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Role of T-type calcium channels in myogenic tone of skeletal muscle resistance arteries

ED VANBAVEL,[1] OANA SOROP,[1] DITTE ANDREASEN,[3]
MARTIN PFAFFENDORF,[2] AND BOYE L. JENSEN[3]
[1]*Departments of Medical Physics and* [2]*Pharmacotherapy, Academic Medical Center, University of Amsterdam, 1100 DE Amsterdam, The Netherlands; and* [3]*Department of Physiology and Pharmacology, University of Southern Denmark-Odense University, DK-5000 Odense, Denmark*

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VanBavel, Ed, Oana Sorop, Ditte Andreasen, Martin Pfaffendorf, and Boye L. Jensen. Role of T-type calcium channels in myogenic tone of skeletal muscle resistance arteries. *Am J Physiol Heart Circ Physiol* 283: H2239–H2243, 2002. First published September 5, 2002; 10.1152/ajpheart.00531.2002.—T-type calcium channels may be involved in the maintenance of myogenic tone. We tested their role in isolated rat cremaster arterioles obtained after CO_2 anesthesia and decapitation. Total RNA was analyzed by RT-PCR and Southern blotting for calcium channel expression. We observed expression of voltage-operated calcium (Ca_V) channels $Ca_V3.1$ (T-type), $Ca_V3.2$ (T-type), and $Ca_V1.2$ (L-type) in cremaster arterioles (n = 3 rats). Amplification products were observed only in the presence of reverse transcriptase and cDNA. Concentration-response curves of the relatively specific L-type blocker verapamil and the relatively specific T-type blockers mibefradil and nickel were made on cannulated vessels with either myogenic tone (75 mmHg) or a similar level of constriction induced by 30 mM K^+ at 35 mmHg. Mibefradil and nickel were, respectively, 162-fold and 300-fold more potent in inhibiting myogenic tone compared with K^+-induced constriction [log(IC_{50}, M): mibefradil, basal -7.3 ± 0.2 (n = 9) and K^+ -5.1 ± 0.1 (n = 5); nickel, basal -4.1 ± 0.2 (n = 5) and K^+ -1.6 ± 0.5 (n = 5); means ± SE]. Verapamil had a 17-fold more potent effect [log(IC_{50}, M): basal -6.6 ± 0.1 (n = 5); K^+ -5.4 ± 0.3 (n = 4); all log(IC_{50}) $P < 0.05$, basal vs. K^+]. These data suggest that T-type calcium channels are expressed and involved in maintenance of myogenic tone in rat cremaster muscle arterioles.

arteriole; $Ca_V3.1$; $Ca_V3.2$; mibefradil; nickel

IN RESPONSE TO PRESSURE, arterioles and small resistance arteries generally develop vasoconstriction in the absence of any extrinsic agonists. This myogenic tone becomes more dominant in smaller vessels (4) and is thought to play a key role in the local control of tissue perfusion. The mechanisms responsible for myogenic tone have been the subject of ongoing studies. It is now well established that myogenic tone is independent of the endothelium in most vascular beds examined (17), even though endothelial products are the primary modulators of myogenic tone. This tone is associated with a rather modest but necessary smooth muscle cell (SMC) depolarization (28) and rise of intracellular calcium (25, 31) in combination with a pressure-induced increase in calcium sensitivity of the contractile filaments (25).

Pharmacological evidence indicates that L-type voltage-operated calcium (Ca_V) channels are significantly involved in pressure-induced calcium influx and tone in several resistance vessels (5, 27). However, steady-state open probability of these channels is quite low at membrane potentials frequently found under basal conditions [−60 to −40 mV at physiological pressures (5, 24)]. The activation range of the smooth muscle L-type channel $Ca_V1.2$ may be shifted by regulation (11), and only a small calcium influx might be needed to maintain an elevated intracellular concentration (19). Alternatively, low-voltage-activated T-type calcium channels may be involved in the calcium influx associated with myogenic tone. These channels activate at more negative potentials and may only partially inactivate at membrane potentials associated with myogenic tone. Two recent studies have indeed shown that mibefradil, a T-type channel antagonist, inhibits myogenic tone in rat cerebral and cremaster vessels at concentrations (IC_{50} 70 and 220 nM) that have been claimed to be specific for T-type over L-type calcium channels (15, 22).

