REVIEW / SYNTHÈSE

Is there a role for T-type Ca^{2+} channels in regulation of vasomotor tone in mesenteric arterioles?[1]

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Abstract: The largest peripheral blood pressure drop occurs in terminal arterioles (<40 μm lumen diameter). L-type voltage-dependent Ca^{2+} channels (VDCCs) are considered the primary pathway for Ca^{2+} influx during physiologic activation of vascular smooth muscle cells (VSMC). Recent evidence suggests that T-type VDCCs are expressed in renal afferent and efferent arterioles, mesenteric arterioles, and skeletal muscle arterioles. T-type channels are small-conductance, low voltage-activated, fast-inactivating channels. Thus, their role in supplying Ca^{2+} for contraction of VSMC has been disputed. However, T-type channels display non-inactivating window currents, which may play a role in sustained Ca^{2+} entry. Here, we review the possible role of T-type channels in vasomotor tone regulation in rat mesenteric terminal arterioles. The $Ca_V3.1$ channel was immunolocalized in VSMC, whereas the $Ca_V3.2$ channel was predominantly expressed in endothelial cells. Voltage-dependent Ca^{2+} entry was inhibited by the new specific T-type blockers *R*(–)-efonidipine and NNC 55-0396. The effect of NNC 55-0396 persisted in depolarized arterioles, suggesting an unusually high activation threshold of mesenteric T-type channels. T-type channels were not necessary for conduction of vasoconstriction, but appear to be important for local electromechanical coupling in VSMC. The first direct demonstration of endothelial T-type channels warrants new investigations of their role in vascular biology.

Key words: T-type channel, voltage-dependent Ca^{2+} channel, microcirculation, vascular smooth muscle, endothelium, pharmacology, conducted vasomotor response.

Résumé : La plus importante baisse de pression artérielle périphérique se produit dans les artérioles terminales (<40 μm). Les canaux Ca^{2+} sensibles au voltage (CCSV) de type L sont considérés comme la principale voie d'entrée des ions Ca^{2+} durant l'activation physiologique des cellules musculaires lisses vasculaires (CMLV). De récents travaux donnent à penser que des CCSV de type T sont exprimés dans les artérioles afférentes et efférentes rénales, les artérioles mésentériques et les artérioles musculaires squelettiques. Les canaux de type T sont des canaux de faible conductance, à faible seuil d'activation, rapidement inactivés. Ainsi, leur rôle dans l'apport de calcium déclenchant la contraction de la CMLV est contesté. Toutefois, les canaux de type T engendrent des courants de fenêtre non inactivant qui pourraient jouer un rôle dans l'entrée soutenue du Ca^{2+}. Ici, nous examinons le rôle possible des canaux de type T dans la régulation du tonus vasomoteur dans les artérioles terminales mésentériques du rat. Le canal $Ca_V3,1$ a été immunolocalisé dans les CMLV, tandis que le canal $Ca_V3,2$ a surtout été exprimé dans les cellules endothéliales (CE). L'entrée du Ca^{2+} sensible au voltage a été inhibée par les nouveaux bloqueurs spécifiques de type T, *R*(–)-éfonidipine et NNC 55-0396. L'effet de NNC 55-0396 a persisté dans les artérioles dépolarisées, ce qui laisse croire à un haut seuil d'activation inhabituel des canaux de type T mésentériques. Les canaux de type T n'ont pas été nécessaires pour la conduction de la vasoconstriction; toutefois, ils semblent jouer un rôle important dans le couplage électromécanique local dans les CMLV. La première démonstration directe des canaux de type T endothéliaux justifie de nouvelles études de leur rôle en biologie vasculaire.

Mots-clés : canal de type T, canal Ca^{2+} sensible au voltage, microcirculation, muscle lisse vasculaire, endothélium, pharmacologie, conduction de la réponse vasomotrice.

