# Dominant role of smooth muscle L-type calcium channel Ca$_v$1.2 for blood pressure regulation

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**Blood pressure is regulated by a number of key molecules involving G-protein-coupled receptors, ion channels and monomeric small G-proteins. The relative contribution of these different signaling pathways to blood pressure regulation remains to be determined. Tamoxifen-induced, smooth muscle-specific inactivation of the L-type Ca$_v$1.2 Ca$^{2+}$ channel gene in mice (SMAKO) reduced mean arterial blood pressure (MAP) in awake, freely moving animals from $120 \pm 4.5$ to $87 \pm 8$ mmHg. Phenylephrine (PE)- and angiotensin 2 (AT2)-induced MAP increases were blunted in SMAKO mice, whereas the Rho-kinase inhibitor Y-27632 reduced MAP to the same extent in control and SMAKO mice. Depolarization-induced contraction was abolished in tibialis arteries of SMAKO mice, and development of myogenic tone in response to intravascular pressure (Bayliss effect) was absent. Hind limb perfusion experiments suggested that 50% of the PE-induced resistance is due to calcium influx through the Ca$_v$1.2 channel. These results show that Ca$_v$1.2 calcium channels are key players in the hormonal regulation of blood pressure and development of myogenic tone.**
*Keywords*: blood pressure/Ca$^{2+}$ channels/RhoA/SMAKO/ smooth muscle cells

## Introduction

Chronically elevated arterial blood pressure leads to many fatal diseases such as myocardial infarction or stroke. This illustrates why a detailed understanding of the key factors involved in blood pressure regulation would be very valuable for modern medicine. Arterial blood pressure is determined by vascular tone, i.e. the contractile activity of vascular smooth muscle cells (SMCs) in the walls of small arteries and arterioles. Regulation of the contractile state of resistance vessels is maintained by an interplay of vasoconstrictor and vasodilator stimuli from circulating hormones, neurotransmitters and also directly by blood pressure (Bayliss effect; Bayliss, 1902). Vascular smooth muscle contraction is triggered by Ca$^{2+}$/calmodulin-dependent phosphorylation of the regulatory myosin light chain. Ca$^{2+}$ influx through ion channels and Ca$^{2+}$ release from intracellular stores have been proposed as the

major source of this activator Ca$^{2+}$ (Davis and Hill, 1999). On the other hand, contractility of vascular smooth muscle is regulated not only by [Ca$^{2+}$]$_i$, but also by membrane potential- and Ca$^{2+}$-independent mechanisms. The small GTPase RhoA and its upstream activators and downstream effectors have been invoked as major players in these Ca$^{2+}$-independent mechanisms (Somlyo *et al.*, 1999). To date, the relative contribution of calcium influx through L-type Ca$^{2+}$ channels to blood pressure regulation and the role of Ca$^{2+}$-independent mechanisms have been discussed highly controversially.

In the microcirculation, Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels has been supposed to play a particularly important role in myogenic reactivity and tone (Davis and Hill, 1999). Vascular voltage-gated Ca$^{2+}$ channels are modulated by several signaling systems and appear to be activated by vasoconstrictors that stimulate the G$_{q/11}$/G$_{12/13}$ signaling pathway, e.g. norepinephrine (Nelson *et al.*, 1988). The autoregulatory Bayliss effect (i.e. constriction of the vessel after an increase of transmural pressure) is probably based on graded membrane depolarization in vessels in response to pressure. Membrane depolarization would open voltage-gated Ca$^{2+}$ channels and increase vasoconstriction, whereas hyperpolarization would close them and induce vasodilatation (Jaggar *et al.*, 1998).

Here, we have investigated the role of the L-type Ca$_v$1.2 Ca$^{2+}$ channel for blood pressure regulation by combining a smooth muscle-specific, temporally controlled gene knockout approach with *in vivo* analysis of blood pressure in awake mice, video-microscopy of isolated resistance-like vessels under isobaric conditions and hind limb perfusion. Our results suggest that the Ca$_v$1.2 Ca$^{2+}$ channel is required for autoregulation and maintenance of vascular tone in response to depolarization and pressure. Agonists activating the vascular G$_{q/11}$ and G$_{12/13}$ pathways activate not only the L-type Ca$_v$1.2 Ca$^{2+}$ channels but also the RhoA pathway in resistance vessels.

## Results

### Generation of SMAKO mice

Mice globally lacking the Ca$_v$1.2 L-type Ca$^{2+}$ channel die *in utero* before day 15 post-coitum (Seisenberger *et al.*, 2000). To circumvent embryonic lethality, the tamoxifen-inducible Cre/loxP recombination system was used to inactivate the Ca$_v$1.2 gene specifically in SMCs (Figure 1A; see Materials and methods for details). SMC-specific Cre/loxP recombination was achieved by expressing the tamoxifen-inducible Cre recombinase under control of the SM22 promoter [SM-Cre ER$^{T2}$(ki) mice; Kuhbandner *et al.*, 2000]. Smooth muscle-specific alpha 1.2 calcium channel knockout (SMAKO) mice before tamoxifen injection are viable, have normal body
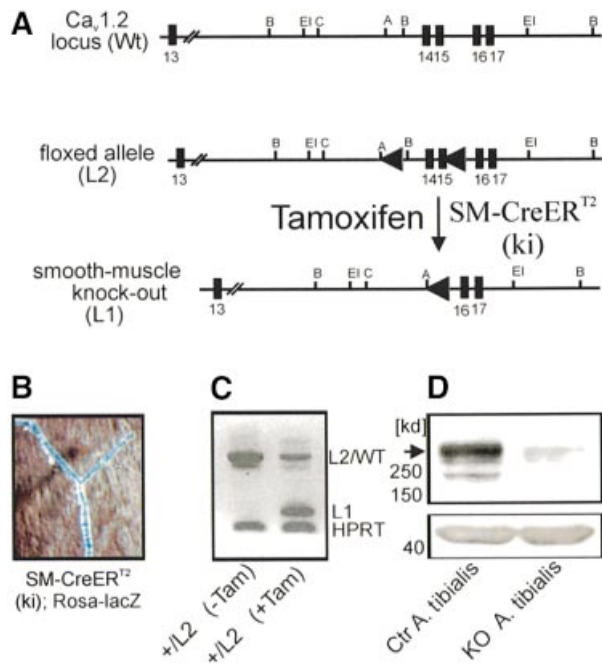
**Fig. 1.** SMC-specific inactivation of the Ca$_v$1.2 gene. (**A**) Schematic representation of the wild-type (WT), the knockout (L1) and the conditional Ca$_v$1.2 alleles (L2). The numbers indicate the exon number. SMC-specific activation of tamoxifen-inducible Cre recombinase [SM-Cre ER$^{T2}$(ki)-Cre] results in the deletion of Ca$_v$1.2 exons 14 and 15. Restriction sites are A, *Acc*65I; B, *Bam*HI; C, *Cla*I; EI, *Eco*RI. (**B**) Detection of *lacZ* gene expression in tibialis arteries of a tamoxifen-treated SM-Cre ER$^{T2}$(ki); Rosa-lacZ mouse. Blue staining indicates Cre-mediated recombination. (**C**) RT–PCR analysis of mesenteric arteries of +/L2 mice before (–Tam) and after (+Tam) tamoxifen treatment. L2/Wt and L1 bands represent L2/wild-type and L1 transcripts, respectively. The L1 band after tamoxifen treatment (+Tam) is generated by Cre-mediated recombination; the remaining L2/WT band is due to the remaining WT allele after recombination. (**D**) Western analysis of proteins from tibialis arteries using an anti-Ca$_v$1.2 antibody demonstrates the absence of Ca$_v$1.2 protein (arrowhead) in vessels from SMAKO mice. β-actin (~43 kDa) was used as loading control.

