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SERINE 68 PHOSPHOLEMMAN PHOSPHORYLATION DURING FORSKOLIN-INDUCED SWINE CAROTID ARTERY RELAXATION

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Abstract

Background—Phospholemman (PLM) is an abundant phosphoprotein in the plasma membrane of cardiac, skeletal, and smooth muscle. It is a member of the FXYD 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 p*hosphospecific anti-PLM antibodies 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—*An antipeptide* antibody "C2" primarily *reacted with* unphosphorylated PLM. *An antipeptide* antibody "CP68" was specific for 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 $[K^+]_o$ depolarization induced a contraction without altering *the C2 or CP68 PLM signal*. Forskolin-induced relaxation of histamine or $[K^+]_o$ 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 contractile agonists increase 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.

Keywords

cyclic AMP; FXYD protein; phospholemman; phosphorylation; vascular smooth muscle

INTRODUCTION

Phospholemman (PLM, also known as FXYD1) 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 phosphorylation at serine 68 (S68) [2,3] while protein kinase C

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(PKC) induces phosphorylation at both serine 63 (S63) and S68 (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 (gamma) 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 MW of ~16kDa 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 isoproterenol in vivo. Its dephosphorylation was inhibited by 10 mM NaF. Boulanger-Saunier, et al. 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 [13]. 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 (PKG). PLM mRNA has been reported in canine aortic, esophageal, and gastric smooth muscle [1]. While these publications suggest that PLM is present and is 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 $[Ca^{2+}]_i$, formation of a $Ca^{2+}{}_4$-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 [17,18,19].

cAMP mediated smooth muscle relaxation occurs via binding of agonists to specific seven membrane spanning receptors (e.g. β 2 adrenergic) which activate adenylyl cyclase via G proteins (e.g. see [20]). Adenylyl cyclase can also be directly and relatively specifically activated by low concentrations of forskolin (e.g., 0.1 to 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 [21,22]. Specific mechanisms for reduction in $[Ca^{2+}]_i$ include i) inhibition of Ca^{2+} mobilization from the sarcoplasmic reticulum [21]; ii) hyperpolarization [23], iii) decreased Ca^{2+} influx through voltage-gated channels [24], and iv) 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 HSP20 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 (MOPS) 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_2HP0_4 was omitted. Dissection of medial strips, mounting and determination of the optimum length (L_o) for stress development at 37 °C was 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-C G T F R S S I R R L S T R R R-COOH was used as the antigen and the resulting rabbit polyclonal antibodies affinity purified as described [28]. To make S68 phosphorylation-specific antibodies (CP68), the 19-amino acid peptide NH_2-D E E E G T F R S S I R R L Sp (68) T R R R-COOH was made with phosphoserine (Sp) at S68. This peptide were 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. PhosphoPLM-specific antibodies were then affinity-purified from the cleaned serum by passage over an immunosorbent containing the phosphoPLM peptide. ELISA showed less than 2% cross reactivity of the phosphoPLM-specific antibody with the dephosphoPLM peptide (Bethyl Labs, Montgomery, TX).

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—40 μg of purified recombinant dephosphorylated PLM (trifluorethanol removed by N_2 evaporation) [29] was incubated in 200 μl 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-^{32}P-ATP. Samples were removed both before and after addition of 1 μl (1.1 μg) of the catalytic subunit of PKA (specific activity 6.3 μmol/min/mg, a generous gift of Tom Lincoln PhD). The reaction was stopped by addition of 1% SDS. *The stoichiometery of PLM phosphorylation was calculated from the ^{32}P activity of the phosphorylated PLM (DPM/mol PLM) divided by the ^{32}P 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 (20g/20ml) at −78° C [27]. The frozen tissue was allowed to slowly thaw to room temperature in the slurry (2 hours), 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). *20 μl 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-phospholemman antibodies (1:10000 for C2 and 1:1000 for CP68). After washing, incubation with secondary antibodies (1:5000 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 $[K^+]_o$ for 10 min followed by the addition of 10 μM 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 (*c.f.* middle panel of Fig. 3). *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 (*c.f.* top panel of Fig. 3).