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Introduction

By coupling excitation with contraction, voltage-dependent Ca^{2+} entry into the cell is a key process in acute microvascular tone regulation. Vascular L-type channels encoded by the $Ca_V1.2$ gene (*CACNA1C*) are activated at potentials positive to about –40 mV (McDonald et al. 1994; Morel et al. 1998; Navedo et al. 2007; Smirnov and Aaronson 1992) and are characterized by their large and long-lasting (hence 'L-type') inward Ca^{2+} currents (Catterall 2000; McDonald et al. 1994). The resting membrane potential in vascular smooth muscle cells (VSMC) in situ is –45 to –35 mV (Knot and Nelson 1998; Loutzenhiser et al. 1997; Stekiel et al. 1986; Welsh and Segal 1998), and the biophysical profile of L-type channels makes them well adapted to a role in regulation of arteriolar tone (Nelson et al. 1990; Smirnov and Aaronson 1992). Most recently, however, expression and functional roles of non-L-type voltage-dependent Ca^{2+} channels (VDCCs) (Table 1) have been reported for arterioles in several different mammalian organs (Feng et al. 2004; Gustafsson et al. 2001; Hansen et al. 2000, 2001; Morita et al. 1999; VanBavel et al. 2002). In the present paper we review the evidence in favor of a role for T-type VDCCs in regulation of arteriolar tone. Functional and molecular biology data suggest that T-type channels are important for excitation–contraction coupling in rat mesenteric terminal arterioles.

Expression and suggested function of T-type channels in arterioles

Expression of T-type channel $Ca_V3.1$ and $Ca_V3.2$ α_1-subunits (Table 1) has been detected by RT-PCR in rat renal arterioles (Hansen et al. 2001; Salomonsson et al. 2002), rat mesenteric terminal arterioles (Braunstein et al. 2009; Gustafsson et al. 2001; Jensen et al. 2004), and rat cremaster small arteries (VanBavel et al. 2002). Recently T-type channel protein was localized by immunocytochemistry in rat afferent (Brueggemann et al. 2005) and mesenteric terminal (Braunstein et al. 2009) arterioles. The functional role of T-type channels in resistance arterioles has thus far been investigated mainly by using the organic VDCC inhibitor mibefradil, which is considered selective for T-type channels at concentrations up to approximately 100 nmol/L (Table 2). At this concentration, mibefradil blocked approximately 50% of the voltage-dependent Ca^{2+} influx and contraction in rabbit afferent and rat juxtamedullary efferent arterioles (Hansen et al. 2001) and approximately 30% of the voltage-dependent Ca^{2+} influx in rat mesenteric terminal arterioles (Jensen et al. 2004). In rat mesenteric terminal arterioles supplying the transparent part of the mesentery, none of the high voltage-activated (HVA) L-, P/Q-, N-, or R-type Ca^{2+} channels were expressed (Gustafsson et al. 2001; Jensen et al. 2004), and a high concentration of mibefradil (10 μmol/L) abolished local and conducted vasoconstriction to local microapplication of current or norepinephrine, suggesting that T-type channels were the primary target (Gustafsson et al. 2001). However, despite mRNA expression of $Ca_V3.1$ and $Ca_V3.2$ in rat interlobular arteries, mibefradil (1 μmol/L) did not block voltage-dependent Ca^{2+} influx, suggesting a predominant role of L-type channels in these resistance vessels (Salomonsson et al. 2002).

Table 1. Nomenclature for pore-forming subunits of voltage-dependent Ca^{2+} channels.

Activation threshold	Channel	Alias	Current (type)
HVA	$Ca_V1.1$	α_{1S}	L
	$Ca_V1.2$	α_{1C}	L
	$Ca_V1.3$	α_{1D}	L
	$Ca_V1.4$	α_{1F}	L
	$Ca_V2.1$	α_{1A}	P/Q
	$Ca_V2.2$	α_{1B}	N
	$Ca_V2.3$	α_{1E}	R
LVA	$Ca_V3.1$	α_{1G}	T
	$Ca_V3.2$	α_{1H}	T
	$Ca_V3.3$	α_{1I}	T

Note: HVA, high voltage-activated; LVA, low voltage-activated. Nomenclature is according to the IUPHAR compendium of voltage-gated ion channels (Catterall et al. 2003).