Expression of recently cloned calcium channels in heterologous cell systems has established the existence of three pore-forming channel subunits with T-type current characteristics (activation at low voltages and rapid inactivation, sensitivity to mibefradil and nickel): $Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$ (previously known as α_{1G}, α_{1H}, and α_{1I}) (6, 20). mRNA for $Ca_V3.1$ and $Ca_V3.2$ and functional involvement in contraction have now been demonstrated in renal afferent and juxtamedullary efferent arterioles (10). mRNA for T-type calcium channels was also found in rat mesenteric arterioles of ~30

Address for reprint requests and other correspondence: E. VanBavel, Dept. of Medical Physics, Academic Medical Center, Univ. of Amsterdam, PO Box 22700, 1100 DE Amsterdam, The Netherlands (E-mail: e.vanbavel@amc.uva.nl).

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μm in diameter (8), whereas the L-type channel $Ca_V1.2$ was absent, and these arterioles also did not constrict to high potassium.

The above information supports a widespread distribution of T-type channel expression in vascular resistance segments and a role for T-type calcium channels in arteriolar function. The purpose of this paper was to investigate T-type channel expression in skeletal muscle resistance vessels and to further explore whether T-type channels are involved in the maintenance of myogenic tone. To address these issues, rat cremaster muscle arterioles were cannulated in vitro, and the sensitivity of myogenic tone to pharmacological antagonists of L- and T-type channels was elucidated. Sensitivity to antagonists was compared in two situations with different membrane potentials: at physiological perfusion pressure and at a low perfusion pressure where the same level of tone was achieved by the addition of potassium to depolarize the membrane potential.

METHODS

Isolation of resistance arteries. Male Wistar rats weighing 250–300 g were briefly anesthetized by CO_2 gas and decapitated. The left and right cremaster muscles were carefully isolated, suspended in buffer [containing (in mmol/l) 130 NaCl, 5 KCl, 2 $CaCl_2$, 10 D-glucose, 20 sucrose, and 10 HEPES; pH 7.4] and mounted in dissection chambers for isolation of vessels at 4°C. For molecular biology, first- and second-order vessel segments with a total length of ~1 cm were isolated from each cremaster. For pharmacology on cannulated vessels, unbranched segments of first-order arterioles were isolated of ~3 mm in length.

RNA isolation, RT-PCR, and Southern blotting. First- and second-order arterioles were cut in ~400-μm pieces and directly added to guanidinium-thiocyanate (4 mol/l) as previously described (1, 2). For homogenization, the fragments were repeatedly triturated through syringes with decreasing dimensions ending with 25 gauge. Yeast tRNA (12 μg) was added as a carrier. Total RNA was extracted by phenol-chloroform extraction, precipitated with isopropanol, and repeatedly washed with 70% ethanol (2). RNA pellets were suspended in diethyl pyrocarbonate-treated water and used for cDNA synthesis as previously described in detail (1). RNA corresponding to 1 mm of vessel length was used for cDNA synthesis.

PCRs were performed with 18-mer DNA oligonucleotides (Invitrogen) for rat T- and L-type Ca_V channels and β-actin as previously described (10). PCR was performed for 32 cycles (Mastercycler, Eppendorf). For a negative control, reverse transcription of total RNA was performed in the absence of reverse transcriptase and then amplified by PCR, and, in separate tubes, water was added instead of cDNA in PCR. For southern blotting, PCR products were separated by agarose gel electrophoresis and blotted to Zeta Probe GT membranes (Bio-Rad) using standard capillary blotting procedures as previously described (1). Hybridization was allowed overnight to a specific probe in vitro labeled with [α-^{32}P]-dCTP; all procedures were according to Sambrook et al. (23). Autoradiography was performed for 2–4 h on Kodak Biomax MS film.

