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Phospholemman does not participate in forskolin-induced swine carotid artery relaxation

Melissa K. Meeks, Shaojie Han, Amy L. Tucker, and Christopher M. Rembold

Cardiovascular Division, Department of Internal Medicine; University of Virginia Health System Charlottesville, Virginia 22908 USA

Running title: Protein phosphorylation and cAMP induced relaxation

Address for correspondence and reprints:

Christopher M. Rembold, M.D. Box 800146 Cardiovascular Division University of Virginia Health System Charlottesville, Virginia 22908-0146 USA +1 434 924 2825 FAX: +1 434 243 0014 Email: crembold@virginia.edu

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ABSTRACT

Background

Phosphorylation of phospholemman (PLM) on ser⁶⁸ has been proposed to at least partially mediate cyclic AMP (cAMP) mediated relaxation of arterial smooth muscle.

Methods

We evaluated the time course of the phosphorylation of phospholemman (PLM) on ser⁶⁸, myosin regulatory light chains (MRLC) on ser¹⁹, and heat shock protein 20 (HSP20) on ser¹⁶ during a transient forskolin-induced relaxation of histamine-stimulated swine carotid artery. We also evaluated the dose response for forskolin- and nitroglycerin-induced relaxation in phenylephrine-stimulated PLM^{-/-} and PLM^{+/+} mice.

Results

The time course for changes in ser¹⁹ MRLC dephosphorylation and ser¹⁶ HSP20 phosphorylation was appropriate to explain the forskolin-induced relaxation and the recontraction observed upon washout of forskolin. However, the time course for changes in ser⁶⁸ PLM phosphorylation was too slow to explain forskolin-induced changes in force. There was no difference in the phenylephrine contractile dose response or in forskolin-induced relaxation dose response observed in PLM^{-/-} and PLM^{+/+} aortae. In aortae precontracted with phenylephrine, nitroglycerin induced a slightly, but significantly greater relaxation in PLM^{-/-} compared to PLM^{+/+} aortae.

Conclusions

These data are consistent with the hypothesis that ser¹⁹ MRLC dephosphorylation and ser¹⁶ HSP20 phosphorylation are involved in forskolin-induced relaxation. Our data suggest that PLM phosphorylation is not significantly involved in forskolin-induced arterial relaxation.

INTRODUCTION

Vascular smooth muscle contraction primarily involves pathways whereby increases in myoplasmic calcium ($[Ca^{2+}]_i$) activate myosin light chain kinase, which phosphorylates myosin regulatory light chains (MRLC) on ser¹⁹ (Murphy and Rembold, 2005). MRLC phosphorylation can also be increased by inhibition of MRLC phosphatase (Somlyo and Somlyo, 2000;Etter *et al.*, 2001)). Increases in ser¹⁹-MRLC phosphorylation cause contraction. We term these processes "activation."

Relaxation can occur by two general mechanisms: 1) "Deactivation" is the reversal of activation involving dephosphorylation of MRLC by either a reduction in $[Ca^{2+}]_i$ or an increase in MRLC phosphatase activity (Rembold 1991;Gerthoffer and Murphy, 1983;Etter *et al.*, 2001). 2) "Force suppression" is relaxation that occurs while MRLC phosphorylation levels remain elevated in the presence of excitatory stimuli (McDaniel *et al.*, 1992;Bárány and Bárány, 1993;Rembold *et al.*, 2001). Phosphorylation of heat shock protein 20 (HSP20, also known as HspB6 (Fan *et al.*, 2005)) on ser¹⁶ (Beall *et al.*, 1997;Beall *et al.*, 1999;Woodrum *et al.*, 1999;Jerius *et al.*, 1999) has been proposed to be the mediator of force suppression (Rembold *et al.*, 2001;Meeks *et al.*, 2005). cAMP-mediated relaxation, e.g. by forskolin treatment, can induce relaxation by both deactivation (Rembold and Chen, 1998) and force suppression (Rembold *et al.*, 2001).

