Is myosin phosphatase regulated *in vivo* by inhibitor-1? Evidence from inhibitor-1 knockout mice

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1. The Ca^{2+} sensitivity of smooth muscle contractility is modulated via regulation of phosphatase activity. Protein phosphatase inhibitor-1 (I-1) is the classic type-1 phosphatase inhibitor, but its presence and role in cAMP-dependent protein kinase (PKA) modulation of smooth muscle is unclear. To address the relevance of I-1 *in vivo*, we investigated smooth muscle function in a mouse model lacking the I-1 protein ($I\text{-}1^{(-/-)}$ mice).

2. Significant amounts of I-1 protein were detected in the wild-type (WT) mouse aorta and could be phosphorylated by PKA, as indicated by ^{32}P-labelled aortic extracts from WT mice.

3. Despite the significant presence of I-1 in WT aorta, phenylephrine and KCl concentration–isometric force relations in the presence or absence of the PKA pathway activator isoproterenol (isoprenaline) were unchanged compared to $I\text{-}1^{(-/-)}$ aorta. cGMP-dependent protein kinase (PKG) relaxation pathways were also not different. Consistent with these findings, dephosphorylation rates of the 20 kDa myosin light chains (MLC_{20}), measured in aortic extracts, were nearly identical between WT and $I\text{-}1^{(-/-)}$ mice.

4. In the portal vein, I-1 protein ablation was associated with a significant ($P < 0.05$) rightward shift in the EC_{50} of isoproterenol relaxation ($EC_{50} = 10.4 \pm 1.4$ nM) compared to the WT value ($EC_{50} = 3.5 \pm 0.2$ nM). Contraction in response to acetylcholine as well as Ca^{2+} sensitivity were similar between WT and $I\text{-}1^{(-/-)}$ aorta.

5. Despite the prevalence of I-1 and its activation by PKA in the aorta, I-1 does not appear to play a significant role in contractile or relaxant responses to any pharmacomechanical or electromechanical agonists used. I-1 may play a role as a fine-tuning mechanism involved in regulating portal vein responsiveness to β-adrenergic agonists.

Regulation of phosphoprotein phosphatases has been shown to be a major component in the Ca^{2+} sensitization of smooth muscle contraction (Somlyo & Somlyo, 2000). The ~19 kDa phosphatase inhibitor-1 (I-1) protein is the classic regulator of type-1 phosphatase activity (Oliver & Shenolikar, 1998) and has been shown to be present in smooth muscle (Elbrecht *et al.* 1990). However, studies of the relevance of I-1 in regulating smooth muscle myosin phosphatase activity *in vitro* and, consequently, smooth muscle function have provided conflicting results (Alessi *et al.* 1992; Tokui *et al.* 1996). These data may be due to the inability of *in vitro* systems to recapitulate the complex regulatory mechanisms acting on the phosphatase, including its regulation by kinases and/or targeting subunits. Thus, an *in vivo* approach is essential to determine the physiological effects of the inhibitor-1 protein. To address this issue, we used the recently developed I-1 knockout ($I\text{-}1^{(-/-)}$) mouse to delimit the role of I-1 in both tonic and phasic smooth muscle contractility.

A major mechanism involved in regulating smooth muscle contractility is the phosphorylation of serine-19 on the 20 kDa myosin light chains (MLC_{20}) (de Lanerolle & Paul, 1991; Hartshorne *et al.* 1998), resulting in the activation of myosin–actin crossbridge cycling and contraction. The phosphorylation status of MLC_{20}, and consequently contraction, is controlled by the dynamic balance between the activities of myosin light chain kinase (MLCK) and

myosin light chain phosphatase (MLCP). MLCK has been well characterized (Gallagher *et al.* 1997) and its activation is Ca^{2+} dependent, involving a Ca^{2+}–calmodulin complex. It is now clear that MLCP is also regulated and this regulation has been suggested to involve the cytosolic I-1 (Somlyo *et al.* 1989).

MLCP is a heterotrimer consisting of a ~37 kDa catalytic subunit (PP1c), a ~20 kDa subunit of unknown function, and a ~110–130 kDa myosin phosphatase targeting subunit (MYPT1) (Shirazi *et al.* 1994; Hartshorne *et al.* 1998). Interaction of PP1c with MYPT1 confers selectivity of PP1c towards the myosin molecule (Alessi *et al.* 1992) and enhances PP1c activity (Shirazi *et al.* 1994; Ichikawa *et al.* 1996). As in other tissues, the targeting subunit may play a direct role in regulating PP1c activity. For instance, in striated muscle, phosphorylation of the glycogen targeting subunit, R_{GL}, results in dissociation of PP1c, and inactivation of the phosphatase by I-1 (Hubbard & Cohen, 1989). By analogy, phosphorylation of MYPT1 by protein kinase C (PKC) may perform a similar function in regulating the interactions between MYPT1 and PP1c (Feng *et al.* 1999; Toth *et al.* 2000). Although the physiological significance of altered MLCP regulation is not known with certainty, myosin phosphatase activity probably plays a paramount role in certain pathological states (Solaro, 2000).