weight, breed normally and are indistinguishable from control littermates. The phenotype changes dramatically after tamoxifen-induced inactivation of the Ca$_v$1.2 gene: between 21 and 28 days after the first tamoxifen injection, SMAKO mice show general signs of severe illness (lowered activity, relieving posture). Post-mortem examination revealed that the mice suffer from a complete ileus (bowel paralysis) combined with urinary retention. SMAKO mice die between 28 and 35 days after tamoxifen injection, probably due to complications of the ileus (peritonitis, shock). The control mice which were also treated with tamoxifen showed no change in phenotype.

By crossing SM-Cre ER$^{T2}$(ki) mice with lacZ reporter mice, we confirmed that Cre-mediated recombination occurs in SMCs of small vessels (Figure 1B). Cre-mediated recombination could also be observed in other vessels and non-vascular tissues containing smooth muscle, e.g. aorta, renal artery, colon, small intestine and urinary bladder, but not in heart or cerebral cortex (not shown).

RT–PCR analysis of cDNA obtained from branches of mesenteric arteries of +/L2 mice after tamoxifen treatment again demonstrated the recombination event (conversion of L2 to L1) in small vessels. The mesenteric arteries of
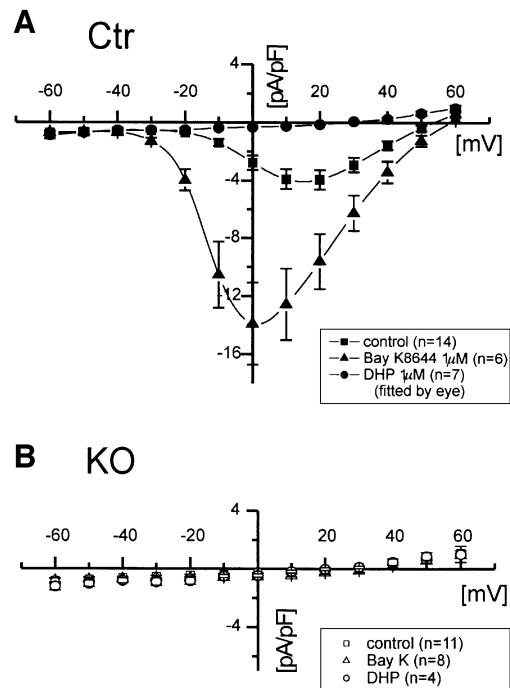
**Fig. 2.** Kinetics and pharmacology of smooth muscle L-type I$_{Ba}$. (**A** and **B**) Results obtained with SMCs isolated from the tibialis artery of control (Ctr) and SMAKO mice (KO), respectively. Current voltage (I–V) relationships were recorded in the absence and presence of 1 μM Bay K 8644 or nisoldipine. The HP was 80 mV. Cells were depolarized to potentials between –60 and +60 mV with 10 mV increments for 100 ms at 0.2 Hz.

these mice still contain the wild-type Ca$_v$1.2 gene (+ or WT allele, respectively) after Cre-mediated recombination (Figure 1C).

### Complete loss of L-type currents in smooth muscle cells from tibialis arteries

To quantify the recombination efficiency in smooth muscle of SMAKO mice, we then analyzed single SMCs by electrophysiology for the presence of L-type Ca$^{2+}$ channels and small, resistance-sized (~100–150 μM) vessels for the presence of Ca$_v$1.2 protein. Immunoblotting of whole-cell protein extracts from the tibialis artery using a Ca$_v$1.2-specific antibody revealed that the Ca$_v$1.2 protein was reduced to <10% in SMAKO mice (Figure 1D). The remaining signal probably represents Ca$_v$1.2 protein from other vascular cells such as endothelium, blood cells and fibrocytes. This assumption is confirmed by the analysis of L-type Ba$^{2+}$ currents (I$_{Ba}$) in freshly isolated control and SMAKO SMCs from tibialis arteries. No L-type current was detected in myocytes from SMAKO mice (*n* = 0/18 SMCs), while 90.5% (*n* = 19/21 cells) of the control SMCs had typical L-type current. The maximal I$_{Ba}$ density was –4.1 ± 0.7 and –14.0 ± 0.4 pA/pF in the absence and presence of 1 μM Bay K 8644, respectively (Figure 2). In three out of eight SMAKO cells, BayK 8644 increased I$_{Ba}$ to –0.8 ± 0.27 pA/pF, which amounts to ~5% of the Bay K 8644 current measured in control cells.