Pharmacological studies. Isolated cremaster muscle arterioles were cannulated on two glass micropipettes using previously reported techniques (25). Vessels were pressurized to 75 mmHg at 34°C, the in vivo temperature of the cremaster muscle, and allowed to develop myogenic tone. Cumulative concentration-response curves were then made for the effect of one of three calcium channel blockers on tone: verapamil, an L-type calcium channel blocker, the analog mibefradil, which is relatively selective for T-type channels, and nickel, which also relatively selectively blocks T-type channels. Only one blocker was used for each vessel. In separate experiments, the effect of these blockers was tested on vessels kept at 35 mmHg, resulting in a lack of myogenic tone, after the induction of preconstriction by 30 mM K^+. On the basis of the potassium equilibrium potential, this concentration was estimated to result in a depolarization to at least −35 mV, where T-type but not L-type channels are inactivated.

Data analysis and statistics. IC_{50} values were determined for each individual concentration-response curve using nonlinear curve fitting with variable Hill slope (GraphPad) and subsequently averaged over the group. Unpaired *t*-tests were used for the comparison of the log(IC_{50}) values on myogenic tone and potassium-induced constriction. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of α_1-units of calcium channels. RNA was extracted from cremaster muscle arterioles of three rats and analyzed along with RNA obtained from an aortic cell line (A7r5) and the rat cerebral cortex, which

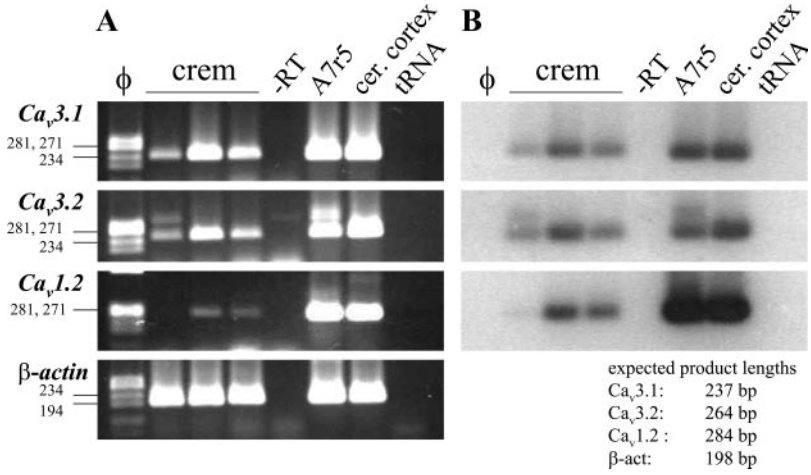

Fig. 1. *A*: RT-PCR amplification products for the T-type voltage-operated calcium (Ca_V) channels $Ca_V3.1$ and $Ca_V3.2$, the L-type channel $Ca_V1.2$, and β-actin (β-Act). *B*: results of Southern blotting of these products. Lanes from *left* to *right*: size marker (ϕX174 DNA/*Hae*III markers), cDNA amplified from cremaster muscle arterioles from 3 separate rats (Crem), negative control [absence of reverse transcriptase (−RT)], cDNA amplified from an aortic smooth muscle cell line (A7r5), positive control where cDNA from the rat cerebral cortex was amplified (Cer cortex), and negative control where carrier tRNA was amplified (tRNA).

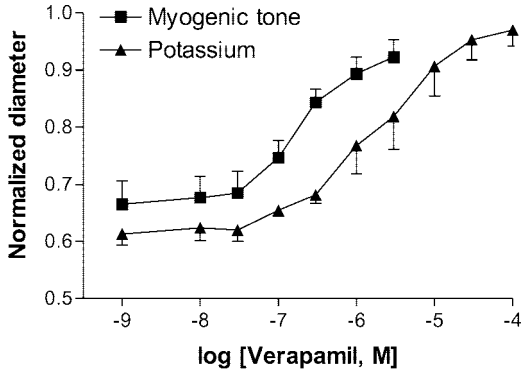

Fig. 2. Inhibition of myogenic tone at 75 mmHg and potassium-induced constriction (30 mM at 35 mmHg) by verapamil. Error bars are SE.