I-1 is an endogenous smooth muscle phosphatase inhibitor that is inactive when dephosphorylated. However, when activated by PKA (Cohen, 1989) or PKG (Hemmings *et al.* 1984; Tokui *et al.* 1996) phosphorylated I-1 specifically inhibits type-1 phosphatase activity. Regulation of myosin phosphatase activity has also been suggested as an important mechanism in the Ca^{2+}-sensitizing properties of agents such as cGMP (Lee *et al.* 1997). Activation of I-1 by PKG suggests a possible role for I-1 in this effect, but this remains unconfirmed *in vivo*. *In vitro* experiments using the purified myosin phosphatase holoenzyme have suggested that the MLCP is relatively insensitive to I-1 (Alessi *et al.* 1992). Contrary to these results, activated (phosphorylated) I-1 has been shown to inhibit myosin phosphatase activity (Mitsui *et al.* 1992). This finding is supported by contractility experiments, performed in β-escin-skinned smooth muscle cells (Tokui *et al.* 1996), which demonstrated that phosphorylated I-1 enhanced contraction at submaximal [Ca^{2+}], suggesting that I-1 may play a role in regulating the Ca^{2+} sensitivity of smooth muscle phosphatase activity *in vivo*. These results seemingly lead to an apparent paradox, since β-adrenergic receptor stimulation, which results in phosphorylation of I-1, is generally associated with smooth muscle relaxation. Oliver & Shenolikar (1998) suggest that I-1 phosphorylation may decrease type-1 phosphatase activity against the inhibitory phosphorylation site on MLCK (Conti & Adelstein, 1981). This would decrease myosin phosphorylation, and provides a path for I-1 phosphorylation in β-adrenergic receptor-mediated relaxation of smooth muscle. However, evidence to support such a conclusion is lacking. Thus, the role of I-1 in the regulation of smooth muscle contractility *in vivo* is not known with certainty.

The potential importance of I-1 in regulating smooth muscle phosphatase activity is underscored by two other mechanisms known to regulate the myosin phosphatase. These involve the phosphorylation of MLCP by Rho-kinase (Fujita *et al.* 1995; Kimura *et al.* 1996) or inhibition of MLCP by the ~17 kDa PKC-activated protein phosphatase inhibitor CPI-17 (Li *et al.* 1998), each representing unique mechanisms for inhibiting myosin phosphatase activity *in vivo* (Somlyo & Somlyo, 2000). These studies suggest that phosphatase regulation via endogenous inhibitor proteins, such as I-1, may represent novel and potentially important participants in smooth muscle function.

To assess the role of I-1 in smooth muscle contractility, we have examined contractility in tonic (aorta) and phasic (portal vein) muscle from WT and I-$1^{(-/-)}$ mice, in response to a variety of electromechanical and pharmacomechanical agonists. Our data suggest that I-1 ablation does not affect contractility in aorta, but may play a subtle role in regulating β-adrenergic receptor responsiveness in the portal vein.

METHODS

Generation of protein phosphatase inhibitor-1 knockout mice

I-$1^{(-/-)}$ mice were generated as previously described (Allen *et al.* 2000). Handling of mice and experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Aortic vessel preparations

Vessels were prepared as previously described (Lalli *et al.* 1997). Briefly, 8- to 10-week-old C57Bl/6 WT and I-$1^{(-/-)}$ mice were killed by asphyxiation in a pre-charged CO_2 chamber. Aortas were immediately dissected, rinsed in cold bicarbonate physiological saline solution (PSS), and connective tissue and fat were removed. Tissues were maintained in a PSS solution containing (mM): 118 NaCl, 4.73 KCl, 1.2 $MgSO_4$, 0.026 EDTA, 1.2 NaH_2PO_4, 2.5 $CaCl_2$, 11 glucose, and 25 $NaHCO_3$; pH when bubbled with 95% O_2–5% CO_2 at 37 °C was 7.4. Experiments on aortic tissue were performed in both endothelium-intact and denuded tissue. Data presented are from denuded aorta only, unless otherwise indicated. In cases of denuded aortas, the endothelium was removed by gently rubbing the vessel between thumb and forefinger. Data were acquired using a Biopac MP100 data acquisition system and analysed using Acqknowledge software.

Western blot analysis

Aortas from both WT ($n = 5$) and I-$1^{(-/-)}$ ($n = 7$) mice were excised, washed, and homogenized in an ice-cold buffer containing (mM): 10 imidazole (pH 7.0), 300 sucrose, 1 DTT, 1 sodium metabisulfite, 100 NaF and 2 EDTA, and protease inhibitors 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg ml^{-1} leupeptin and 10 μg ml^{-1} soybean trypsin inhibitor, pH 7.0. Brain samples from both groups were prepared using an identical buffer. Protein concentrations were

determined by the BioRad method, comparing samples *versus* a BSA standard. Homogenates were subjected to SDS-PAGE, blocked with 5% non-fat milk in Tris-buffered saline + 0.2% Tween-20 (TBST), and incubated with a rabbit, polyclonal I-1-specific antibody (generated in-house and provided by Paul Greengard) for 1 h. Blots were then incubated for 1 h with anti-rabbit secondary antibodies. Membranes were subjected to Enhanced Chemiluminescence PLUS detection reagents (Amersham) and exposed to film for appropriate times.

Isolation of aortic extracts

Extracts from five WT and five I-1$^{(-/-)}$ aortas were obtained by homogenizing each with a ground glass pestle in 10 volumes of ice-cold buffer containing (mM): 40 Tris-HCl pH 7.5, 300 NaCl, 5 DTT, 5 EDTA and 0.2 PMSF, with 1% nonidet P-40, 10 μg ml^{-1} RNAse, 10 μg ml^{-1} DNAse, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin, and phosphatase inhibitors 50 nM deltamethrin and 2 nM okadaic acid. Extracts were spun at 50 000 *g* for 20 min and the supernatant saved for MLC_{20} dephosphorylation assays or ^{32}P-labelling.

Radiolabelling of I-1 in aortic extracts

Extracts from WT or I-1$^{(-/-)}$ aorta (70 μl) were radiolabelled in a solution containing 11.5 mM $MgSO_4$, 5 μg of the catalytic subunit of PKA purified from bovine heart (Sigma Chemical Co.) and 0.1 mM γ-labelled ^{32}P-ATP (2200 c.p.m. pmol^{-1}) in a final volume of 100 μl (1 h at room temperature). The reaction was stopped by the addition of 5 μl of 0.5 M EDTA. An aliquot (7 μl) was removed, boiled in SDS sample buffer, and subjected to SDS-PAGE and autoradiography.