The calcium channel blocker nisoldipine (1 μM) blocked I$_{Ba}$ in control SMCs by 93% (Figure 2) but did not change the current of SMAKO myocytes (Figure 2).

There was no difference in membrane capacitance between SMCs from control and SMAKO mice, indicating that gene inactivation did not affect cell size and morphology ($22 \pm 1.6$ pF, $n = 19$, in control and $19.5 \pm 1.6$ pF, $n = 18$, in SMAKO mice).

The electrophysiological data and the immunoblots confirm the notion that tamoxifen-induced activation of the Cre recombinase effectively inactivated the $Ca_v1.2$ gene in arterial SMCs.

### Lack of $Ca_v1.2$ attenuates blood pressure and response to vasoconstrictors

We followed the changes in mean arterial blood pressure (MAP) in individual mice during and after tamoxifen-induction of the Cre recombinase by telemetry (Figure 3A). In general, inactivation of the $Ca_v1.2$ gene resulted in a decrease of MAP from $120 \pm 4.5$ to $87 \pm 8$ mmHg (Figure 3B). Tamoxifen treatment had no short- or long-term effects on MAP in control animals ($n = 3$, not shown). The MAP was decreased further in control and SMAKO mice to ~60 mmHg 1 h after i.p. injection of 0.5 mg/kg$_{body\text{-}weight}$ isradipine (Figure 3B and C). This additional drop in MAP indicates that $Ca_v1.2$ channels at other, non-vascular sites could contribute to the regulation of blood pressure. Blockade of cardiac $Ca_v1.2$ channels, for example, could lead to blood pressure reduction by a negative inotropic action. Furthermore, the effect of dihydropyridines such as isradipine on blood pressure is attributed not only to their blocking action on smooth muscle L-type $Ca^{2+}$ channels, but also to release of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) from the vascular endothelium, which will result in vessel relaxation and thus blood pressure reduction (Fisslthaler *et al.*, 2000; Hirasawa and Pittman, 2003).

Although treatment with the $\alpha$-adrenergic agonist phenylephrine (PE) and with angiotensin 2 (AT2) increased MAP in both genotypes, the blood pressure response to these compounds was significantly lower in SMAKO mice, indicating that G-protein-coupled pathways leading to smooth muscle contraction either directly (e.g. via channel modulation by phosphorylation) or indirectly (e.g. via membrane potential changes) affect the $Ca_v1.2$ L-type calcium channel (control mice: $160 \pm 2$ mmHg, PE; $159 \pm 2$ mmHg, AT2; SMAKO mice: $117 \pm 4$ mmHg, PE; $119 \pm 4$ mmHg, AT2; $P < 0.05$). We inferred from these results that both receptors signaled not only through the $Ca_v1.2$ channel but also by an increase in the release of $Ca^{2+}$ from intracellular stores or through activation of the RhoA pathway. Therefore, we tested whether or not the residual basal MAP obtained in the presence of isradipine could be lowered further by inhibition of the Rho pathway. One hour after i.p. injection of a high dose of the p160 Rho-kinase inhibitor Y-27632 (30 mg/kg$_{body\text{-}weight}$), MAP in control and SMAKO mice pre-treated with isradipine was reduced further from $58 \pm 3$ to $40 \pm 1.5$ mmHg and from $66 \pm 6$ to $50 \pm 8$ mmHg, respectively (Figure 3B and C).

### Blunted vasoconstrictor effect in vessels from SMAKO mice

The results shown so far indicated that the L-type $Ca^{2+}$ channel is functionally absent in vascular SMCs from SMAKO mice. To support this notion further, we
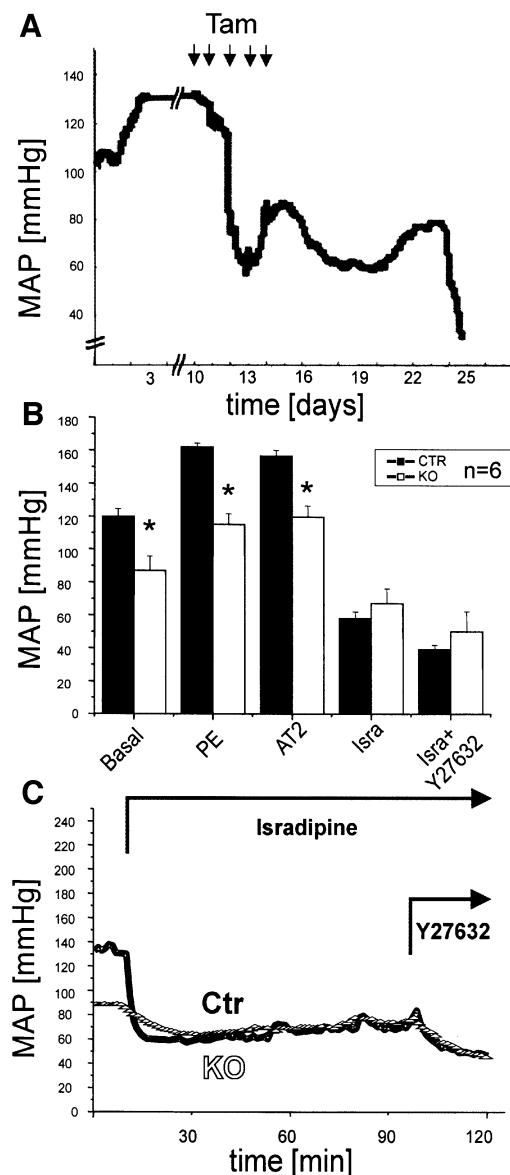


**Fig. 3.** Reduced arterial blood pressure in SMAKO mice. Blood pressure was measured in conscious, freely moving mice by telemetry. (**A**) Original tracing showing the effect of tamoxifen application (1 mg/day for 5 days) leading to Cre-mediated $Ca_v1.2$ gene recombination on MAP in a SMAKO mouse. (**B**) Basal blood pressure and effect of an i.p. bolus (200 μl) injection of PE (10 μg/kg), AT2 (5 μg/kg), isradipine (Isra; 0.5 mg/kg) and Y-27632 (30 mg/kg) on MAP in awake control (Ctr) and SMAKO (KO) mice analyzed 21–28 days after tamoxifen injection. (**C**) Original tracing of an experiment statistically analyzed in (B). Arrows indicate time of i.p. injection of compounds.