Phospholemman (PLM, also known as FXYD1) was also proposed to be involved in cAMP-mediated smooth muscle relaxation (Rembold *et al.*, 2005). There are four papers describing the physiology of PLM in smooth muscle. The first three predate the naming of PLM but describe a plasma membrane phosphoprotein that copurified with Na,K-ATPase (Boulanger-Saunier C. *et al.*, 1985), was phosphorylated on one site by agents that increase cAMP and PKA (Boulanger-Saunier C. *et al.*, 1985;Sarcevic *et al.*, 1989), was phosphorylated on a second site by contractile agonists and phorbol esters (Boulanger-Saunier C. *and* Stoclet, 1987), and was not phosphorylated by protein kinase G (PKG) (Sarcevic *et al.*, 1989). With phosphospecific PLM antibodies (Rembold *et al.*, 2005), we found that: 1) histamine stimulation induced a swine carotid artery contraction associated with a small increase in ser⁶⁸ PLM phosphorylation. 3) Nitroglycerin-induced relaxation was not associated with a further increase in ser⁶⁸ PLM

phosphorylation. 4) Pretreatment with 1 µM forskolin for 15 min significantly reduced swine carotid contraction induced by zero extracellular Na^+ , suggesting that forskolin reduced $[Na^+]_i$ These findings suggested a potential role for ser⁶⁸ PLM phosphorylation in cAMP-mediated relaxation of arterial smooth muscle.

The goal of this study was to determine the role of PLM in cAMP-mediated arterial smooth muscle relaxation. We tested whether the phosphorylation status of PLM, HSP20, and MRLC correlated with forskolin-induced swine carotid artery relaxation and the recontraction observed with washout of forskolin. We also tested whether genetic absence of PLM in PLM^{-/-} mice alters forskolin and nitroglycerin-induced relaxation in aortae compared to the aortae of PLM^{+/+} mice. PLM^{-/-} mice were previously found to develop modest cardiac hypertrophy without an alteration in blood pressure compared to $PLM^{+/+}$ mice (Jia *et al.*, 2005).

MATERIAL AND METHODS

Swine carotid experiments: Swine carotid artery rings, obtained from an abattoir, were mounted, stretched to their optimal length, pharmacologically treated, frozen, and analyzed for PLM phosphorylation, HSP20 phosphorylation, and MRLC phosphorylation as previously described (Rembold et al., 2001;Rembold et al., 2005).

Mouse aorta preparation: Dr. Amy Tucker engineered and provided PLM^{-/-} and PLM^{+/+} mice (Jia et al., 2005). Under an University of Virginia approved IACUC protocol, mice were euthanized with CO₂ followed by cervical dislocation. Segments of mouse infrarenal abdominal aortae (length ~1.5 mm) were dissected and mounted on a Mulvany-Halpern dual wire myograph (Danish Myotechnology model 610M, Aarhus, Denmark). The physiological saline solution (PSS) contained (in mM) 140 NaCl, 4.7 KCl, 1.2 Na₂HPO₄, 1.6 CaCl₂, 1.2 MgCl₂ 5.6 D-glucose; 2.0 morpholinopropane sulfonic acid buffer (pH 7.4 at 4 or 37 °C), and 0.02 ethylenediamine N,N'-tetraacetic acid (EDTA) to chelate trace heavy metal ions. Tissues were stretched to a resting force of ~1 gram-weight as described in the results. Changes in extracellular $[K^+]_0$ were made by stoichiometric replacement of Na⁺ with K⁺. Since the tissues

were small, individual tissue weights could not be accurately measured. Therefore, force was normalized to the steady state force induced by 109 mM K^+ depolarization.

[cAMP] measurement: Two mm long segments of abdominal aortae were incubated freely floating as described in the results, then frozen in acetone/dry ice, thawed slowly, homogenized in 0.1 M HCl, and analyzed for [cAMP] by radioimmunoassay as described (McDaniel *et al.*, 1991).

Statistics: Differences were evaluated with Student's unpaired t test and significance was defined as p < 0.05.

RESULTS

We performed a time course of forskolin-induced relaxation to test whether ser^{68} phosphorylation of phospholemman (PLM) was involved in forskolin-induced relaxation. For a phosphorylation to be involved in the relaxation, the change in phosphorylation should precede or parallel the forskolin-induced relaxation and the reverse change in phosphorylation should precede or parallel the recontraction observed when forskolin is washed out (Rembold *et al.*, 2001).