Radioactive labelling of MLC_{20}

Bacterially expressed rat aorta MLC_{20} (80 μg) was phosphorylated in a final volume of 100 μl in a solution containing 100 mM NaCl, 1 mM DTT, 10 mM $MgSO_4$, 0.1 mM γ-labelled ^{32}P-ATP (2200 c.p.m. pmol^{-1}) and 20 mM Tris, pH 7.5, for 30 min at room temperature with 0.1 μg of bacterially expressed, catalytically active, truncated MLCK. The reaction was stopped by adding EDTA (final concentration, 15 mM).

MLC_{20} dephosphorylation rates

Dephosphorylation assays were performed by combining 45 μl of phosphorylated MLC_{20} with 40 μl of WT or I-1$^{(-/-)}$ extract and incubating at room temperature. Aliquots of the reaction mixture were removed at 0, 2.5, 5, 10, 20, 40 and 60 min, separated by SDS-PAGE and stained with Coomassie brilliant blue. Bands corresponding to MLC_{20} were excised and counted.

Portal vein force measurements

Portal veins were mounted isometrically in a longitudinal orientation, as previously described (Sutliff *et al.* 1999). Tissues were maintained in PSS as described for the aorta. One loop of the portal vein was attached to a triangle-shaped holder and the other loop was attached to a Harvard force transducer (Harvard Apparatus, Hollistom, MA, USA). Experiments were conducted after optimal tension was achieved by adjusting the length of the vessel until maximal peak–peak oscillations were observed. Data were acquired and analysed as stated for the aorta.

Statistical analysis

All values are expressed as means ± standard error of the mean (S.E.M.). Statistical comparisons between WT and I-1$^{(-/-)}$ mice were made by performing two-way repeated measures ANOVA (Stewart-Newman-Kuels) or Student's unpaired *t* test, where applicable. Concentration–response curves were generated using Logistic Fit from Origin software, and fitting parameters were averaged. Statistical significance was imparted at $P < 0.05$; *n* refers to the number of mice used in each experimental group.

Table 1. Dimensions of WT and I-1$^{(-/-)}$ aorta and portal vein

	WT	I-1$^{(-/-)}$
Aorta		
n	10	10
Weight (mg)	0.90 ± 0.05	0.90 ± 0.04
Length (mm)	5.34 ± 0.17	5.23 ± 0.12
Thickness (mm)	0.075 ± 0.004	0.074 ± 0.003
CSA (mm^2)	0.79 ± 0.05	0.76 ± 0.03
Portal vein		
n	10	10
Weight (mg)	0.32 ± 0.05	0.36 ± 0.05
Length (mm)	3.29 ± 0.12	3.59 ± 0.17
CSA (mm^2)	0.09 ± 0.01	0.09 ± 0.01

Weight, length and cross-sectional area (CSA) were similar between WT and I-1$^{(-/-)}$ aorta or portal vein. CSA in aorta was calculated by the equation: CSA = [(2 × weight)/(1.06 × circumference)]. CSA in portal vein was calculated by the equation: weight/[(1.06 × length). Wall thickness was estimated in aortas by the equation: Thickness = weight/(1.05 × length × circumference).

RESULTS

Inhibitor-1 is present in the mouse aorta

Aorta from WT and protein phosphatase I-1$^{(-/-)}$ mice were indistinguishable by their gross anatomical parameters (Table 1). I-1 has been reported to be present in smooth muscle of higher animal species (Elbrecht *et al.* 1990) but has not been examined in the mouse aorta. Although the molecular mass of I-1 is ∼19 kDa, this protein has been shown to migrate at ∼26–29 kDa on SDS-PAGE (Allen *et al.* 2000), probably due to its abnormally low detergent-binding properties. Western blot analysis of WT and I-1$^{(-/-)}$ aorta or brain homogenates (included as positive and negative controls) revealed an immunoreactive band only in the WT samples (Fig. 1*A*), demonstrating the presence of I-1 in aortic smooth muscle from WT mice. To determine whether I-1 can be phosphorylated by PKA in mouse aorta, aortic extracts from WT and I-1$^{(-/-)}$ mice were incubated with PKA in the presence of [γ-^{32}P]ATP. Autoradiograms of these extracts revealed a robust signal at ∼29 kDa only in WT aorta (Fig. 1*B*), consistent with the phosphorylation of I-1 under these conditions.

Effects of I-1 ablation on aorta contractility

Administration of phenylephrine (PE), an α-adrenergic agonist, results in activation of the phosphatidylinositol pathway and induces smooth muscle contraction (Gong *et al.* 1995). We anticipated that increased phosphatase activity against MLC_{20}, due to ablation of I-1, would attenuate and/or desensitize PE-induced contractions. This would lead to a rightward shift in the PE concentration–force relationship. Steady-state isometric force and sensitivity to PE were indistinguishable between WT and I-1$^{(-/-)}$ aorta (Fig. 2). The time to 50% maximal contraction/force (TC_{50}) was also similar between the WT and I-1$^{(-/-)}$ aorta (37.3 ± 2.67 *vs.* 45.5 ± 5.2 s, $n = 5$,

$P > 0.05$). Since I-1 is only active when phosphorylated by PKA or PKG, functional differences between WT and I-1$^{(-/-)}$ mice should be accentuated under conditions in which I-1 is activated. To ensure that I-1 is phosphorylated under these conditions and thus active in our assay, PE concentration–force relations were generated after pretreatment of aortas with isoproterenol (300 nM). Following isoproterenol pretreatment, the PE–force relations were shifted to the right (decreased sensitivity) in both groups (Fig. 2*B*). However, isoproterenol treatment did not reveal any contractile differences between WT and I-1$^{(-/-)}$ aorta. The TC_{50} values for PE following 300 nM isoproterenol were 40.5 ± 7.6 and 38.3 ± 4.9 s, for WT ($n = 6$) and I-1$^{(-/-)}$ ($n = 6$) aorta, respectively ($P > 0.05$).