measured isometric and isobaric force development in aortic rings and pressurized tibialis arteries, respectively. The contribution of the $Ca_v1.2$ channel to the regulation of arterial tone by G-protein-coupled receptors was evaluated by examining the effects of PE and AT2 on arterial diameter and force development. PE caused an increase of $1.6 \pm 0.3$ mN/mm in isometric force development normalized to vessel length in the control (Figure 4A and C). In contrast, PE increased the force only by $0.7 \pm 0.15$ mN/mm in aortic rings from SMAKO mice (Figure 4B and C). Similarly, reduction of vessel diameter in response to PE and AT2 in tibialis artery sections pressurized to 60 mmHg from SMAKO mice was
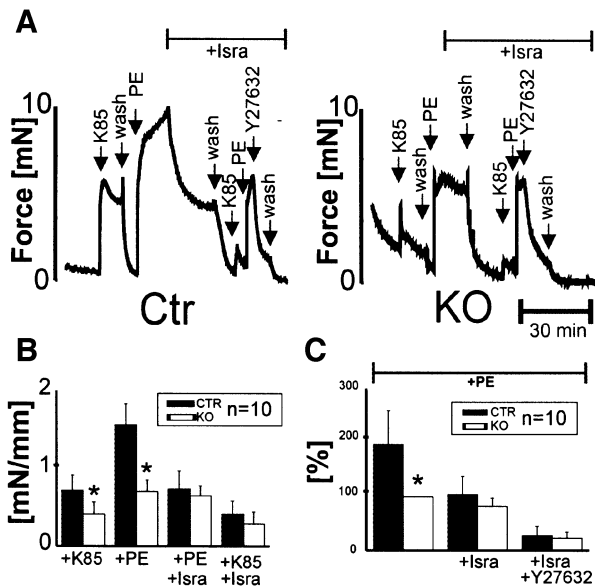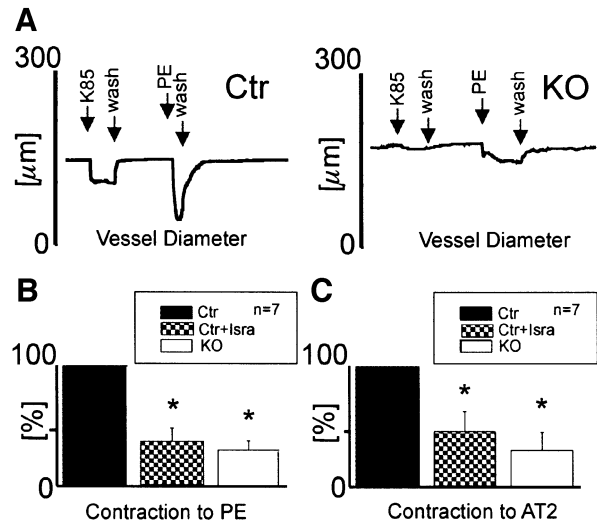
**Fig. 4.** Decreased isometric force generation of aortic rings from SMAKO mice. (**A**) Original tracings and (**B**) statistical analysis of contractions of isolated aortic rings from control (Ctr) and SMAKO (KO) mice. Force generation in response to 85 mM K+ (K85) and 1 μM PE measured with or without 1 μM isradipine (Isra) or 5 μM Y-27632 as indicated. Force in (B) is normalized to vessel length. (**C**) Relaxation of aortic rings pre-contracted with 1 μM PE by 1 μM isradipine and 5 μM Y-27632. Force levels are expressed as a percentage of the maximum contractile force in SMAKO mice. *$P < 0.05$ versus the same condition in control mice.



**Fig. 5.** Tibialis arteries from SMAKO mice show a blunted response to vasocontracting agonists. (**A**) Original tracings showing the effect of superfusion with 85 mM K+ (K85) and 1 μM PE on luminal diameter in vessels from control (Ctr) and SMAKO (KO) mice. (B and C) Contractile response of tibialis arteries from control mice before and after application of 1 μM isradipine (Isra) and from SMAKO mice. Vessels were superfused with (**B**) 1 μM PE or (**C**) 500 nM AT2. *$P < 0.01$.

significantly ($P < 0.01$) attenuated to $28 \pm 7$ and $30 \pm 15\%$ of the control value, respectively (Figure 5A–C). After application of isradipine (1 μM), the responses to PE and AT2 reached the value of SMAKO mice in both models of vessel contraction (Figures 4A–C and 5A–C). Again, we evaluated the effect of the Rho-kinase inhibitor Y-27632 on $G_{q/11}/G_{12/13}$-mediated vessel contraction in the absence of $Ca_v1.2$ protein (SMAKO mice) or after pharmacological blockade of the L-type calcium channels by 1 μM isradipine. Under both conditions, Y-27632 almost completely relaxed aortic rings pre-contracted with PE (Figure 4A, B and D).

### Arterial myogenic tone is absent in SMAKO mice

Elevation of intravascular pressure constricts small arteries (Bayliss, 1902; Knot and Nelson, 1998). This autoregulatory behavior of vessels, also termed myogenic tone, has been supposed to play a key role in regulation of tissue blood flow *in vivo* (Johnson *et al.*, 1981). We investigated myogenic tone using video-microscopy of pressurized resistance-sized tibialis artery preparations. Vasoconstriction induced by elevating intravascular pressure was almost totally inhibited in SMAKO vessels compared with control arteries (control mice: $16.5 \pm 0.4\%$ myogenic tone at 90 mmHg; SMAKO mice: $2.2 \pm 1\%$ myogenic tone; $P < 0.01$; Figure 6A). Isradipine treatment of control vessels reduced myogenic tone to the level found in SMAKO mice ($2.5 \pm 0.55\%$ myogenic tone).