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-^{32}P-ATP. Different amounts of phosphorylated and dephosphorylated PLM (exposed to γ-^{32}P-ATP without PKA) were loaded on SDS gels, blotted, analyzed for ^{32}P activity, and *immunoblotted with both antibodies*. Increasing ^{32}P activity was observed with increasing amount of PKA-treated PLM (Fig. 1, top panel). Calculated stoichiometry revealed that ~50% of PLM was phosphorylated by PKA treatment. *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 to* that observed with dephosphorylated PLM.

Swine carotid medial smooth muscle rings were first equilibrated and then contracted with 10 μM histamine or 40 mM $[K+]_o$ for 10 min. Some were then relaxed with 0.1, 0.3, 1, 3, or 10 μM 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 immunoreactivity to the two PLM antibodies determined on immunoblots. As detailed in the 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 the C2 antibody immunoreactivity normalized to the intensity of 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. This normalization procedure allows comparison of changes in the C2 and CP68 antibody intensity on different immunoblots.* Fig. 2 shows a representative immunoblot of tissues stimulated with histamine, and Fig. 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 (lane 2 in Fig. 2 and the filled symbols in Fig. 3). 10 μM histamine induced a large decrease in *the* C2 *signal*, a small increase in *the* CP68 *signal*, and a maximal contraction (lane 3 in Fig. 2 and open squares in Fig. 3). *Adding* forskolin *to histamine stimulated tissues was* associated with *no change in the C2 signal, dose dependent increases in the* CP68 *signal* (lanes 4–7 in Fig. 2), and *dose dependent reduction in* force (Fig. 3).

40 mM $[K+]_o$ depolarization induced a maximal contraction without significant changes in *the* C2 or CP68 *signal* (open circles in Fig. 3). *Adding* forskolin to *high* $[K^+]_o$ *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 $[K+]_o$-depolarized tissues.

The relation between the CP68/KF ratio and active force is shown in Fig. 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 serine 68 PLM phosphorylation.

We evaluated whether agents that increase [cGMP] also induce PLM phosphorylation. 10 μM histamine 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 intracellular $[Na^+]$ ($[Na^+]_i$). $[Na^+]_i$ 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 Ca^{2+} channels are blocked, contraction induced by removal of $[Na^+]_o$ should result from the increase in $[Ca^{2+}]_i$ that occurs from Ca^{2+} 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 $[Ca^{2+}]_i$, 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 (Figure 6). Pretreatment with 1 μM forskolin for 15 min significantly reduced the zero Na^+ contraction compared to a control that was not treated with forskolin.

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 [12,13,14] was likely PLM. We found that *the* CP68 PLM *signal* correlated with cAMP, but not cGMP mediated relaxation (Figs. 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 PLM phosphorylation, either via histamine induced activation of PKC or PKA. Pretreatment with 1 μM 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 is specific for S68 PLM phosphorylation and that the C2 antibody is at least mostly specific for dephosphorylated PLM. PKA treatment of recombinant PLM increased *the* CP68 *signal* and decreased *the* C2 *signal* (Fig. 1). Since *dephosphorylated PLM did not produce a* CP68 *signal*, we suggest that the CP68 antibody is specific for S68 phosphorylation. The specificity of the C2 antibody could not be clearly delineated since PKA treatment induced only ~50% phosphorylation. Nevertheless, the C2 signal decreased by ~50% with PKA treatment, suggesting that the C2 antibody is at least mostly specific for dephosphorylated 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 (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, forksolin 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 (PKG). 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. β2 adrenergic stimuli, presumptively via increasing [cAMP], increased Na,K-ATPase activity in smooth muscle [34]. Fay and Moore [35] found that β2 adrenergic stimuli in smooth muscle reduced $[Na^+]_i$ suggesting an increase in Na,K-ATPase activity.