Caution must be exercised when interpreting data obtained with mibefradil at concentrations above 100 nmol/L. For example, in rat cremaster resistance arteries, mibefradil blocked myogenic tone and depolarization-induced vasoconstriction without inhibiting the associated intracellular $[Ca^{2+}]$ increase, suggesting that these effects were not due to T-type or L-type channel blockage (Potocnik et al. 2000). The most conclusive evidence for the nonspecific action of mibefradil comes from work utilizing a smooth muscle-targeted, conditional knockout mouse model of the $Ca_V1.2$ L-type channel to show that the antihypertensive action of mibefradil was entirely due to an effect on L-type channels (Moosmang et al. 2006). Thus, the general role of T-type channels for vasomotor tone regulation is controversial, mainly because of the possibly unspecific actions of mibefradil (Cribbs 2006). Nevertheless, there is considerable independent evidence that T-type channels do play a role in regulating renal efferent arteriolar tone (Feng et al. 2004; Hansen et al. 2001; Hayashi et al. 2007) and mesenteric arteriolar tone (Braunstein et al. 2009; Gustafsson et al. 2001; Jensen et al. 2004). The role of T-type channels in efferent arteriolar tone has recently been reviewed elsewhere (Hayashi et al. 2007), and in this review the emphasis will be on evidence from the mesenteric microcirculation.

How does the biophysical profile of T-type channels match a role in vascular smooth muscle contraction?

Three different T-type channels have been cloned, of which $Ca_V3.1$ and $Ca_V3.2$ are commonly expressed in peripheral tissues, such as the cardiovascular system, and $Ca_V3.3$ is primarily expressed in the brain. Recombinant T-type channels are characterized by activation at relatively hyperpolarized potentials (threshold voltage (V_{th}) –70 mV; *I–V* peak –30 mV), fast inactivation (τ 11–16 ms at –10 mV for $Ca_V3.1$ and $Ca_V3.2$), and small single-channel currents (7–11 pS) (Perez-Reyes 2003). Inactivation of recombinant T-type channels is highly voltage-dependent, with half-maximal inactivation (h_∞) at –72 mV (Perez-Reyes 2003). For the above reasons, T-type channels are often referred to as low voltage-activated (LVA) channels with tiny and transient (hence 'T-type') currents. Considering the most

Table 2. Comparison of VDCC inhibitor efficacy (IC_{50}) on macroscopic calcium currents in mammalian cells (thus excluding frog oocytes and insect cells) at room temperature.

VDCC inhibitor	Channel or current	IC_{50}, μmol/L	V_H, mV (bath solution mmol/L Ba^{2+} or Ca^{2+})	Cell type	Reference
Nimodipine	L-type (HVA)	0.006	–90 (5 Ca^{2+})	mHL-1	Xia et al. 2004
	T-type (LVA)	7.3	–90 (5 Ca^{2+})	mHL-1	Xia et al. 2004
	NICC	~10[a]	–80 (10 Ba^{2+})	rMA-SMC	Morita et al. 2002
Nifedipine	$Ca_V1.2$ ($\alpha_{1C\text{-}b}$)	0.01	–100 (10 Ba^{2+})	CHO	Morel et al. 1998
	$Ca_V3.2$	1.2	–100 (1.8 Ca^{2+})	HEK293	Lee et al. 2006
	T-type (LVA)	3	–100 (20 Ca^{2+})	rAO-SMC	Akaike et al. 1989
Mibefradil	$Ca_V1.2$	3	–60 (2 Ca^{2+})	HEK	Jimenez et al. 2000
	$Ca_V3.1$	0.12–0.14	–60 (10–20 Ba^{2+})	HEK293	Morita et al. 2002; Klugbauer et al. 1999
	$Ca_V3.2$	0.069	–80 (2 Ca^{2+})	HEK293	Martin et al. 2000
	NICC	0.3	–60 (10 Ba^{2+})	rMA-SMC	Morita et al. 2002;
NNC 55-0396	L-type (HVA)	>>100[b]	–40 (10 Ca^{2+})	INS-1	Huang et al. 2004
	$Ca_V3.1$	6.8	–70 (10 Ca^{2+})	HEK293	Huang et al. 2004
R(–)-efonidipine	$Ca_V1.2$	1000	–60 (5 Ba^{2+})	BHK	Furukawa et al. 2004
	$Ca_V3.1$	0.1	–60 (5 Ba^{2+})	BHK	Furukawa et al. 2004
Pimozide	$Ca_V1.2$	0.1	–60 (20 Ba^{2+})	PC12	Ito et al. 1996
	$Ca_V3.1$	0.035	–100 (2 Ca^{2+})	HEK	Santi et al. 2002
	$Ca_V3.2$	0.054	–100 (2 Ba^{2+})	HEK	Santi et al. 2002
Nickel	L-type	324	–50 (1.8 Ba^{2+})	rbU-SMC	Bradley et al. 2004
	$Ca_V3.1$	250	–90 (10 Ba^{2+})	HEK293	Lee et al. 1999
	$Ca_V3.2$	12	–90 (10 Ba^{2+})	HEK293	Lee et al. 1999
	NICC	68	–60 (5 Ba^{2+})	gpMA-SMC	Morita et al. 1999