Elevation of intravascular pressure depolarizes the SMCs within the arterial wall (Brayden and Nelson, 1992). To link the lack of $Ca_v1.2$ channels in tibialis arteries from SMAKO mice with the compromised myogenic tone response, we examined the effect of depolarization by 60 mM K+ on the diameter of SMAKO and control arteries at constant pressure. Membrane depolarization constricted tibialis arteries from control mice by $73 \pm 20\%$. Depolarization-induced contraction was absent in SMAKO and in isradipine-treated vessels, indicating that depolarization activates $Ca_v1.2$ $Ca^{2+}$ channels leading to increased intracellular $Ca^{2+}$ and vasoconstriction (Figure 6B). Similarly, depolarization by 85 mM K+ in isometric aortic ring preparations caused significantly less force development in SMAKO than in control mice ($0.42 \pm 0.2$ versus $0.73 \pm 0.2$ mN/mm; $P < 0.01$; Figure 4A–C).

### Multiple roles of $Ca_v1.2$ channels in the microcirculation

The contractile activity of vascular SMCs in the walls of small arteries and arterioles is the major determinant of blood pressure (Jackson, 2000). To test whether our results found in larger vessels could also be applied to microcirculatory hemodynamics, we used the perfused hind limb model (Figure 7). Resistance at 100 mmHg perfusion pressure was reduced to ~60% in SMAKO compared with control hind limbs (Figure 7B), indicating the loss of autoregulatory myogenic tone (Brandes *et al.*, 2000). Isradipine treatment of control preparations reduced peripheral vessel resistance in the hind limbs to SMAKO levels (control mice: $16.2 \pm 0.9$ mmHg/ml/min; control mice + isradipine: $8.8 \pm 1.4$ mmHg/ml/min; SMAKO mice: $10.3 \pm 0.9$ mmHg/ml/min; $P < 0.01$). Perfusion pressure changes to bolus application of 100 μl of 1 mM PE were blunted by isradipine treatment and in SMAKO mice [change in perfusion pressure (ΔPerfusion pressure) in control mice: $68 \pm 19$ mmHg; in control mice + isradipine: $27 \pm 3$ mmHg; in SMAKO mice: $35.5 \pm 5$ mmHg; $P < 0.01$; Figure 7A, C and D], indicating that ~50% of the PE-induced resistance increase was
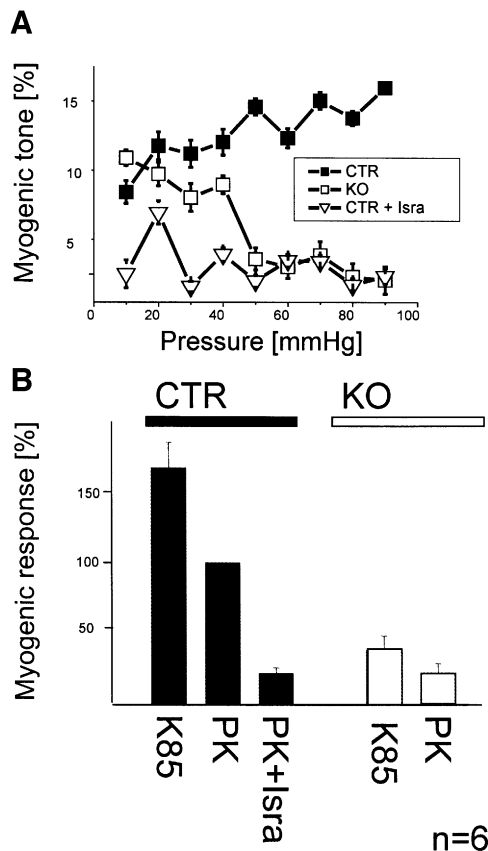
**Fig. 6.** Pressure- and depolarization-induced myogenic tone is absent in SMAKO mice. (**A**) Myogenic responses of tibialis arteries of SMAKO (KO) mice and control mice (Ctr) before and after treatment with isradipine (Isra). Myogenic tone is expressed as a percentage of the difference to the passive vessel diameter in $Ca^{2+}$-free solution at each given pressure. (**B**) Myogenic response at 60 mmHg of control (Ctr) and SMAKO (KO) tibialis arteries superfused with 85 mM $K^+$ (85K) or 5.6 mM $K^+$ (PK) with or without 1 µM isradipine (Isra). The myogenic response is shown as a percentage of the diameter in control vessels at 5.6 mM $K^+$ concentration.

causally linked to calcium influx through $Ca_v1.2$ channels. The Rho-kinase inhibitor Y-27632 decreased the PE-induced resistance increase further by 45% in SMAKO mice (SMAKO mice + Y-27632: 15.8 ± 2 mmHg; control mice + isradipine + Y-27632: 11.9 ± 2 mmHg; $P < 0.01$; Figure 7C and D), supporting a role for RhoA signaling in the microvasculature.

## Discussion

The present study reports for the first time on a tissue-specific inactivation of the $Ca_v1.2$ gene in SMCs by using the temporally controlled Cre/loxP system. With this technique, we were able to analyze the contribution of the smooth muscle L-type $Ca_v1.2$ channel to blood pressure regulation in the absence of calcium channel blockers. The genetic approach is superior to pharmacological experiments for various reasons. Higher concentrations of calcium channel blockers will affect the cardiac $Ca_v1.2$ channel *in vivo* and thereby decrease cardiac contractility and blood pressure. Dihydropyridines such as isradipine act voltage dependently (for a review see Hofmann *et al.*,
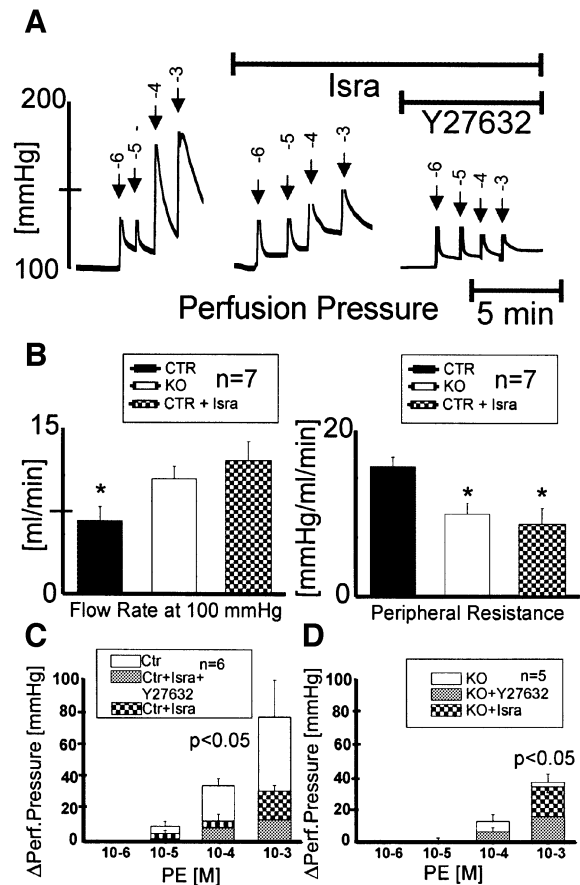