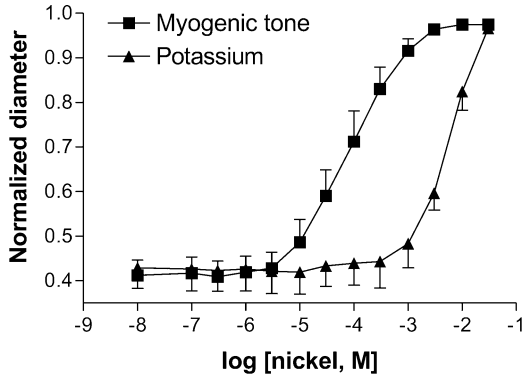

Fig. 4. Inhibition of myogenic tone at 75 mmHg and potassium-induced constriction (30 mM at 35 mmHg) by nickel.

served as a positive control. As can be seen in Fig. 1*A*, amplification products for T-type channels $Ca_V3.1$ and $Ca_V3.2$ were detected in all three cremaster vessel preparations by RT-PCR amplification for 32 cycles. Southern blotting and hybridization further corroborated the identity of the amplification products as being $Ca_V3.1$ and $Ca_V3.2$ cDNAs (Fig. 1*B*). In contrast, L-type channel $Ca_V1.2$ mRNA was not found in one of the cremaster vessels and was at the limit of detection by ethidium bromide staining in both other preparations. However, the increased sensitivity provided by the Southern blot assured expression and identity of $Ca_V1.2$ in these preparations (Fig. 1*B*). Expression of all three subunit mRNAs was also detected in the rat aortic SMC line A7r5 and in RNA samples isolated from the rat cerebral cortex using 50 ng total RNA as template for RT-PCR. Amplification products were only obtained in the presence of reverse transcriptase and cDNA in the RT-PCR, confirming the mRNA origin of the amplification products. Reverse transcription of carrier yeast tRNA followed by PCR for each subunit did also not result in detectable amplification products (Fig. 1, tRNA lane).

Pharmacological studies. Nineteen vessels were studied in the myogenic tone group, having an average passive inner diameter of 145 ± 3 μm at 75 mmHg. When maintained at this pressure, the vessels developed myogenic tone, resulting in a reduction of the normalized inner diameter to 0.57 ± 0.03 (mean ± SE, n = 19). Myogenic tone was sensitive to verapamil, mibefradil, and nickel; concentration-response curves are indicated in Figs. 2–4. Log(IC_{50}) values are indicated in Table 1. Fourteen slightly smaller vessels (passive inner diameter at 75 mmHg: 134 ± 3 μm, $P < 0.05$) were kept at 35 mmHg. The vessels remained passive at this diameter, probably due to myogenic inhibition of tone. A stable level of preconstriction was induced by 30 mM K^+, resulting in a reduction of the normalized diameter to the same level as in the previous series: 0.54 ± 0.03 (P = not significant, basal versus K^+). All three calcium channel blockers were able to inhibit the potassium-induced tone, as indicated by the concentration-response curves in Figs. 2–4 and the IC_{50} values in Table 1. A comparison of the inhibition of basal versus potassium-induced tone shows marked differences: both mibefradil and nickel were very potent inhibitors of basal but not of potassium-induced tone. Thus a 162-fold difference in IC_{50} was found for mibefradil, whereas the IC_{50} for nickel was 300-fold lower on myogenic tone compared with potassium-induced constriction. Verapamil, an L-type calcium channel blocker, 17-fold more potently blocked myogenic tone compared with potassium-induced tone (see Table 1).

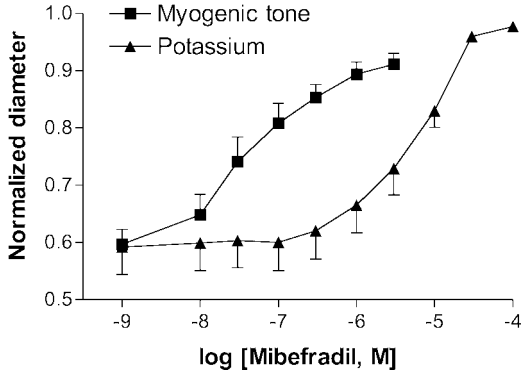

Fig. 3. Inhibition of myogenic tone at 75 mmHg and potassium-induced constriction (30 mM at 35 mmHg) by mibefradil.