Activation of swine carotid artery rings with 10 µM histamine for 30 min increased ser⁶⁸-PLM phosphorylation, ser¹⁹-MRLC phosphorylation, and contractile force without significantly altering ser¹⁶-HSP20 or ser¹⁵⁷-HSP20 phosphorylation (30 min in Fig. 1, the dashed lines represent the values of each measured after 30 min in histamine). Addition of 1 µM forskolin to the histamine stimulated tissue from 30 to 45 min increased ser¹⁶-HSP20 phosphorylation and decreased ser¹⁹-MRLC phosphorylation in parallel with the relaxation. In contrast, ser⁶⁸-PLM phosphorylation only significantly increased after 15 min of forskolin treatment, indicating a slower change in ser⁶⁸-PLM phosphorylation than a change in force. Washout of forskolin in the continued presence of histamine reduced ser¹⁶-HSP20 phosphorylation and increased ser¹⁹-MRLC phosphorylation in parallel with the recontraction. Ser⁶⁸-PLM phosphorylation remained elevated during the recontraction. During forskolin-induced relaxation and recontraction, ser¹⁵⁷-HSP20 phosphorylation remained high and did not significantly change.

Figure 1

Prior to evaluation of PLM^{-/-} abdominal aortae, we evaluated how to set tissue length (diameter) in mouse infrarenal aorta. Fig. 2 shows the 109 mM K⁺-induced length-tension relation of wild type (PLM^{+/+}) aortae. Despite increasing passive force to values ~700% higher than active force, no descending limb of the length-tension relation was observed. This response differs significantly from that observed in the swine carotid, where there is a descending limb of the length-tension relation and maximal active force is observed when passive force is ~10% of active force (Herlihy and Murphy, 1973). Since the length-tension relation did not define an optimal length for force development, we chose to set a length based on a physiologic arterial pressure of 100 mmHg and the Laplace equation for wall stress. Laplace states that wall stress = pressure x vessel internal radius = 100 mmHg x 0.4 mm = 1.33×10^4 N m⁻² x 4×10^{-4} m = 5.33 N m⁻¹. Therefore, for a 2 mm long vessel, wall stress = 5.33 N m⁻¹ x 2×10^{-3} m = 1.1×10^{-2} N = 1.1 gram-weight. We therefore set passive force to be ~1 gram-weight to attain a resting wall stress approximating an arterial pressure of 100 mmHg.

Figure 2

We then studied contractile and relaxant behavior in PLM^{-/-} and PLM^{+/+} mouse infrarenal aortae. There was no difference in the phenylephrine contractile dose response observed in the PLM^{-/-} and PLM^{+/+} aortae (Fig. 3A). In aortae precontracted with 10 μ M phenylephrine, forskolin induced a relaxation, and there was no difference in the forskolin dose response observed in the PLM^{-/-} and PLM^{+/+} aortae (Fig. 3B). In aortae precontracted with 10 μ M phenylephrine, nitroglycerin induced a slightly greater relaxation in PLM^{-/-} aorta compared to PLM^{+/+} aortae (P<0.05, Fig. 3C).

Figure 3

In a separate experiment, two mm long segments of abdominal aorta from four PLM^{-/-} and four PLM^{+/+} mice were incubated freely floating in 10 μ M phenylephrine for 2 min followed by addition of 1 μ M forskolin for 30 min. Tissues were then frozen for measurement of [cAMP]. [cAMP] did not significantly differ in PLM^{-/-} aortae (147 ± 56 pmol ml⁻¹, mean ± 1 SEM) when compared to PLM^{+/+} aortae (334 ± 147 pmol ml⁻¹, p=0.15).