Note: VDCC, voltage-dependent Ca^{2+} channels; V_H, holding potential; HVA, high voltage-activated; LVA, low voltage-activated; NICC, nifedipine-insensitive HVA T-type-like Ca^{2+} channels; $Ca_V1.2$ ($\alpha_{1C\text{-}b}$), vascular isoform of $Ca_V1.2$; mHL, mouse HL-60-differentiated monocytes; rMA or gpMA, rat or guinea pig mesenteric artery; SMC, smooth muscle cells (freshly dispersed); CHO, chinese hamster ovary; HEK, human embryonic kidney; rAO, rat aortic (in primary culture); INS, insulinoma; BHK, baby hamster kidney; PC, pheochromocytoma; rbU, rabbit urethra.

[a]10 μmol/L nimodipine blocked 40% of the current.

[b]100 μmol/L NNC 55-0396 had no detectable effect on L-type current in an insulin-secreting cell line.

prominent features of recombinant T-type channels are activation and inactivation at hyperpolarized potentials coupled with fast inactivation, how does this profile match a role in sustained Ca^{2+} entry in VSMC having in vivo resting membrane potentials in the range from –45 to –35 mV? In the following review, we explore the possibility that a small, non-inactivating T-type window current in the physiologic range of membrane potentials is the answer to that question.

Window currents are defined by the narrow range of potentials at which the steady-state activation and inactivation curves overlap leaving less than 1% of the channels open and able to generate a non-inactivating current with a typical amplitude of one tenth of the maximal current (McDonald et al. 1994; Perez-Reyes 2003). To this end, a T-type window current at hyperpolarized potentials (–75 to –45 mV, peak –57 mV) was responsible for the sustained Ca^{2+} entry that triggers human myoblast fusion (Bijlenga et al. 2000). In human $Ca_V3.2$ channels expressed in HEK293 cells, the window currents predicted from the overlap between steady-state activation and inactivation curves was in the voltage range from –60 to –40 mV (Kaku et al. 2003). At present, there is no evidence for window currents through recombinant T-type channels at or above the depolarized potentials corresponding to the resting membrane potential (–45 to –35 mV) in VSMC in situ. However, rat and guinea pig mesenteric small arteries expressed a non-L-type Ca^{2+} channel with T-type-like pharmacology, rapid inactivation, and window currents in the voltage range from –50 to –20 mV (Morita et al. 1999, 2002). Whether this nifedipine-insensitive Ca^{2+} channel (NICC) corresponds to the T-type channel(s) expressed in rat mesenteric terminal arterioles has not been resolved. We speculated that transient activation of T-type channels followed by a small window current in a narrow voltage range would be detectable in our Ca^{2+} influx experiments, as previously found in human myoblasts (Bijlenga et al. 2000). Table 3 shows the peak and plateau Ca^{2+} responses to increasing [KCl] measured in rat mesenteric terminal arterioles. The peak values increased with increasing [KCl], whereas the plateau values increased at moderate [KCl] and then declined at higher [KCl]. The simplest interpretation is that the plateau values correspond to sustained Ca^{2+} influx, consistent with a window current in the physiologic range of membrane potentials, and the peak values correspond to transient activation of Ca^{2+} channels. Although these data are not conclusive because of the lack of simultaneous V_m measurement, they suggest that a window current is responsible for the sustained Ca^{2+} influx during high-K^+ application in rat mesenteric arterioles.