DISCUSSION

This study shows that T-type calcium channel subunits $Ca_V3.1$ and $Ca_V3.2$ are expressed in rat cremaster muscle arterioles and that mibefradil and nickel were far more potent inhibitors of myogenic tone com-

Table 1. *Effect of calcium channel blockers on myogenic tone and K^+-induced constriction*

Calcium Channel Blocker	Log(IC_{50}, M) Myogenic tone (75 mmHg)	Log(IC_{50}, M) K^+ (30 mM) constriction (35 mmHg)	Significance
Verapamil	−6.63 ± 0.16 (n=5)	−5.40 ± 0.33 (n=4)	<0.01
Mibefradil	−7.31 ± 0.19 (n=9)	−5.10 ± 0.11 (n=5)	<0.00001
Nickel	−4.08 ± 0.18 (n=5)	−1.60 ± 0.49 (n=5)	<0.001

Results are means ± SE; n = no. of rats. Values are unitless log (IC_{50}), where IC_{50} is expressed in molars. Significance was measured by unpaired t-tests.

pared with potassium-induced tone. While we appreciate that both blockers may also affect L-type calcium channels, the two decades higher potency on myogenic tone compared with potassium-induced constriction suggests specific actions in the current study. Together, these data point at a role for T-type calcium channels in the development and maintenance of myogenic tone.

Care should be taken in relating the expression of $Ca_V3.1$ and $Ca_V3.2$ at the messenger level to their possible role in cell function, because the applied RT-PCR was not quantitative and because messenger and protein levels may well diverge. Also, the RT-PCR was performed on the whole vascular wall. While there is no evidence for expression of voltage-dependent calcium channels in endothelial cells, and while it seems highly unlikely that the perivascular nerve endings contain any mRNA, we cannot fully exclude the possibility that part of the messenger signal we found for the T-type channels originated from other cells than SMCs. Immunohistochemical evidence could further substantiate expression and localization of these channels, but this awaits the further development of antibodies. We attempted to obtain electrophysiological evidence for these channels in SMCs isolated from cremaster arterioles. However, in our hands, the digestion protocols, which included papain, resulted in cells that had neither L- nor T-type currents and also did not contract to high potassium concentrations. The single study we are aware of that did successfully measure calcium currents in these specific cells (29) was not aimed at discriminating between both channels. In that study, using holding potentials of −80 mV, no clear calcium or barium currents were observed below −50 and −20 mV, respectively, arguing against functional involvement of T-type channels under those conditions. Further patch-clamp experiments will be required here. However, T-type calcium currents have been demonstrated in vascular smooth muscle cells of other sources (7, 21).

In our study, nickel blocked myogenic tone with a threshold concentration of ~10 μM and an IC_{50} of 0.1 mM. These values are very similar to the threshold and IC_{50} reported for inhibition of peak current in expressed $Ca_V3.1$ channels, whereas $Ca_V3.2$ channels had a higher sensitivity for nickel (16). Nickel also blocks expressed $Ca_V1.2$ currents, but at a much higher concentration [<100 μM (30)]. Thus our data indicate that specifically $Ca_V3.1$ could be involved in the maintenance of myogenic tone, in accordance with the observed expression of this channel. The role of $Ca_V3.2$, which we also found to be expressed, remains to be elucidated.