DISCUSSION

Phospholemman

Phospholemman (PLM) a 72 amino acid sarcolemmal protein that is abundant in heart, skeletal, and smooth muscle (Palmer *et al.*, 1991). PLM has a physical and functional association with membrane ion transporters such as the Na,K-ATPase (Crambert *et al.*, 2002;Feschenko *et al.*, 2003) and the Na-Ca exchanger (NCX1) (Zhang *et al.*, 2003;Mirza *et al.*, 2004). These findings suggest that PLM may have functional roles similar to other members of the FXYD family of proteins (Sweadner and Rael, 2000). For example, the γ (gamma) subunit of Na,K-ATPase (FXYD2) shares significant homology with PLM (Sweadner and Rael, 2000) that could explain the effect of PLM on Na,K-ATPase activity (Mercer *et al.*, 1993). In vitro, protein kinase A (PKA) induces PLM phosphorylation at ser⁶⁸ while protein kinase C (PKC) induces phosphorylation at both ser⁶³ and ser⁶⁸ (Walaas *et al.*, 1994;Mounsey *et al.*, 1999). As noted in our previous paper (Rembold *et al.*, 2005) and in Fig. 1, histamine and forskolin additively increased ser⁶⁸ PLM phosphorylation, likely from histamine-induced PKC activation and forskolin-induced PKA activation, respectively.

We found that the time course of ser⁶⁸ PLM phosphorylation was too slow to correlate with either the forskolin-induced relaxation (30 to 45 min in Fig. 1) or the recontraction observed when forskolin was washed out (45 to 105 min in Fig. 1). We also found that genetic absence of PLM (the PLM^{-/-} mouse) was not associated with an alteration in the dose response of forskolin-induced relaxation of abdominal aorta (Fig. 3B). We tested whether a difference in forskolin-induced increases in [cAMP] could explain the absence of a forskolin dose response (perhaps the PLM^{-/-} aorta had a compensatory larger forskolin-induced increase in [cAMP] to compensate for the absence of PLM in the PLM^{-/-} aorta). However, we found that the forskolin-induced increase in [cAMP] in the PLM^{-/-} mouse did not significantly differ from the PLM^{+/+} mouse ([cAMP] actually trended lower in the PLM^{-/-} mouse compared to the PLM^{+/+} mouse). These data suggest that PLM is not involved in cAMP-mediated arterial relaxation in the mouse aorta or swine carotid.

We cannot rule out the possibility that the genetic absence of PLM increased the importance of other cAMP-mediated relaxation mechanisms. Reported cAMP smooth muscle

relaxant mechanisms include i) inhibition of Ca^{2+} mobilization from the sarcoplasmic reticulum (Lincoln *et al.*, 1996); ii) hyperpolarization (Rembold and Chen, 1998), iii) decreased Ca^{2+} influx through voltage-gated channels (Knot *et al.*, 1996), iv) activation of plasma membrane Ca^{2+} pumps (Raeymaekers and Wuytack, 1996), and force suppression associated with ser¹⁶-HSP20 phosphorylation (Rembold *et al.*, 2001). Alternatively, it is possible that our normalization of forces by K⁺-induced contraction was inappropriate. Ideally, we should normalize force by tissue cross-sectional area. However, these tissues are too small to accurately measure weight. Arguing against a normalization artifact was the lack of a difference in the phenylephrine dose response in the PLM^{-/-} compared to PLM^{+/+} aortae (Fig. 3A). This lack of a difference in phenylephrine dose response suggests that contractile mechanisms were not altered by the genetic absence of PLM.

We did find that the genetic absence of PLM in the PLM^{-/-} mouse was associated with a modest accentuation in nitroglycerin-induced relaxation (Fig. 3C). This is surprising given our finding that nitroglycerin did not induce ser⁶⁸ PLM phosphorylation in the swine carotid (Rembold *et al.*, 2005). These results suggest that PLM, despite the absence of nitroglycerin-induced ser⁶⁸ phosphorylation, may be involved in cGMP-mediated relaxation by an unknown mechanism. Further research is required to determine the mechanism how PLM is involved in cGMP-mediated relaxation.

HSP20 and MRLC phosphorylation

We found that the time course of ser¹⁹ MRLC dephosphorylation and the time course of ser¹⁶ HSP20 phosphorylation paralleled forskolin-induced relaxation (Fig. 1). These data suggest that forskolin-induced relaxation could be caused by the reduction in ser¹⁹ MRLC phosphorylation and/or the increase in ser¹⁶ HSP20 phosphorylation. Ser¹⁵⁷-HSP20 phosphorylation did not change during contraction, relaxation, or recontraction, suggesting that ser¹⁵⁷-HSP20 phosphorylation does not have a role in forskolin-induced relaxation. Further research is ongoing in our laboratory to determine whether ser¹⁶ HSP20 phosphorylation is required for forskolin-induced relaxation.