However, Borin found that cAMP increased $[Na^+]_i$ in isolated smooth muscle cells [36]. Na,K-ATPase knockout mice have been made, but are lethal, with death occurring just after birth. Neonatal aortae from α2-Na,K-ATPase −/− mice were less sensitive to forskolin-induced relaxation when compared to α2-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 suggestive 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 (reviewed in [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 $[Na^+]_i$, also could increase Ca^{2+} extrusion via forward Na-Ca exchange. This would also reduce $[Ca^{2+}]_i$ and cause smooth muscle relaxation, however, this 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 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 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+} ($[Ca^{2+}]_o$) [40]. Overexpression of PLM produces myocytes with a blunted $[Ca^{2+}]_o$ 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 $[Ca^{2+}]_o$ response. If NCX1, the cardiac Na-Ca exchanger, is over expressed in peri-infarction myocytes, a normal $[Ca^{2+}]_o$ 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 $[Ca^{2+}]_i$ after release of SR 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 $[Ca^{2+}]_i$ 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 phosphorylation, 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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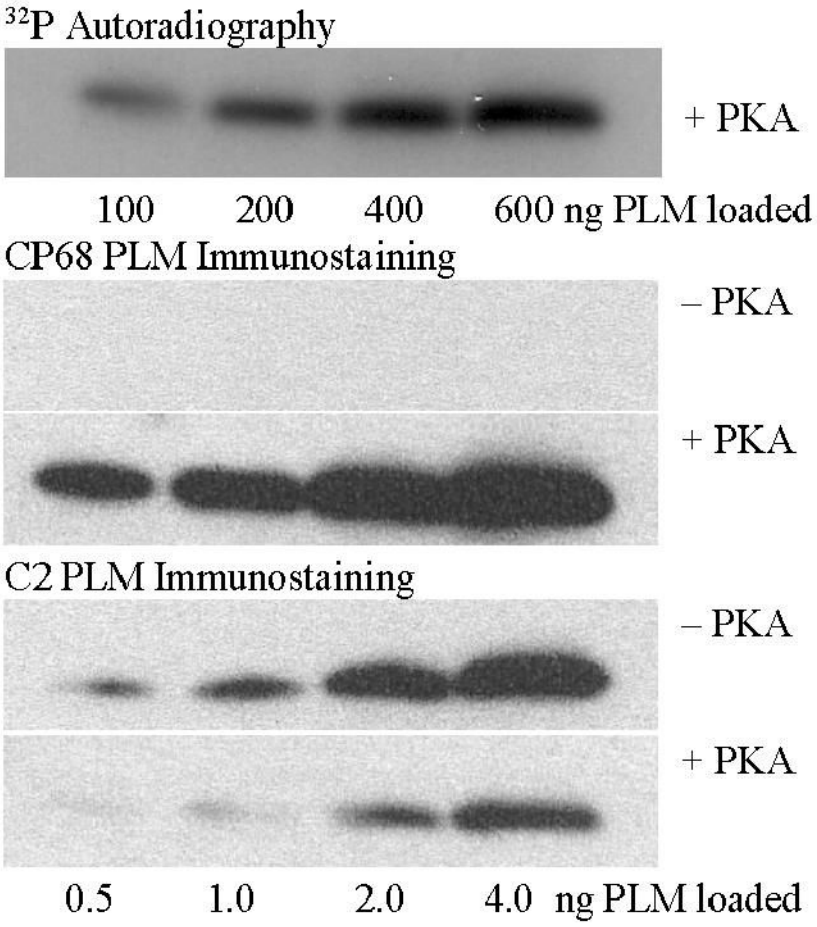

Figure 1. Specificity of the anti-PLM antibodies.