Pharmacologic properties of voltage-dependent Ca^{2+} channels in mesenteric arterioles

In our laboratory, rat mesenteric terminal arterioles from

Table 3. Peak and plateau Ca^{2+} responses (fura-2 ratio) to increasing [KCl] in rat mesenteric terminal arterioles (n = 7) (L.J. Jensen, unpublished data).

[KCl], mmol/L	E_K, mV	Peak R, % of baseline	Plateau R, % of baseline
6	–81	100	100
50	–28	106.9±1.47	105.7±1.49*
75	–17	109.8±2.09	106.2±1.53*
110	–8	110.8±2.35	102.2±0.84*

Note: E_K, equilibrium potential for K^+ calculated assuming $[K^+]_{cell}$ = 150 mmol/L; peak R, maximum fura-2 ratio within 0–20 s from stimulation; plateau R, steady-state response measured 90 s after stimulation. *, Significant at $p < 0.05$ vs. peak R (by signed rank test, paired observations).

2 separate regions have been under study. One is the terminal arterioles (<40 μm lumen diameter) located in the transparent part of the mesentery, which is a thin sheet consisting of a double-layered serous membrane containing lymph vessels, connective tissue, fibroblasts, and nerves. The second region is the terminal arterioles (<40 μm) located in the mesenteric fat pad adjacent to the intestinal (ileum) wall. The pharmacologic profile of voltage-dependent Ca^{2+} entry and the expression profile of VDCCs were investigated in arterioles isolated from both regions. The $Ca_V1.2$ (L-type) α_1-subunit (Gustafsson et al. 2001) and several HVA β-subunits (β_{1b}, β_2, β_3) (Jensen et al. 2004) were not expressed at the mRNA level in arterioles from the transparent part of the mesentery, whereas the L-type α_1-subunit was detected at both the mRNA and protein level in arterioles from the mesenteric fat pad. It is not possible to generalize the pharmacologic observations found in either of the 2 regions to all mesenteric arterioles because the specificities of both T-type and L-type blockers are not ideal (Table 2). If we deal with the data obtained in each region of the mesenteric arteriolar tree separately, however, the common observation is that T-type channels are expressed and important for electromechanical coupling in mesenteric arterioles. $Ca_V3.1$ and $Ca_V3.2$ T-type channels were expressed at the mRNA level in both of the arteriolar preparations. In addition, in terminal arterioles from the mesenteric fat pad, $Ca_V3.1$ protein was immunolocalized in VSMC (Fig. 1), whereas $Ca_V3.2$ immunostaining was predominant in endothelial cells, but also present in VSMC (Braunstein et al. 2009) (Fig. 2).