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FIGURE LEGENDS

Figure 1. The time course of ser⁶⁸-PLM phosphorylation (top panel), ser¹⁵⁷-HSP20 phosphorylation (second panel), ser¹⁶-HSP20 phosphorylation (third panel), ser¹⁹-MRLC phosphorylation (fourth panel), and force (bottom panel) in swine carotid contracted with histamine, relaxed with forskolin, and recontracted by washout of forskolin in the continued presence of histamine. Nine sets of swine carotid artery tissues were stimulated with 10 µM histamine for a total of 105 min starting at 0 min in the figure. 1 µM forskolin was added to the histamine for only 15 min, from 30 to 45 min in the figure. Tissues from each set were frozen at various times (data points left to right): 1) prior to histamine stimulation (labeled rest), 2) after 30 min of 10 μ M histamine stimulation alone, 3) when forskolin induced a ~25% relaxation (3.3 \pm 0.5 min after forskolin addition), 4) when forskolin induced a $\sim 50\%$ relaxation (4.7 \pm 0.9 min after forskolin addition), 5) 15 min after addition of forskolin, 6) when washout of forskolin in the presence of histamine induced a recontraction to $\sim 50\%$ of maximal force (11.5 ± 1.9 min after forskolin washout), 7) when washout of forskolin in the presence of histamine induced a recontraction to ~75% of maximal force (23 ± 3.4 min after forskolin washout), and 60 min after washout of forskolin in the presence of histamine. Force was normalized to that previously elicited by 109 mM extracellular K^+ . Data are means ± 1 SEM with n = 6-9. Symbols without error bars reflect data where the SEM was smaller than the symbol size. PLM phosphorylation was measured with a phosphospecific antibody and is normalized to the phosphorylation observed with histamine stimulation alone. MRLC and HSP20 phosphorylation was measured by isoelectric focusing and is reported as mol phosphorylation per mol protein. The dashed lines are the values with 10 µM histamine alone.

Figure 2. The length tension relationship of $PLM^{+/+}$ mouse abdominal aortae. Eight $PLM^{+/+}$ abdominal aortae from 5-month old mice were mounted on wire myographs. Aortae were repeatedly contracted with 109 mM extracellular K⁺ at varying tissue lengths. Passive force (open circles) was the minimal force measured when tissues were released to a given length from a longer length when bathed in PSS (K⁺ 4.7 mM). Active force (filled circles) was the peak force measured upon activation with 109 mM K⁺ at each length. Data are presented as mean ± 1 SEM. Symbols obscure some of the smaller error bars. The vertical bar indicates the length at 1 gram-weight.

Figure 3. Comparison of contractile and relaxant responses in PLM^{-/-} and PLM^{+/+} mouse aortae. Eleven PLM^{-/-} (open circles) and ten PLM^{+/+} (filled circles) aortae from 5-month old mice were mounted on wire myographs. After setting resting force to ~1 gram-weight, aortae were contracted with 109 mM extracellular K⁺ to determine the force for later normalization. After washout of high K⁺, aortae were contracted with cumulatively increasing concentrations of phenylephrine as shown in the panel A (forces normalized to the prior K^+ contraction). After washout of phenylephrine, aortae were then contracted to an intermediate force with 10 µM phenylephrine and relaxed with cumulatively increasing concentration of forskolin as shown in panel B (forces normalized to the 10 µM phenylephrine contraction). After washout of phenylephrine and forskolin, aortae were contracted to an intermediate force with 10 µM phenylephrine and relaxed with cumulatively increasing concentration of nitroglycerin as shown in panel C (forces normalized to the 10 µM phenylephrine contraction). In approximately half of the arteries, the order of forskolin- and nitroglycerin-induced relaxation was switched. Data are presented as mean ± 1 SEM with * indicating a significant difference between the PLM^{-/-} and PLM^{+/+} aortae by unpaired t test. Symbols obscure some of the smaller error bars.

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