Alternatively, smooth muscle contraction can be elicited by high potassium solutions causing membrane depolarization and a resulting influx of extracellular Ca^{2+} (receptor independent). If I-1 regulates MLCP activity, KCl-induced contractions in I-1$^{(-/-)}$ mice should be attenuated and/or rightward shifted. However, the KCl concentration–isometric force relations were similar between WT and I-1$^{(-/-)}$ aorta (Fig. 3*A*). TC_{50} values in response to KCl-induced contractions were similar between WT and I-1$^{(-/-)}$ aorta (72.4 ± 22.7 *vs.* 66.5 ± 13.3 s, $n = 5$, $P > 0.05$). Isoproterenol, which activates I-1, has been reported to diminish the contractile response to KCl administration in a manner different from agonist-induced contractions.

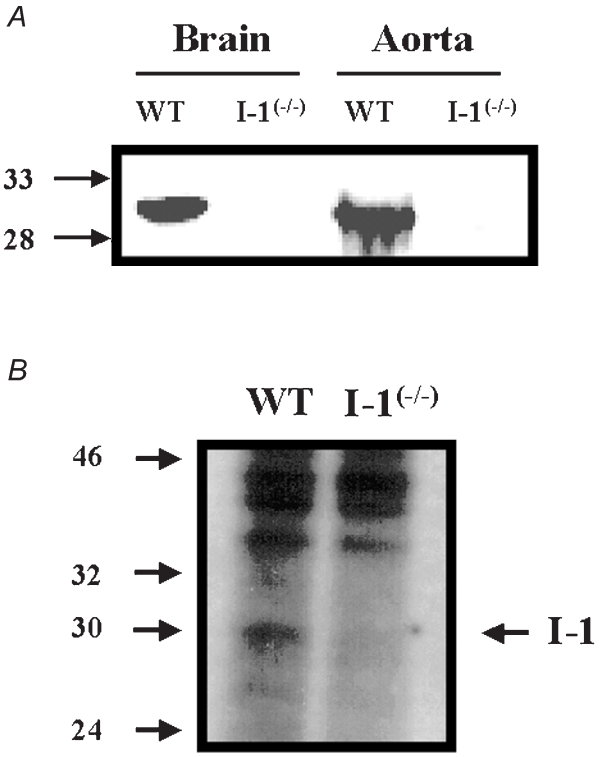

Figure 1. Protein phosphatase inhibitor-1 is present and phosphorylated in mouse aorta

A, homogenates for both wild-type (WT) and I-1$^{(-/-)}$ aorta (100 μg) were subjected to Western blot analysis. Homogenates (10 μg) from WT brain (lane 1) and I-1$^{(-/-)}$ brain (lane 2) were run as positive and negative controls, respectively. An immunoreactive band at ~29 kDa in both WT aorta and brain (lanes 1 and 3) was absent in the I-1$^{(-/-)}$ tissues (lanes 2 and 4). *B*, extracts phosphorylated by PKA in the presence of [γ-^{32}P]ATP were subjected to SDS-PAGE and autoradiography. WT extracts contained a signal at ~29 kDa, which was absent in the I-1$^{(-/-)}$ extracts, consistent with the phosphorylation of I-1 under these conditions.

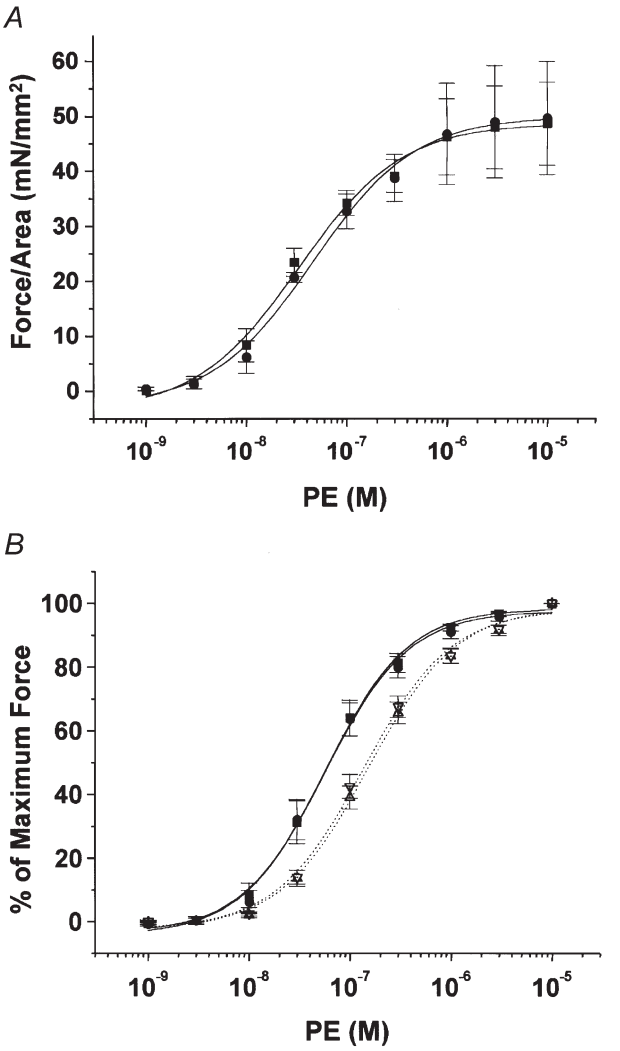

Figure 2. Phenylephrine concentration–isometric force relations

Receptor-mediated responses to phenylephrine (PE) were generated in WT (●) and I-1$^{(-/-)}$ (■) denuded aortas. *A*, force/area responses to phenylephrine were not different between the two groups ($P > 0.05$). *B*, percentage maximal force was unchanged and pretreatment with isoproterenol (dotted lines) resulted in a rightward shift in both WT (▽) and I-1$^{(-/-)}$ (△) curves to a similar extent ($P > 0.05$, $n = 4$ for all groups). Values represent means ± S.E.M.