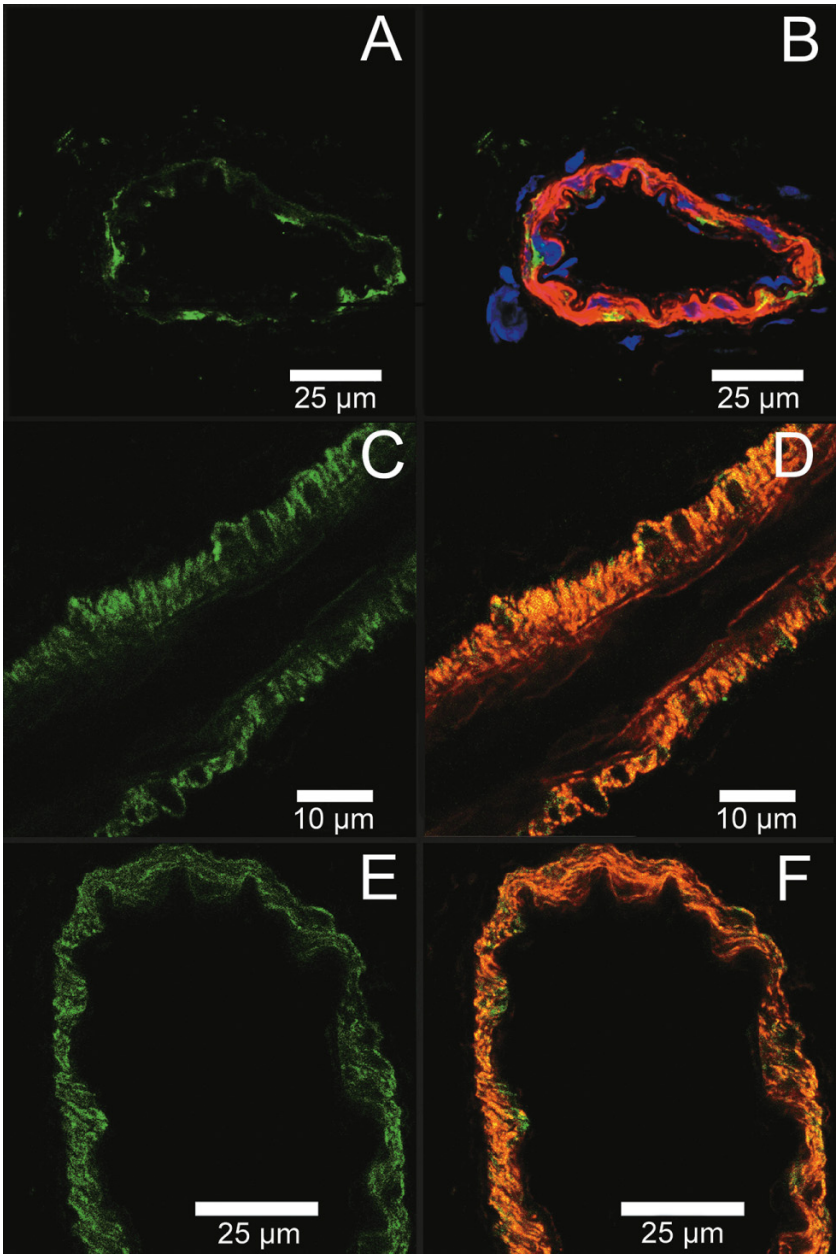
In arterioles from the transparent part of the mesentery, protein expression of T- and L-type channels was not investigated. In this preparation we instead applied several highly specific neurotoxin blockers of HVA channels and monitored the effect on KCl-induced Ca^{2+} increase (Jensen et al. 2004) (Fig. 3). Calciseptine (500 nmol/L), which is a highly specific antagonist of L-type channels (de Weille et al. 1991), had no effect on the Ca^{2+} responses to KCl in mesenteric arterioles. In rat afferent arterioles serving as a positive control for the efficacy of this toxin on vascular L-type channels, 500 nmol/L calciseptine inhibited the Ca^{2+} response by 47% (Jensen et al. 2004). These data supported our notion that the $Ca_V1.2$ L-type channel is not expressed in arterioles from this mesenteric region (although this was not confirmed by immunocytochemistry). In apparent contradiction to this, we found that 1 μmol/L nifedipine, a concentration sufficient to completely block vascular L-type channels (Morel et al. 1998), inhibited the KCl-induced Ca^{2+} response by approximately 40%. This may, however, be simply explained by a nonspecific action on T-type channels, since micromolar concentrations of the dihydropyridines nimodipine and nifedipine also affect T-type channels (Table 2).

Application of 100 nmol/L ω-agatoxin IVA, a highly specific neurotoxin blocker of P/Q-type VDCCs (Mintz et al. 1992), did not inhibit the KCl-induced Ca^{2+} responses in these arterioles (Jensen et al. 2004) (Fig. 3). This was supported by our finding that message for the $Ca_V2.1a$ or $Ca_V2.1b$ splice variants forming functional P- and Q-type Ca^{2+} channels was also not expressed (Jensen et al. 2004). Next we tested the effects of 500 nmol/L ω-conotoxin MVIIA and 100 nmol/L SNX-482, two highly specific neurotoxin blockers of N-type (Feng et al. 2003) and R-type (Newcomb et al. 1998) VDCCs, respectively. Because these 2 blockers showed no effects on the KCl-induced Ca^{2+} responses (Fig. 3), we concluded that none of the HVA Ca^{2+} channels were expressed in mesenteric terminal arterioles from the transparent part of the mesentery (Jensen et al. 2004).