**Fig. 7.** SMAKO mice analyzed in a perfused hind limb model show reduced tone and vasoconstrictor responses. Both hind limbs were perfused via the infrarenal aorta abdominalis with modified Krebs–Henseleit solution. (**A**) Original tracing showing the effect of bolus (200 µl) applications of PE on perfusion pressure in the absence or presence of 1 µM isradipine (Isra) and 5 µM Y-27632 in a control mouse. Numbers above the arrows indicate the concentration of the agonist applied with each bolus (log mol). Flow rate was adjusted before application of PE to compensate perfusion pressure reduction by the vasorelaxing compounds. (**B**) Parameters of the perfused hind limb of control mice with and without 1 µM isradipine and of SMAKO mice. Peripheral resistance is expressed as perfusion pressure divided by flow rate. (**C** and **D**) Vasocontraction response at constant flow to bolus (200 µl) applications of PE (log mol) in the absence or presence of 1 µM isradipine (Isra) and 5 µM Y-27632 in (C) control mice (Ctr) or (D) SMAKO mice. Vasocontraction response is shown as ΔPerfusion pressure [mmHg] (change in perfusion pressure) at constant flow.

1999), which makes it difficult to block L-type $Ca^{2+}$ channels in isolated cells or tissues at physiological membrane potentials. Furthermore, L-type $Ca^{2+}$ channel blockers have multiple unwanted side effects. For example, L-type $Ca^{2+}$ channel antagonists have been shown to induce release of EDHF (Fisslthaler *et al.*, 2000) from vascular endothelium and to attenuate production of the vasoconstricting endothelin-1 in endothelial cells (Yakubu and Leffler, 2002). All these mechanisms will interfere with both *in vivo* and *in vitro* experiments using L-type $Ca^{2+}$ channel blockers.

A concern with any tissue-specific knockout approach utilizing the Cre/lox technique is the selectivity and efficiency of the deletion. Based on western analyses, on the electrophysiological results and the effect of high concentrations of isradipine, we estimate the efficiency of

the vascular $Ca_v1.2$ deletion to be close to 100%. Although it is possible that a small subset of vascular SMCs still expressed $Ca_v1.2$ channels, the physiological analyses with SMAKO vessels and SMCs clearly show that $Ca_v1.2$ protein was functionally absent in SMAKO mice.

Intracellular $Ca^{2+}$ plays a pivotal role in regulating electromechanical coupling in muscle, including vascular smooth muscle. L-type $Ca^{2+}$ channels are activated in the membrane potential range found in microvascular smooth muscles. This makes them particularly suited to couple vessel membrane depolarization to contraction (Davis and Hill, 1999). Our results indeed support the concept that the $Ca_v1.2$ channel is required to couple pressure-induced membrane potential changes to the myogenic response, because the autoregulatory Bayliss effect and membrane potential-activated smooth muscle contractions were absent in SMAKO mice.

As the population of ion channels in SMCs is most probably heterogeneous, in various vascular beds the contribution of $Ca_v1.2$ channels to the regulation of myogenic tone could be different. For example, it has been shown that T-type $Ca^{2+}$ channels are involved in maintenance of myogenic tone in renal (Hansen *et al.*, 2001) and cremaster muscle arterioles (VanBavel *et al.*, 2002). However, the functional significance of these findings remains unclear as these studies primarily rely on the use of the T-type $Ca^{2+}$ channel blockers mibefradil and $Ni^{2+}$, which also affect L-type calcium channels (Lacinová *et al.*, 1995; Klugbauer *et al.*, 1999). Further studies with SMAKO mice might help to reveal the role of T-type $Ca^{2+}$ channels in smooth muscle.

In agreement with the loss of the autoregulation of myogenic tone, basal MAP was reduced by 33 mmHg in SMAKO mice. Why do SMAKO mice still have a functional, albeit attenuated blood pressure regulation? Arterial blood pressure is not only determined by autoregulatory myogenic mechanisms, but also by an interplay of vasoconstrictors (most importantly AT2 and norepinephrine) and vasodilators such as Prostacyclin, NO and EDHF. The vasoconstricting effect of PE and AT2 was reduced but not abolished. Apparently, these hormones use multiple pathways to induce smooth muscle contraction, including the calcium-dependent and -independent pathway. The mechanism leading to membrane depolarization and activation of L-type calcium channels in response to PE, AT2 or pressure increase is not clear. A number of possibilities have been suggested such as activation of a non-specific cation conductance or a $Ca^{2+}$-dependent chloride conductance. Further studies with $Ca_v1.2$-deficient mice will help clarify this mechanism. The hind limb perfusion experiments suggested that 50% of the PE-induced resistance is due to calcium influx through the $Ca_v1.2$ channel. Only 20% of the resistance was caused by the calcium-independent pathway involving Rho-kinase. Rho/Rho-kinase-mediated signaling has been suggested to play a key role in the regulation of force and velocity of actomyosin cross-bridge cycling in smooth muscle by inhibition of myosin phosphatase-mediated dephosphorylation of the regulatory light chain of myosin II (Somlyo *et al.*, 1999). Recent evidence suggests that the modulation of vascular smooth muscle $Ca^{2+}$ sensitivity by RhoA is a major determinant of vascular tone in resistance arteries and hence an important factor in the control of systemic blood pressure. Different vasoconstrictors have been shown to exert their effects at least in part through activation of the RhoA signaling cascade (Sakurada *et al.*, 2001; Sauzeau *et al.*, 2001; Bolz *et al.*, 2003). Furthermore, pharmacological inhibition of Rho kinase normalized blood pressure in three genetically different rat models of hypertension, while it only caused a slight fall in blood pressure in control rats (Uehata *et al.*, 1997). Residual vasoconstriction or blood pressure changes in response to G-protein-coupled receptor agonists in SMAKO mice were clearly dependent on Rho-kinase signaling, both *in vivo* and in the microcirculation. These experiments confirm the notion that, in addition to $Ca_v1.2$ $Ca^{2+}$ channel activation by membrane potential changes, the $Ca^{2+}$ channel-independent Rho-kinase pathway contributes to blood pressure regulation by G-protein-coupled vasoconstrictors.