This is based on the observation that the desensitization occurs in the absence of alterations in intracellular Ca^{2+} concentrations or sensitivity of MLCK to Ca^{2+}–CaM (Tang *et al.* 1992). KCl-induced contractions following isoproterenol pretreatment (300 nM) did result in desensitization to KCl (rightward shift), but were similar in WT and I-$1^{(-/-)}$ aorta, suggesting that I-1 does not play a role in this desensitization (Fig. 3*B*). The TC_{50} values were not different between the WT and I-$1^{(-/-)}$ aorta (94.2 ± 10.6 *vs.* 97.4 ± 13.4 s, $n = 5$, $P > 0.05$).

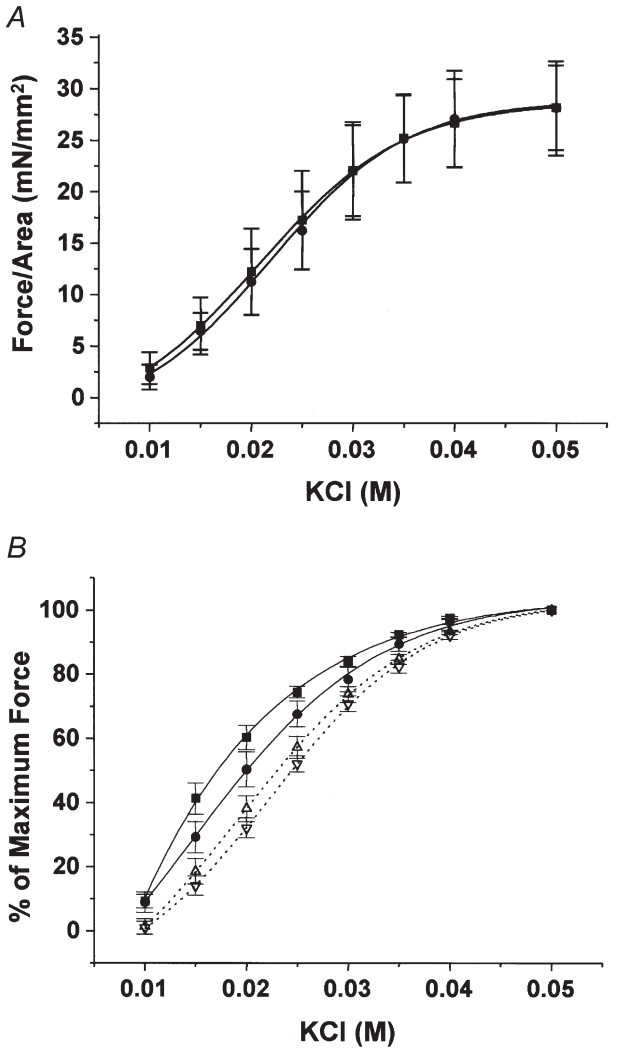

Figure 3. KCl concentration–isometric force relations

Responsiveness to KCl was assessed in WT (●, $n = 4$) and I-$1^{(-/-)}$ (■, $n = 4$) denuded aortas, in parallel. *A*, KCl did not affect the absolute force per area generated in either group ($P > 0.05$). *B*, percentage maximal response was also not different between the two groups. Pretreatment with isoproterenol (dotted lines) resulted in a rightward shift of the concentration curves in a similar manner in WT (▽) and I-$1^{(-/-)}$ (△) aorta ($P > 0.05$). Values represent means ± S.E.M.

I-1 in aortic relaxation

I-1 is a substrate for PKA (Huang & Glinsmann, 1976) and PKG (Tokui *et al.* 1996), both of which invoke smooth muscle relaxation. Based on these results, we examined whether I-1 might play a role in the response to vasodilators. To assess the role of I-1 in relaxation, aortas were contracted with an EC_{50} concentration of PE (300 nM) and relaxed with increasing concentrations of either forskolin (PKA mediated) or sodium nitroprusside (SNP; PKG mediated). If the myosin phosphatase is more active in the I-$1^{(-/-)}$ aortas, they should be sensitized to relaxation. Relaxation in response to forskolin was nearly

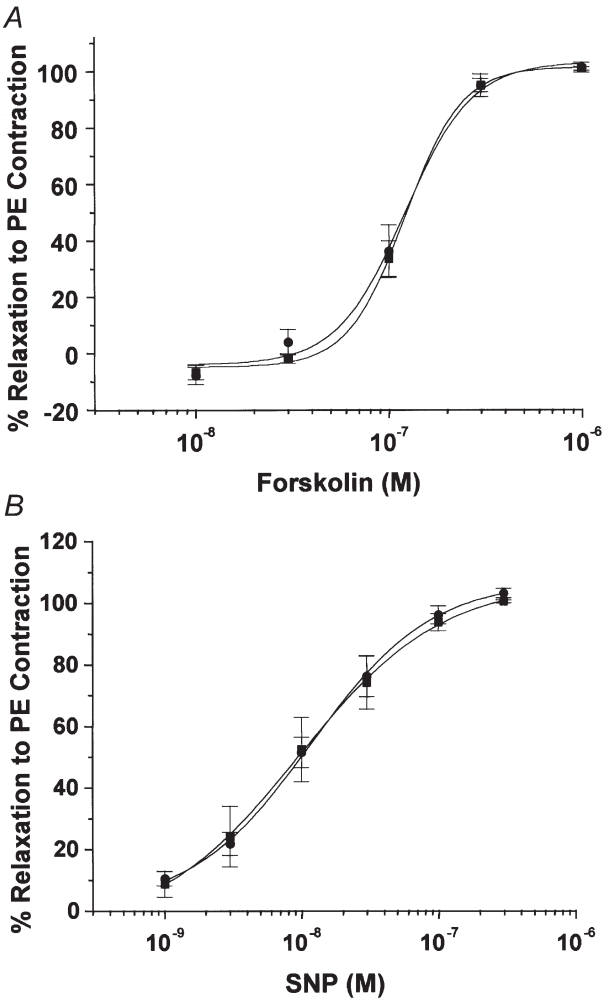

Figure 4. PKA-mediated (forskolin) and PKG-mediated (SNP) concentration–relaxation relations

Denuded aortas from WT (●, $n = 4$) and I-$1^{(-/-)}$ (■, $n = 4$) mice were contracted with a submaximal concentration of PE and subsequently relaxed with increasing concentrations of forskolin (*A*) or sodium nitroprusside (*B*). Relaxation in response to either agonist, expressed as a percentage of PE-induced contraction, was nearly identical between the two groups ($P > 0.05$). Values represent means ± S.E.M.