The mibefradil concentration required to inhibit the KCl-induced Ca^{2+} responses by 50% was between 100 nmol/L and 1 μmol/L. Pimozide (500 nmol/L), another effective blocker of both L- and T-type channels (Table 2), inhibited the KCl-induced Ca^{2+} responses by 85%. The effects of mibefradil and pimozide were highly use-dependent, which was probably due to a preferential binding of the drugs to the inactivated state of the Ca^{2+} channel (Jensen et al. 2004). In addition, mibefradil (10 μmol/L) abolished local and conducted vasoconstriction to local current application onto arterioles in the transparent part of the mesentery in vivo (Gustafsson et al. 2001). T-type channels were therefore, by default, the only likely candidates as mediators of voltage-dependent Ca^{2+} entry and electromechanical coupling in these arterioles (Gustafsson et al. 2001; Jensen et al. 2004).

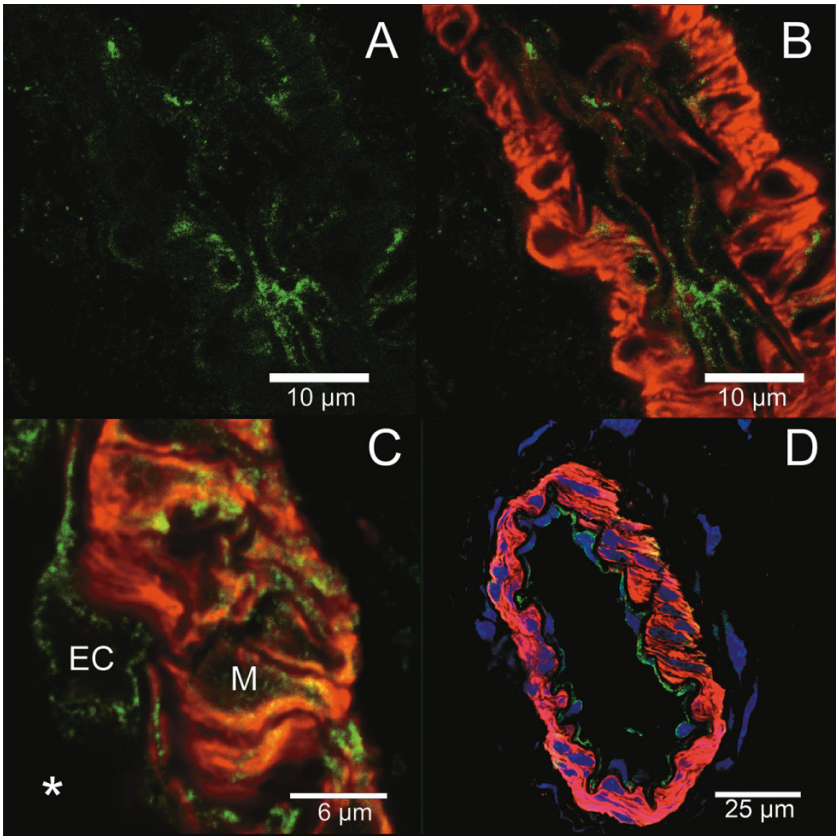
In a separate study we investigated the effects of 2 newly developed, specific blockers of T-type channels on local and remote Ca^{2+} responses to local depolarizing KCl application (Braunstein et al. 2009) (Fig. 4). This study was performed in terminal arterioles isolated from the mesenteric fat pad, as these vessels, which supply both adipose tissue and the iliac wall, should be subject to vasomotor tone regulation. Both NNC 55-0396 (Huang et al. 2004) and R(–)-efonidipine (Furukawa et al. 2004) are reported to be specific inhibitors of recombinant T-type channels with either no or very little effect on L-type channels (Table 2). The concentration of NNC 55-0396 required to inhibit the KCl-induced Ca^{2+} responses by 50% was between 100 nmol/L and 1 μmol/L. This concentration is well below the IC_{50} (~7 μmol/L) re-