Clearly, the SMAKO mouse represents a unique model, wherein the complex interplay of blood pressure-regulating molecules can be analyzed in detail.

## Materials and methods

All experiments were conducted in accordance with the 'Guide for the use and care of laboratory animals' and approved by the Regierung von Oberbayern.

### *Conditional inactivation of the $Ca_v1.2$ gene in smooth muscle cells*

As described previously (Seisenberger *et al.*, 2000), two different $Ca_v1.2$ alleles were generated by Cre-mediated recombination in embryonic stem cells (L1 and L2; Figure 1A). In L1, exons 14 and 15 which encode the IIS5 and IIS6 transmembrane segments and the pore loop in domain II were deleted. Additionally, this deletion causes an incorrect splicing from exon 13 to part of an intron upstream of exon 16, and thereby generates a premature stop codon in exon 16 and a loss of function allele. L2 contains the 'floxed' exons 14 and 15 and encodes a functional $Ca_v1.2$ gene. To generate SMAKO mice, the $Ca_v1.2^{L2/L2}$ mouse (i.e. a mouse homozygous for the L2-allele) was crossed with a mouse expressing a tamoxifen-inducible Cre recombinase under control of the SM22 promoter [SM-Cre $ER^{T2}(ki)$] (Kuhbandner *et al.*, 2000). The resulting $Ca_v1.2^{L2/L2}$, SM-Cre $ER^{T2}(ki)^{Cre/Cre}$ mice were then mated with $Ca_v1.2^{+/L1}$ mice (i.e. mice carrying one L1-allele and one wild-type allele) to obtain the smooth muscle-specific knockout $Ca_v1.2^{L1/L2}$, SM-Cre $ER^{T2}(ki)^{+/Cre}$ (i.e. SMAKO mice) and control animals [$Ca_v1.2^{+/L2}$, SM-Cre $ER^{T2}(ki)^{+/Cre}$; control]. The background mouse strain was C57BL/6. Both lines were viable and showed no gross abnormalities. To induce smooth muscle-specific Cre recombination (conversion of L2 to L1 allele *in vivo*), adult SMAKO and control mice were treated with freshly prepared tamoxifen solution (Sigma) by i.p. injection once a day for 5 days at a dosage of 1 mg a day. Tamoxifen was dissolved in miglyol oil (Caelo) at a concentration of 10 mg/ml.

### *RT–PCR on mRNA of mesenteric arteries*

Mesenteric sheets containing small branches of mesenteric arteries were isolated and cleaned of connective tissue. Poly(A) mRNA was isolated from the vessels using Dynabeads Oligo(dT)$_{25}$ (Dynal Biotech). The following buffers were used: GTC buffer [4 M guanidine thiocyanate, 20 mM Na-acetate pH 5.4, 0.1 mM dithiothreitol (DTT), 0.5% lauroyl sarcosinate (w/v), 6.5 µl/ml mercaptoethanol], binding buffer (100 mM Tris–HCl pH 8.0, 20 mM EDTA, 400 mM LiCl) and washing buffer (10 mM Tris–HCl pH 8.0, 0.15 M LiCl, 1 mM EDTA). The mRNA was eluted with diethylpyrocarbonate (DEPC)-treated water. Random hexamer primers and Superscript Reverse Transkriptase II (Life Technologies) were used for cDNA synthesis. The following primers were used: for PCR amplifying $Ca_v1.2$ (E13 5′-aCAGCC-AATAAAGCCCTCCT-3′ and Lef 1 5′-GGCTTCTCCATCACC-TCCTGTT-3′) and for HPRT (QG 197 5′-GTAATGATCAGT-CAACGGGGGGAC-3′ and QG 198 5′-CCAGCAAGCTTGCAA-CCTTAACCA-3′).

### Western blot analysis and lacZ staining

The tibialis ateries were pulverized under liquid nitrogen and boiled in 2% SDS/50 mM Tris for 10 min. The resulting homogenates (80 µg of protein) were separated by 7% SDS–PAGE, blotted on a PVDF membrane (Millipore) and probed with a $Ca_v1.2$-specific antibody (Chemicon). Equal loading of slots was ascertained by the use of a monoclonal β-actin antibody (Abcam). Antibodies were visualized by the ECL system (NEN). LacZ staining was done as described (Kuhbandner et al., 2000).

### Smooth muscle cell isolation

The vessel dissection and cell isolation procedures were slightly modified from the procedures described by Kleppisch et al. (1995). Briefly, control and SMAKO littermates were killed by cervical dislocation and arteries were cut into 2 mm segments and placed in $Ca^{2+}$-free isolation solution of the following composition: 60 mmol/l NaCl, 85 mmol/l sodium glutamate, 5.6 mmol/l KCl, 2 mmol/l $MgCl_2$, 10 mmol/l glucose, 10 mmol/l HEPES pH 7.4. After a 10 min equilibration (37°C), artery segments were placed in $Ca^{2+}$-free isolation solution (37°C) containing 1 mg/ml albumin, 0.7 mg/ml papain and 1 mg/ml DTT. After 40 min exposure to papain, artery segments were placed for 10–15 min in a second isolation solution containing 0.1 mmol/l $CaCl_2$ and a collagenase type F and hyaluronidase mixture (1 mg/ml each). The tissue was subsequently washed twice (10 min each) in $Ca^{2+}$-free isolation solution and triturated with a polished wide-bore pipet. Cells were stored on ice and used the same day.