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). It shows increasing radioactivity with increasing amount of PLM loaded; calculated stoichiometry revealed that ~50% of PLM was phosphorylated. The second and third panel show immunoblots with the CP68 antibody. *Dephosphorylated PLM was not detected by the CP68 antibody. PKA-treated PLM was detected by the CP68 antibody in proportion to the amount of PKA-treated PLM loaded.* The fourth and fifth panels show immunoblots with the C2 antibody. *Dephosphorylated PLM was detected by the C2 antibody in proportion to the amount of dephosphorylated PLM loaded. There was less C2 signal from PKA-treated PLM when compared to dephosphorylated PLM, suggesting the C2 antibody primarily immunoreacts with dephosphorylated PLM.*.

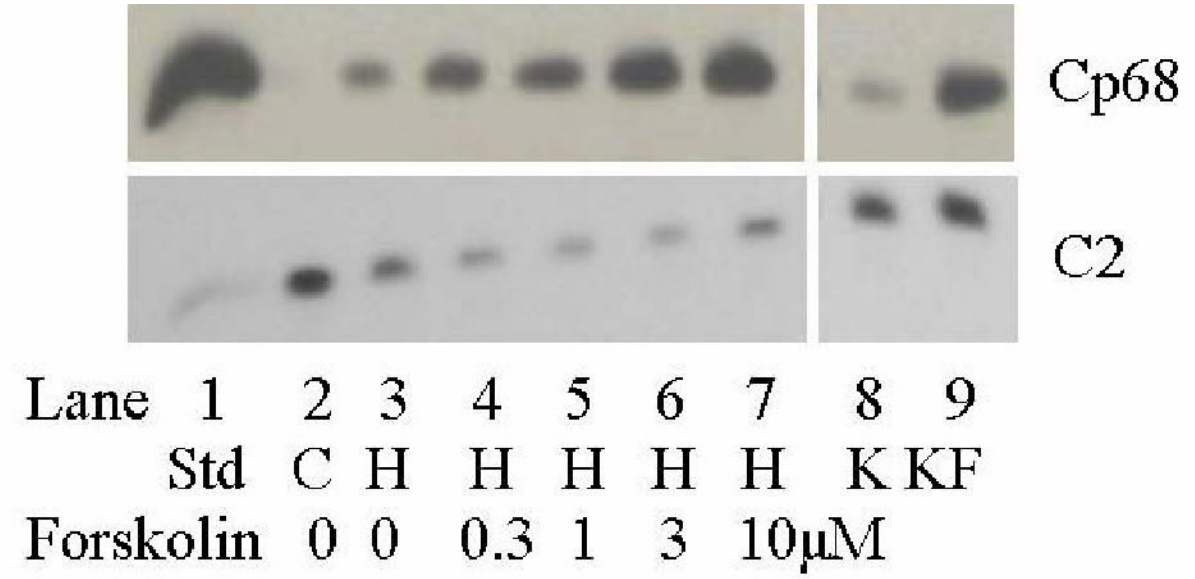

Figure 2. ***Changes in the*** **CP68 (top panel) and C2 (bottom panel) PLM** ***signals*** **in swine carotid artery tissue homogenates.**

Lane 1 (from left) 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* Fig. 3 as detailed in the 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, respectively. Lanes 8–9 are the K and KF standards (see methods). *The CP68 signal (reflecting S68 PLM phosphorylation, top panel)* revealed small increases with histamine alone and larger dose dependent increases with forskolin. *The C2 signal (reflecting unphosphorylated PLM, lower panel),* showed decreases with histamine treatment and no further change with *addition of* forskolin.