Table 2. Parameters of portal vein contractility from WT and I-1$^{(-/-)}$ mice

Parameter	WT	I-1$^{(-/-)}$
Amplitude of force (mN mm^{-2})	20.24 ± 1.53	15.27 ± 1.36*
Tension–time integral (mN s mm^{-2})	245.1 ± 21.1	195.5 ± 20.0
+dF/dt (mN s^{-1} mm^{-2})	62.21 ± 4.41	67.37 ± 9.09
−dF/dt (mN s^{-1} mm^{-2})	−42.99 ± 2.04	−45.89 ± 4.44
Frequency (min^{-1})	2.46 ± 0.15	2.17 ± 0.12
Isoproterenol EC$_{50}$ (nM)	3.5 ± 0.2	10.4 ± 1.4*

Baseline measurements were obtained from 10 WT and 10 I-1$^{(-/-)}$ isometrically mounted portal veins, in parallel. ±dF/dt values were obtained from 4 WT and 4 I-1$^{(-/-)}$ portal veins. Parameters are listed on the left with units of measure listed in parentheses. Baseline values are expressed as means ± S.E.M. Statistical significance (Student's unpaired t test) is indicated by an asterisk (* $P < 0.05$).

identical between the WT and I-1$^{(-/-)}$ aortas (Fig. 4*A*). Interestingly, a small, but significant, decrease in the time to half-maximal relaxation (TR$_{50}$) in response to forskolin was observed in the I-1$^{(-/-)}$ aortas (WT 173.7 ± 15.1 s *vs.* I-1$^{(-/-)}$ 103.7 ± 12.5 s, $n = 9$, $P < 0.05$). Relaxation in response to SNP was also not different between WT and I-1$^{(-/-)}$ aorta (Fig. 4*B*). Additionally, SNP was without effect on the rates of relaxation (TR$_{50}$) in WT and I-1$^{(-/-)}$ aorta (39.0 ± 4.3 *vs.* 36.3 ± 2.7 s, $n = 6$, $P > 0.05$). In addition to the relaxant effects of forskolin and SNP, we further examined whether I-1 was involved in relaxation in response to ACh. WT and I-1$^{(-/-)}$ aortas responded in a similar manner to increasing ACh concentrations (data not shown).

MLC$_{20}$ dephosphorylation rates in WT and I-1$^{(-/-)}$ aortic extracts

Although I-1 is present in the aorta (Fig. 1), no obvious effects of I-1 ablation were evident in aortic contraction or relaxation. Thus, we directly examined myosin phosphatase activity by examining rates of MLC$_{20}$ dephosphorylation in aortic homogenates from WT and I-1$^{(-/-)}$ mice, in the presence of 1 μM forskolin. Forskolin was included in this assay in order to fully phosphorylate (activate) I-1, maximizing the inhibitory activity of I-1. The phosphorylation status of I-1 was maintained during the assay by inclusion of the phosphatase inhibitors okadaic acid (2 nM) and deltamethrin (50 nM) to inhibit type-2A and type-2B phosphatase, respectively, the two classes of phosphatases responsible for dephosphorylating (inactivating) I-1 (Oliver & Shenolikar, 1998). The time courses of MLC$_{20}$ dephosphorylation, measured by the release of ^{32}P from radiolabelled myosin light chains, were similar between the WT and I-1$^{(-/-)}$ extracts in the presence of forskolin (Fig. 5), suggesting that I-1 is not regulating myosin phosphatase activity.

Role of I-1 in portal vein smooth muscle

Phasic smooth muscles are distinct from tonic smooth muscles in several of their basic intrinsic properties. Thus, we investigated the effects of I-1 ablation in the spontaneously contracting portal veins from WT and I-1$^{(-/-)}$ mice under basal conditions and in response to a variety of pharmacological agents. Portal veins from WT and I-1$^{(-/-)}$ mice were indistinguishable by their gross anatomical parameters (Table 1). If I-1 is involved in the inhibition of phosphatase activity in portal vein,

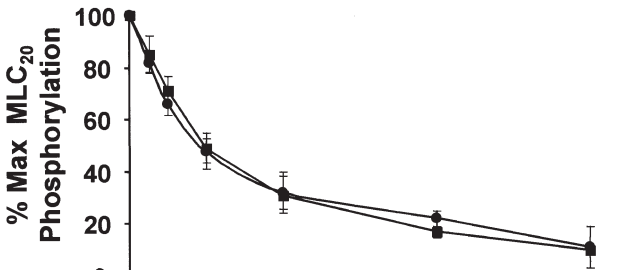

Figure 5. MLC$_{20}$ dephosphorylation rates

^{32}P-labelled MLC$_{20}$ dephosphorylation rates were measured in aortic extracts from WT (●, $n = 5$) and I-1$^{(-/-)}$ (■, $n = 5$) mice treated with 50 μM forskolin. Type-2A (2 nM okadaic acid) and type-2B (50 nM deltamethrin) phosphatase inhibitors were included to maintain the phosphorylation status of I-1 during the assay. Values represent means ± S.E.M. of duplicate experiments.