### Electrophysiology

Whole-cell currents were recorded at room temperature using fire-polished electrodes with resistances of 2.7–3.5 MΩ. Pipettes were filled with 112 mM CsCl, 1 mM $MgSO_4$, 3 mM $Na_2$-aTP, 5 mM HEPES, 10 mM EGTA pH 7.4. Extracellular bath solution was 104 mM NaCl, 5.4 mM CsCl, 20 mM TEACl, 5 mM $BaCl_2$, 1 mM $MgCl_2$, 1 mM $NaH_2PO_4$, 10 mM glucose, 5 mM HEPES pH 7.4. The holding potential (HP) was 80 mV. To measure current–voltage (I–V) relationships for $I_{Ba}$ of L-type $Ca^{2+}$ channel, trains of test pulses were applied once every 5 s for 100 ms from –60 to 60 mV with 10 mV increments. Data were collected and stored on an EPC-9 computer under control of Pulse software (HEKA electronics). Total cell membrane capacitance was determined by compensation mechanisms of the EPC9 computer and used as a measurement of membrane area. Stock solutions were: Bay K 8644 10 mM in ethanol; nisoldipine 20 mM in ethanol. On the experimental day, stock solutions were freshly diluted to the indicated concentrations with the extracellular solution.

### Telemetric blood pressure recordings

Male SMAKO and control littermate mice (8–12 weeks old) were treated with tamoxifen solution (Sigma) by i.p. injection once a day for 5 days at a dosage of 1 mg a day. Animals were kept on a 12 h light/dark cycle. Blood pressure signals from the aortic arch were measured in conscious, unrestrained animals with surgically implanted, miniaturized telemetry devices (Datascience Corp.). For long-term measurements of MAP, mice were implanted with the transmitter, allowed to recover for 2 weeks and then treated with tamoxifen. Immediately after implantation of the transmitter, mice were returned to their home cages (placed on top of telemetry receivers), where they continued to be monitored daily throughout the study for general condition, body weight, food and water intake, state of surgical wound healing, and any signs of morbidity. MAP, heart rate and locomotor activity were recorded continuously until 28 days after tamoxifen injection. For short-term measurements of MAP, mice pre-treated with tamoxifen (21–28 days before) were implanted with the transmitter, allowed to recover from anesthesia and then directly measured when fully awake. Vasoactive compounds were applied i.p. as a bolus (200 µl) in phosphate-buffered saline (PBS). Sixty second MAP recordings were obtained every 90 s for 15 min before and until 120 min after drug administration. Recordings obtained after a stable drug effect was observed were used for statistical analysis.

### Measurement of arterial diameter

Tibialis arteries were quickly isolated, dissected free of connective tissue and transferred to a arteriograph (Danish Myosystems) filled with ice-cold physiological saline solution (PSS) containing: 119 mmol/l NaCl, 4.7 mmol/l KCl, 24.0 mmol/l $NaHCO_3$, 1.2 mmol/l $KH_2PO_4$, 1.6 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 0.023 mmol/l EDTA and 11.0 mmol/l glucose pH 7.4. The vessels were cannulated with glass pipets and secured with nylon thread. The arteriograph was then placed on the stage of an inverted microscope, and the artery visualized with a monochrome CCD camera coupled to a calibrated video system to measure arterial diameter. The arteries were slowly pressurized to 60 mmHg under no-flow conditions and warmed to 37°C while being continuously superfused (5 ml/min) with PSS bubbled with 95% $O_2$/5% $CO_2$ (pH 7.35–7.40 in the bath). After an equilibration period of ~20 min, arteries showed stable myogenic tone at 60 mmHg. Afterwards, the effect of different pharmacological agents on vessel diameter was evaluated. Obtaining the maximal dilated diameter in a calcium-free PSS concluded all experiments. Pressure–diameter curves were obtained by increasing the pressure in 10 mmHg steps from 10 to 100 mmHg. Pressure–diameter curves were repeated in the presence of calcium-free PSS.

### Organ bath experiments with aortic rings

Aortae were dissected, cut into segments (2–3 mm), and mounted on wire triangles. Segments were placed in organ chambers (Danish Myosystems), connected to force transducers, and gradually stretched to 1 g. Studies were performed in modified Krebs–Henseleit (KH) buffer (118.3 mmol/l NaCl, 4.7 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l $KH_2PO_4$, 25 mmol/l $NaHCO_3$, 0.026 mmol/l EDTA, 11.1 mmol/l glucose pH 7.40 aerated with 95% $O_2$/5% $CO_2$).

### Perfused hind limb

After sacrifice, the infrarenal aorta was prepared, and a catheter (1 mm, Hugo Sachs, Germany) was introduced, advanced to the iliac arteries and tied with a 6/0 prolene stitch (Ethicon). The inferior caval vein was slit open longitudinally to prevent venous congestion. A roller pump was used to perfuse the hind limb constantly with filtered KH solution. A pressure transducer and a compliance chamber were connected to a side port of the perfusion system. The flow rate was gradually increased to achieve a perfusion pressure of ~100 mmHg. At this perfusion pressure, a considerable amount of spontaneous myogenic tone is present, which made pre-constriction unnecessary. When a substance produced a pronounced change in resistance, flow rate was adjusted. When a stable pressure plateau was reached, PE was applied in increasing concentrations as a bolus (100 µl) in KH solution, in the presence or absence of inhibitors (isradipine, Y-27632). For inhibitor studies, agonist-induced responses were recorded in the absence and then in the presence of the inhibitor to facilitate paired comparison of the results. Vasodilator responses were measured as changes in perfusion pressure.

### Data analysis

Data are given as mean values $\pm$ SEM. Statistical significances were evaluated by either ANOVA followed by Dunnet's ad hoc tests for unpaired comparisons, or by paired Students' t-test when comparing data obtained in the same cell.

## Acknowledgements

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