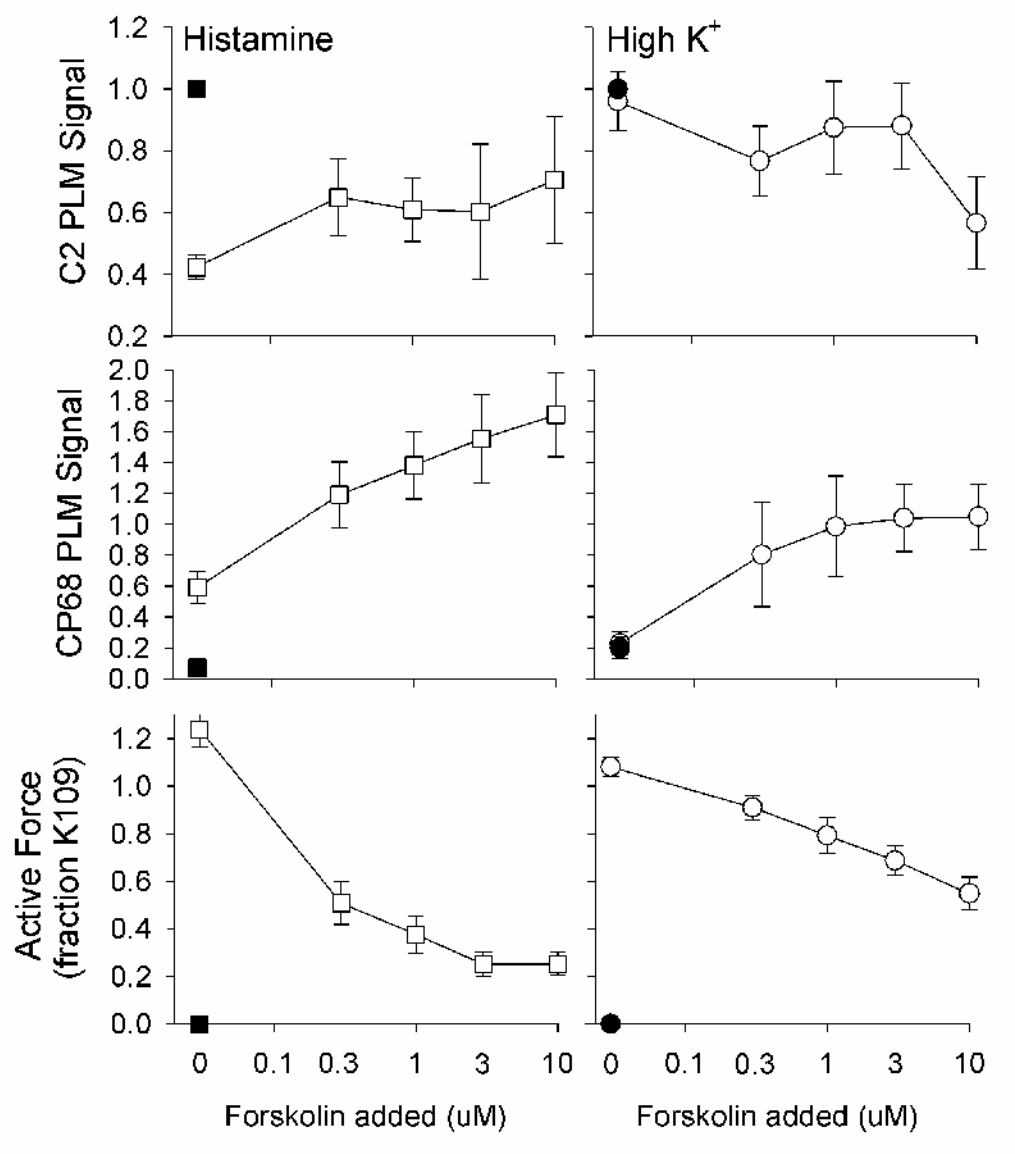

Figure 3. Biochemical events occurring in forskolin relaxed swine carotid artery activated by histamine (left panels) or high $[K^+]_o$ depolarization (right panels).

Immunoblot intensity was normalized as described in the methods and is presented as mean ± 1 SEM with n=4–5. Tissues were activated with 10 μM histamine (open squares) or 40 mM $[K^+]_o$ (open circles) and then relaxed *by addition of* various [forskolin] as described on the abcissa. Unstimulated control tissues are the shown as filled squares or circles. Unphosphorylated PLM was estimated as the C2 *signal* (top panels), putative S68 PLM phosphorylation was estimated as the CP68 *signal* (middle panels), and force as percent of a prior 109 mM $[K^+]_o$ contraction (bottom panels). Some error bars are obscured by the symbols.