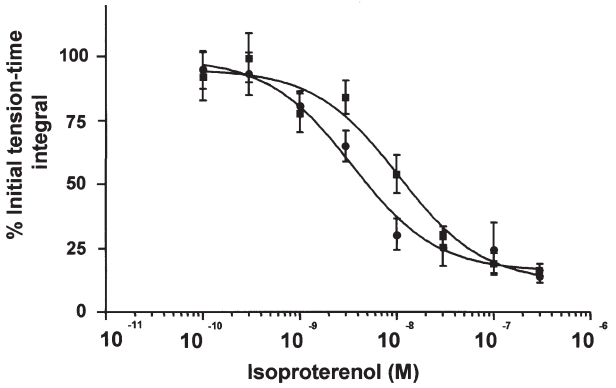

Figure 6. Concentration–tension–time integral relations in response to isoproterenol in WT and I-1$^{(-/-)}$ portal vein

Spontaneous mechanical activity in response to isoproterenol was measured in WT (●, $n = 10$) and I-1$^{(-/-)}$ (■, $n = 10$) portal veins. The concentration of isoproterenol resulting in 50% relaxation was significantly rightward shifted ($P < 0.05$) in I-1$^{(-/-)}$ portal veins, compared to the WT. Values represent means ± S.E.M.

increased phosphatase activity would result in decreased force generation and rate of force development ($+dF/dt$), while the rate of force decline ($-dF/dt$) would be accelerated in the I-1$^{(-/-)}$ preparations. Although values for the amplitude of force generation were decreased in I-1$^{(-/-)}$ mice, the tension–time integral, $\pm dF/dt$, or frequency in spontaneously contracting portal veins from WT and I-1$^{(-/-)}$ mice were similar (Table 2).

To determine the effects of β-adrenergic stimulation in WT and I-1$^{(-/-)}$ portal veins, concentration–relaxation relations were examined in response to isoproterenol. Following isoproterenol administration, portal vein contractility, expressed as a percentage of the basal tension–time integral, was decreased in both the WT and I-1$^{(-/-)}$ preparations (Fig. 6). Although the maximal relaxation in response to isoproterenol was similar between WT and I-1$^{(-/-)}$ portal vein, the concentration required to achieve half-relaxation (EC_{50}) was significantly higher in I-1$^{(-/-)}$ portal vein (Table 2), indicating a desensitized response to β-adrenergic stimulation in the I-1$^{(-/-)}$ mice. In addition, portal vein Ca^{2+} sensitivity was measured by depolarizing the portal veins and exposing them to increasing concentrations of Ca^{2+}. If I-1 is involved in a Ca^{2+}-sensitizing effect, through inhibition of MLCP, uninhibited myosin phosphatase in the I-1$^{(-/-)}$ portal vein should result in a rightward shift in the Ca^{2+} concentration–isometric force relations. Ca^{2+}-elicited contractions were similar between the two groups (Fig. 7*A*). Pretreatment with isoproterenol (300 nM) resulted in a near-parallel Ca^{2+} desensitization of portal veins, as previously observed in response to cGMP (Lee *et al.* 1997), in both WT and I-1$^{(-/-)}$ portal veins. Finally, the effect of I-1 gene ablation on ACh-mediated contraction of portal vein preparations was examined. The tension–time integral for portal veins from WT and I-1$^{(-/-)}$ mice increased with increasing concentrations of ACh. However, no differences in ACh responsiveness were observed between the WT and I-1$^{(-/-)}$ portal veins (Fig. 7*B*).

DISCUSSION

The discovery that MLCP activity, and consequently MLC_{20} dephosphorylation, could be regulated in response to various agents has initiated a search for novel proteins involved in modulating the MLCP. In this study, we investigated the role of protein phosphatase inhibitor-1 (I-1), a known inhibitor of the type-1 phosphatase, in tonic (aorta) and phasic (portal vein) smooth muscle contraction and relaxation. I-1 has been shown to regulate myosin phosphatase activity *in vitro* and I-1 gene-targeted mice provide us with the opportunity to directly examine the role of I-1 in smooth muscle contractility *in vivo*.

Our original hypothesis was that I-1 plays an important role in the regulation of the smooth muscle phosphatase activity, specifically of the myosin-associated phosphatase, and thus is a regulator of smooth muscle contractility. This hypothesis was based on the findings that I-1 can regulate MLCP *in vitro* and that other inhibitors of myosin phosphatase, such as Rho-kinase and CPI-17, are important in regulating smooth muscle contractility (Somlyo & Somlyo, 2000). In addition, I-1 has been suggested to play a role in the Ca^{2+}-sensitization observed in response to agonists such as cGMP (Somlyo *et al.* 1989).

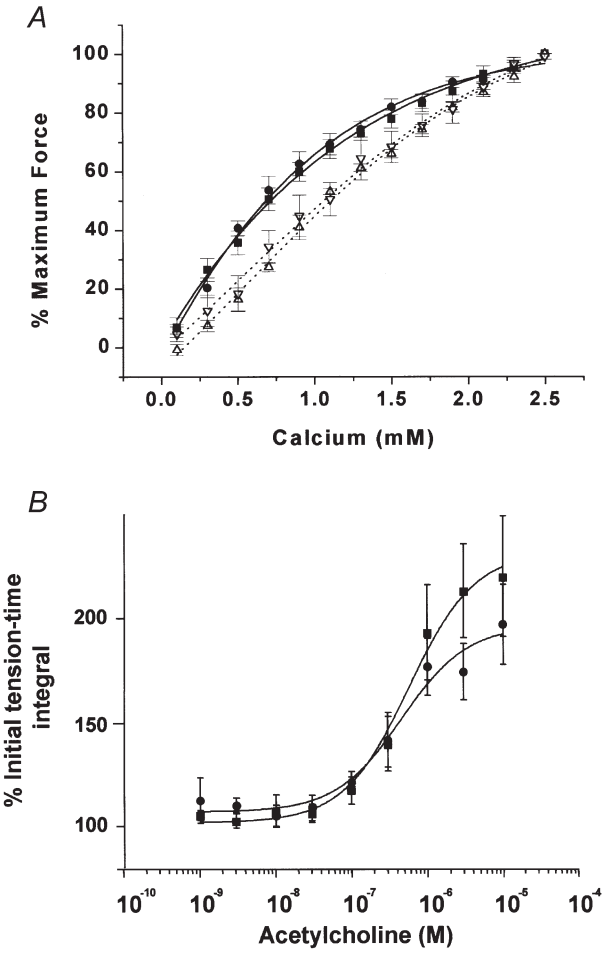

Figure 7. Percentage maximal force or tension–time integral from portal vein in response to increased calcium or ACh concentrations