Mibefradil has been put forward (3, 18) and challenged (22) as a relatively selective T-type calcium channel blocker. Mibefradil inhibited Ba^{2+} current through expressed $Ca_V3.1$ at a holding potential of −60 mV with an IC_{50} at 0.12 μM (12), whereas the effects using calcium as the charge carrier were similar (14). Two recent studies addressed the effects of mibefradil on myogenic tone. Lam et al. (15) found mibefradil to inhibit myogenic tone at 60 mmHg with an IC_{50} of 70 nM in rat middle cerebral arteries. Potocnik et al. (22) studied the effects of mibefradil on rat cremaster arterioles and observed inhibition of myogenic tone at 70 mmHg with an IC_{50} of 0.22 μM. The current study on the same vessel type and perfusion at 75 mmHg found a similar IC_{50} for mibefradil at 0.17 μM. Thus these studies find effects of mibefradil on myogenic tone that are, based on the electrophysiology of the cloned channel, consistent with an inhibition of $Ca_V3.1$. However, Potocnik et al. (22) argued that the effects of mibefradil on myogenic tone may not be carried through T-type calcium channels. In that study (22), mibefradil up to 10 μM did not lower the intracellular calcium concentration and failed to prevent the rise in calcium upon pressure steps and application of high potassium, even though the contractile response was inhibited. The authors suggested that mibefradil could have intracellular effects on calcium sensitivity rather than blockade of T-type calcium channels.

The clear difference in effects of mibefradil and nickel on pressure-induced versus potassium-induced tone could relate to the membrane potential. Alternatively, pressurization might directly affect the opening of the T-type calcium channels. Also, we cannot fully rule out effects of these blockers on the nonselective cation channels recently shown to be involved in pressure-induced depolarization (26). An inhibitory effect of both blockers on nonselective cation conductance has recently been demonstrated on visceral SMCs (13). Whether the difference in potency of verapamil on myogenic versus potassium-induced tone reflects direct modulation of the L-type calcium channels or other effects of this blocker remains to be established.

Whereas in our study the low potency of nickel and mibefradil on K^+-induced constriction is in accordance with the involvement of L-type calcium channels, other studies do indicate a higher sensitivity of depolarization-induced arteriolar constriction to mibefradil and/or nickel (10, 22) also in the cremaster arterioles that we studied (22). The protocols, however, were different: we first established a stable potassium-induced contraction and then performed a cumulative concentration-response series of the blockers, whereas others tested the effect of a sudden switch to high potassium in the presence of varying concentrations of the blockers. Comparison of the above studies suggests that potassium-induced contraction could be initiated by calcium influx through T-type channels, whereas its maintenance requires L-type channels. Maintenance of myogenic tone, however, was shown here to depend on T-type calcium channels.

Gustafsson et al. (8) studied the contribution of L- and T-type calcium channels in local and conducted vasoconstriction of rat mesenteric arterioles of ~30 μm in diameter. These authors found the constriction to topical norepinephrine and current stimulation to be fully insensitive to the L-type channel blockers nifedipine and nimodipine. Also, the L-type channel $Ca_V1.2$ was not expressed, and the arterioles did not contract to high concentrations of potassium. In contrast,

$Ca_V3.1$ and $Ca_V3.2$ were expressed, and nickel and mibefradil suppressed the local and conducted constrictions to norepinephrine and current injection. Possible side effects of the blockers on L-type currents can be excluded here because those channels were not expressed. Hansen et al. (9, 10) found expression of $Ca_V1.2$, $Ca_V3.1$, $Ca_V3.2$, and the P-/Q-type $Ca_V2.1$ in rat afferent arterioles. Each of the calcium channel blockers calciseptine (specific for L-type channels), ω-agatoxin IVA (P-/Q-type blocker), mibefradil, and nickel fully inhibited contraction of cannulated rabbit afferent arterioles to 100 mM K^+ at concentrations believed to reflect specific actions. Interestingly, T- and L-type calcium channels were not found in cortical efferent arterioles. Those studies point at a highly regulated differential distribution of Ca_V channels along the renal vasculature and at the significance of T-type calcium channels. The current study and those of Hansen et al. (9, 10) suggest that a cooperative action of multiple types of calcium channels is required for the maintenance of constriction. It is not clear how this correlates with a simple parallel arrangement of the channels in the cell membrane. Intracellular calcium gradients or heterogeneity within the SMC population could provide explanations.

In conclusion, T-type calcium channels were expressed at the messenger level in rat cremaster arterioles. Pharmacological evidence supports their contribution to myogenic tone.

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