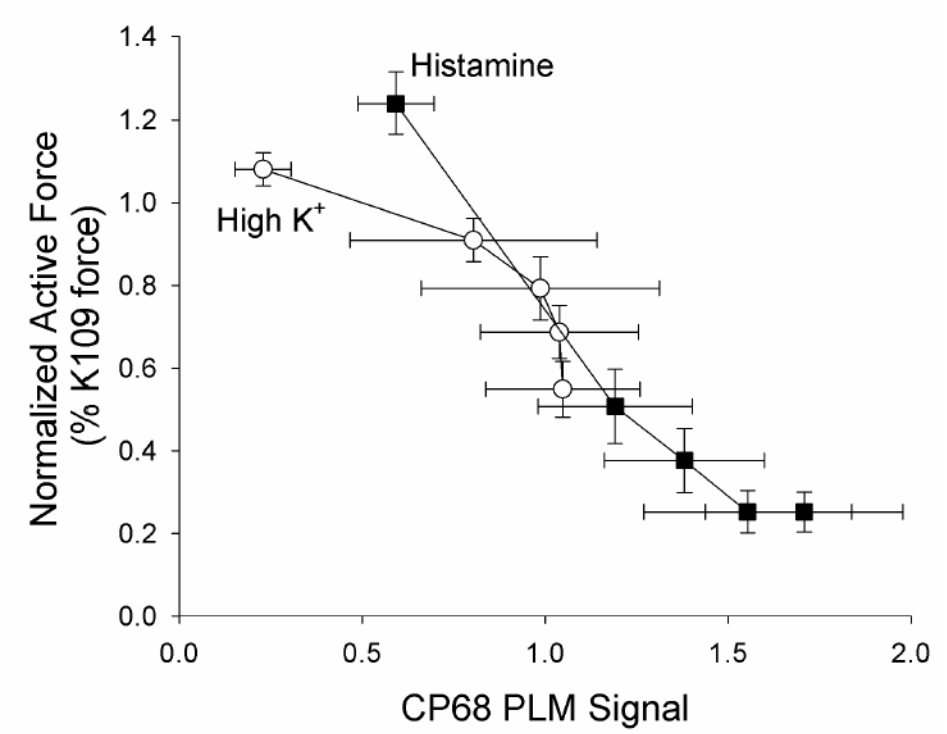

Figure 4. Dependence of contractile force on the CP68 *signal* (putative S68 PLM phosphorylation).
Data are replotted from Fig. 3. Histamine stimulated tissues are shown as filled squares and high $[K^+]_o$ shown as open circles. There was a significant correlation between the *the* CP68 *signal* and force (top panel).