Portal vein contraction in response to calcium (*A*) or ACh (*B*) was measured in WT (●, $n = 5$) and I-1$^{(-/-)}$ (■, $n = 5$) mice. Portal vein contractility in WT and I-1$^{(-/-)}$ mice was similar at every calcium concentration ($P > 0.05$). Pretreatment with isoproterenol (dotted lines), to fully activate I-1, resulted in a parallel rightward shift in the calcium dose–response curve in both the WT (▽) and I-1$^{(-/-)}$ (△) portal veins. Contraction in response to ACh, expressed as a percentage of basal values, increased in a comparable manner in both WT (●, $n = 5$) and I-1$^{(-/-)}$ (■, $n = 5$) portal vein ($P > 0.05$). Values represent means ± S.E.M.

The major finding of this study is that, although I-1 was detected in smooth muscle (Fig. 1*A*) and phosphorylated by PKA (Fig. 1*B*), ablation of I-1 did not have any major affects on either aorta or portal vein contractility *in vivo*. These findings are supported by nearly identical MLC_{20} dephosphorylation rates in WT and I-$1^{(-/-)}$ aorta. Although the *in vivo* substrate for the phosphatase is native myosin, these studies on the dephosphorylation of MLC_{20} strongly suggest that the phosphatase activities in the WT and I-$1^{(-/-)}$ mice are equivalent. The absence of a difference between WT and I-$1^{(-/-)}$ tissues was not due to inactive (dephosphorylated) I-1 in the WT, since experiments performed in the presence of known activators of I-1 (isoproterenol/forskolin) also showed no effect.

Relaxation induced by PKA or PKG, both of which activate I-1, was also unaffected by I-1 ablation. These data demonstrate that I-1 is not an essential component of smooth muscle contractility under basal conditions or following β-adrenergic stimulation in the aorta. We did observe a small, but significant, rightward-shifted (desensitized) response to isoproterenol in I-$1^{(-/-)}$ portal veins. This finding was unique to the portal vein and is not consistent with I-1 inhibiting type-1 phosphatase activity against myosin. At least two potential mechanisms could explain this finding. In the absence of I-1, increased phosphatase activity and the resulting dephosphorylation of the inhibitory phosphorylation site on MLCK (Oliver & Shenolikar, 1998) would increase MLCK activity, impairing relaxation. Alternatively, I-1 could regulate phosphatase activity in the sarcoplasmic reticulum, as described for striated muscle (MacDougall *et al.* 1991). If so, increased SR phosphatase activity would result in activation (dephosphorylation) of the inhibitor of the SR Ca^{2+}-ATPase phospholamban, leading to slower sequestration of Ca^{2+} (Lalli *et al.* 1999). Although the physiological significance of this finding is not clear, these data suggest that I-1 may exist as a fine-tuning mechanism in response to β-catecholamines in the portal vein. In addition, we also observed a rightward shift in the Ca^{2+} response following addition of isoproterenol (desensitized) in WT portal vein, as previously described for cGMP (Lee *et al.* 1997). However, the I-$1^{(-/-)}$ portal vein curve was shifted in a similar manner, excluding I-1 as a component of the Ca^{2+}-desensitizing effect.

Collectively, our data suggest that I-1 does not play a major role in either tonic or phasic smooth muscle contractility *in vivo*. The non-effect of I-1 ablation on MLC_{20} dephosphorylation rates between WT and I-$1^{(-/-)}$ aorta supports *in vitro* studies (Alessi *et al.* 1992), which demonstrated only a modest effect of I-1 on inhibiting the myosin phosphatase holoenzyme activity in avian smooth muscle ($< 30\%$; Alessi *et al.* 1992). The lack of effect of I-1 ablation on either aorta or portal vein contractility compels us to speculate on the relevance of this protein in smooth muscle, given its relative abundance. A role for I-1 in smooth muscle may be in regulating enzymes involved in glycogen metabolism (Cohen, 1989). However, despite numerous *in vitro* reports demonstrating a role for I-1 in this process, studies from skeletal muscles of I-$1^{(-/-)}$ mice showed no effect on the activities of glycogen synthase and glycogen phosphorylase (Scrimgeour *et al.* 1999), challenging I-1's role in this process. Alternatively, there are several lines of evidence that suggest the presence of another myosin phosphatase (Pato & Adelstein, 1980; Erdodi *et al.* 1989; Takai *et al.* 1989; Nomura *et al.* 1992), probably a type-2A enzyme. In this case, inhibition of the myosin type-1 phosphatase by I-1 may represent only a minor fraction of the total myosin phosphatase activity. With respect to the portal vein, the rightward shift in the isoproterenol-induced relaxation in I-$1^{(-/-)}$ tissues suggests that I-1 may serve as a fine-tuning mechanism in regulating contractility in response to β-adrenergic agonists. The difference between the PKA effects of I-1 protein ablation in aorta and portal vein may reflect inherent differences in the myosin-targeting subunits or catalytic subunits themselves in each tissue (Hartshorne, 1998; Hartshorne *et al.* 1998). In the light of the significant amount of I-1 present, other functions for I-1 in vascular smooth muscle are a strong possibility, but remain elusive.

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