLM phosphorylation and less relaxation in tissues depolarized with high $[K^+]_o$ than in tissues stimulated with histamine (fig. 3). This may be related to the increase in S68 PLM phosphorylation induced by histamine alone. There was a highly significant inverse correlation between S68 PLM phosphorylation and force (fig. 4). This suggests that S68 PLM phosphorylation could be involved in cAMP-dependent relaxation. However, such a correlation does not definitively establish PLM as a determinant of the resulting relaxation. Further experiments are planned to evaluate whether S68 PLM phosphorylation is a regulator of smooth muscle force.

We also evaluated whether cGMP-mediated relaxation involves S68 PLM phosphorylation. Many substrates for PKA are also substrates for protein kinase G. We find that nitroglycerin-induced relaxation was not associated with changes in S68 PLM phosphorylation

(fig. 5). This suggests that PLM can only be involved in cAMP-mediated relaxation.

There is a physical and functional association of PLM with membrane ion transporters such as the Na,K-ATPase [6, 7] and the Na-Ca exchanger (NCX1) [8, 9]. Based on our data and these studies, we hypothesize that PLM may be involved in smooth muscle regulation via regulation of Na,K-ATPase. β_2 -Adrenergic stimuli, presumptively via increasing [cAMP], increased Na,K-ATPase activity in smooth muscle [34]. Fay and Moore [35] found that β_2 -adrenergic stimuli in smooth muscle reduced $[Na^+]_i$, suggesting an increase in Na,K-ATPase activity. However, Borin [36] found that cAMP increased $[Na^+]_i$ in isolated smooth muscle cells. Na,K-ATPase knockout mice have been made, but are lethal, with death occurring just after birth. Neonatal aortae from α_2 -Na,K-ATPase $-/-$ mice were less sensitive to forskolin-induced relaxation when compared with α_2 -Na,K-ATPase $+/+$ mice [37]. We also find that forskolin inhibited the swine carotid contraction induced by removal of $[Na^+]_o$ (fig. 6), suggesting that forskolin reduced $[Na^+]_i$, possibly by stimulation of Na,K-ATPase activity. It must be noted that zero $[Na^+]_o$ studies are only suggesting that $[Na^+]_i$ is reduced. We plan to study more direct measures of Na,K-ATPase activity in the future. Nevertheless, these data support the hypothesis that cAMP-mediated relaxation at least partially involves Na,K-ATPase, likely by increasing its activity.

Specifically, we hypothesize that PKA-mediated phosphorylation of PLM on S68 increases the activity of Na,K-ATPase. Since Na,K-ATPase moves 3 Na^+ outward and 2 K^+ inward for every ATP consumed, it will produce a small hyperpolarization from the excess outward Na^+ flux [for a review, see ref. 38]. This hyperpolarization reduces Ca^{2+} influx through L-type Ca^{2+} channels, and is one method whereby increases in Na,K-ATPase activity could reduce $[Ca^{2+}]_i$ and cause smooth muscle relaxation. A second mechanism is that increases in Na,K-ATPase activity, via decreases in $[Na^+]_i$, could also increase Ca^{2+} extrusion via forward Na-Ca exchange. This would also reduce $[Ca^{2+}]_i$ and cause smooth muscle relaxation; however, it would be associated with increased Ca^{2+} efflux rather than the decrease in Ca^{2+} influx expected with hyperpolarization. We found that forskolin induced a hyperpolarization in rat tail artery [23] and a decrease in Mn^{2+} influx in swine carotid artery [39], suggesting that hyperpolarization may be the dominant mechanism in some smooth muscles.

PLM has a role in the regulation of cardiac contractility by $[Ca^{2+}]$ as well. In cultured rat heart myocytes, the

myocytes near an area of infarction have an abnormally blunted contractile response to extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$) [40]. Overexpression of PLM produces myocytes with a blunted $[\text{Ca}^{2+}]_o$ response similar to those observed after myocardial infarction [28]. Intriguingly, PLM is one of a few genes whose expression level rises after myocardial infarction [41], suggesting that PLM could be the mediator of the blunted $[\text{Ca}^{2+}]_o$ response. If NCX1, the cardiac Na-Ca exchanger, is over expressed in peri-infarction myocytes, a normal $[\text{Ca}^{2+}]_o$ response is observed [42]. A unifying explanation would be that PLM inhibits NCX1. Supporting this were findings that PLM overexpression reduced currents through NCX1 and slowed the decline in $[\text{Ca}^{2+}]_i$ after the release of sarcoplasmic reticulum stores with caffeine [28]. Co-overexpression of NCX1 with PLM restored the currents and Ca^{2+} dynamics to normal [8]. Moreover, reduction of PLM expression using anti-sense oligonucleotides increased NCX1 currents and sped the decline in $[\text{Ca}^{2+}]_i$ after caffeine [9]. Most recently, Ahlers et al. [43] found a physical and functional interaction between PLM and NCX1 co-expressed in HEK293 cells. The importance of S68, the site of PKA phosphoryla-

tion, was underscored by the finding that a phosphodeficient PLM mutant S68A lost the ability to inhibit Na-Ca exchange despite persistent physical association.

These data demonstrate that PLM is functional and present in smooth muscle and is phosphorylated by physiologic stimuli. We propose that S68 PLM phosphorylation may have a role in cAMP-, but not cGMP-mediated smooth muscle relaxation, possibly by activation of the Na,K-ATPase activity. The data also demonstrate relative specificity for the C2 and CP68 PLM antibodies for dephosphorylated and S68 phosphorylated PLM, respectively.

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