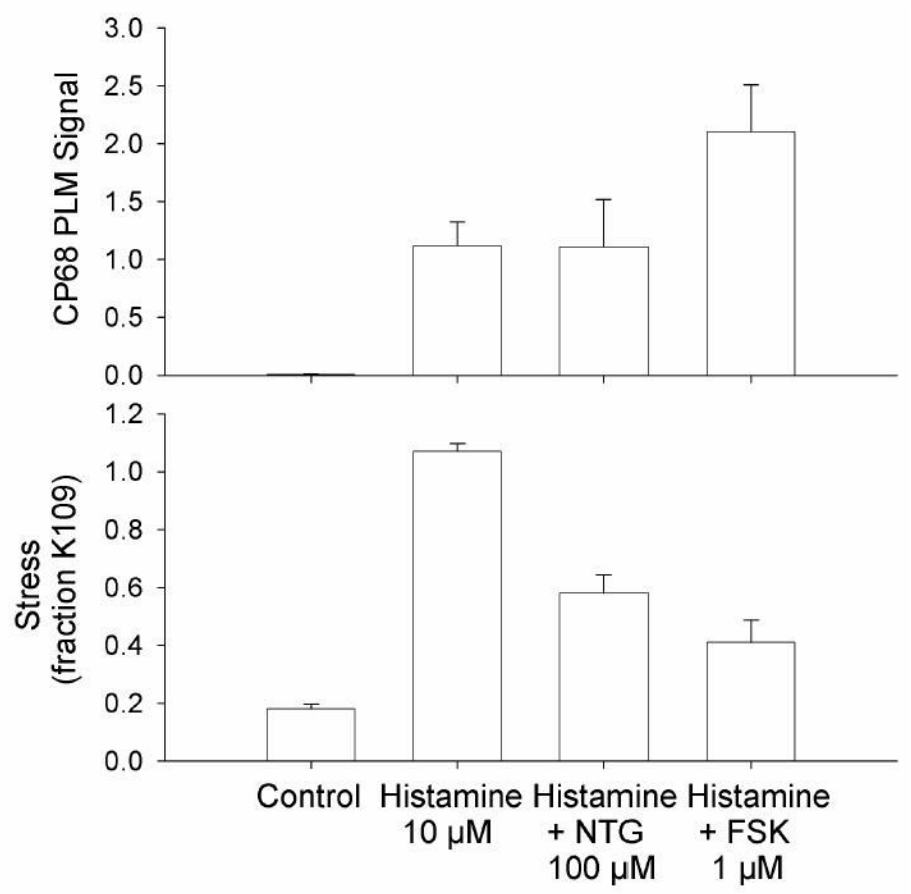

Figure 5. Biochemical events occurring with forskolin or nitroglycerin induced swine carotid artery relaxation.

Immuoblot intensity was normalized as described in the methods and is presented as mean ± 1 SEM with n=4–5. Tissues were unstimulated (control), contracted with 10 μM histamine for 60 min, contracted with 10 μM histamine for 10 min and relaxed by addition of 100 μM nitroglycerin for 50 min, or contracted with 10 μM histamine for 10 min and relaxed by addition of 1 μM forskolin for 50 min. Putative S68 PLM phosphorylation was estimated as the CP68 *signal* (top panel) and force as percent of a prior 109 mM $[K^+]_o$ contraction (bottom panel).

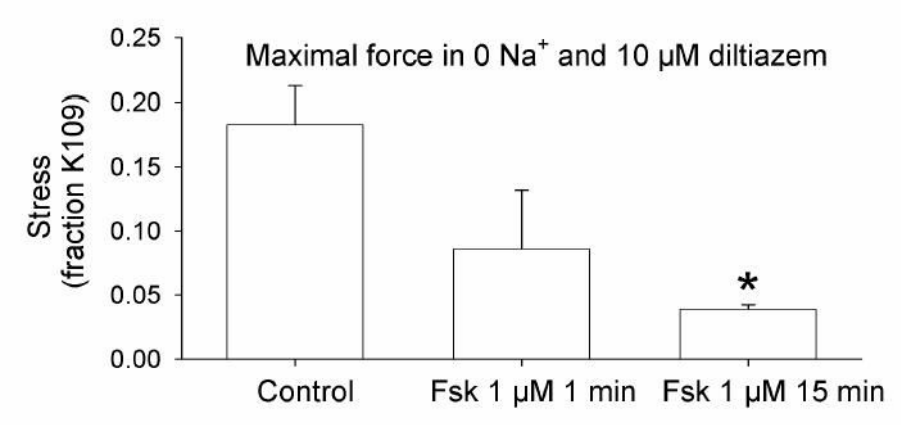

Figure 6. Forskolin dependent contractile response to removal of extracellular [Na^+].

Four sets of tissues were incubated in 10 μM 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 μM) was either not added (control), added 1 min prior to removal of $[Na^+]_o$, or added 15 min prior to to removal of $[Na^+]_o$. The resulting peak contraction is shown as mean ± 1 SEM and analyzed by an ANOVA with Student-Newman-Keuls testing with * indicating a significant difference compared to no forskolin. Forskolin pretreatment for 15 min significantly reduced the zero Na^+ contraction.