Fig. 1. (A) Immunolocalization of $Ca_V3.1$ T-type Ca^{2+} channel (green) in transverse section of an arteriole from the rat mesenteric fat pad. (B) Same image (overlay) with staining of actin (red) and nuclei (blue) included. Note that $Ca_V3.1$ staining appears only in VSMC. No green staining is seen around nuclei of endothelial cells, which can be identified as the actin-negative cells lining the vessel lumen. (C) $Ca_V3.1$ localization (green) in longitudinal section of a mesenteric arteriole is clearly visible in the single layer of VSMC. (D) Same image (overlay) with actin staining shown in red. (E) $Ca_V3.1$-specific staining (green) is shown in rat small mesenteric artery having multiple VSMC layers. (F) Same image (overlay) with actin staining shown in red. (From Braunstein et al. 2009, reproduced with permission of J. Vasc. Res., Vol. 46, p. 145, © 2008 Karger Publishers.)



ported for inhibition of recombinant $Ca_V3.1$ channels (Huang et al. 2004). However, since the intracellular Ca^{2+} concentration is the net result of Ca^{2+} channel activation, Ca^{2+} release, and Ca^{2+} transporter activities, it cannot be expected that IC_{50} values obtained for whole-cell Ca^{2+} currents are quantitatively similar to those obtained for Ca^{2+} influx data. *R*(–)-efonidipine also blocked the KCl-induced Ca^{2+} responses with almost half-maximal inhibition (43%) observed at a concentration of 10 µmol/L (Braunstein et al. 2009) (Fig. 4). Although *R*(–)-efonidipine is a potent inhibitor of recombinant $Ca_V3.1$ T-type channels at the concentrations used in our study (Table 2), this drug appears to be much less potent in mesenteric terminal arterioles. This discrepancy could be explained by low solubility, as we were not able to dissolve *R*(–)-efonidipine in physiologic saline solution (PSS) at concentrations higher than 33 µmol/L (Braunstein et al. 2009). However, the use of *R*(–)-fonidipine as a selective inhibitor of T-type channels may be ques-

Fig. 2. (A) Immunolocalization of $Ca_V3.2$ protein (green) is clearly visible in the lumen of a terminal arteriole from the rat mesenteric fat pad. (B) Same image (overlay) with actin staining shown in red. (C) In this close-up image of an arteriolar wall (*, lumen of vessel), $Ca_V3.2$-specific staining (green) is clearly visible in an endothelial cell (EC, identified as the actin-negative cell facing the lumen (*)). Scattered green staining ($Ca_V3.2$) is visible in the media (M), which is identified by intense actin staining (red). (D) A similar staining pattern is observed in rat small mesenteric artery. Note that EC nuclei (blue) are surrounded by green ($Ca_V3.2$) staining. (From Braunstein et al. 2009, reproduced with permission of J. Vasc. Res., Vol. 46, p. 146, © 2008 Karger Publishers.)



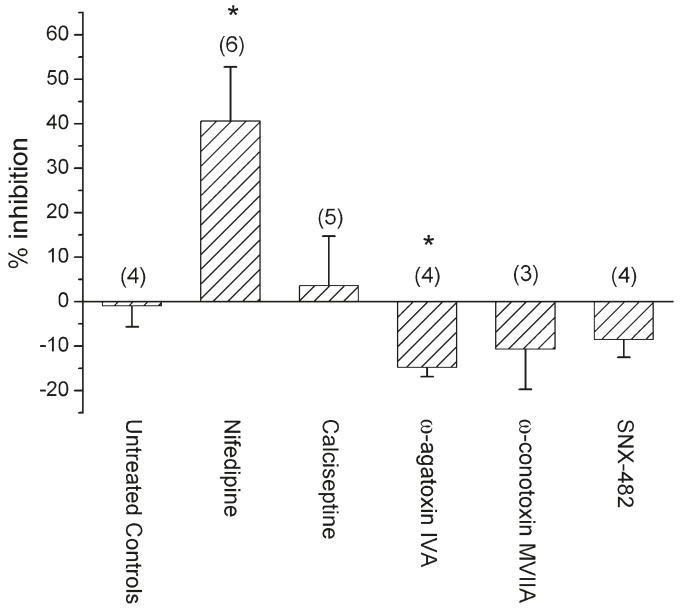
tioned because of inconsistencies between results obtained with efonidipine and its enantiomers in different studies. For example, efonidipine blocked recombinant L-type channels with an IC_{50} value of approximately 2 nmol/L in one study (Lee et al. 2006), whereas in another study using a similar voltage protocol (Furukawa et al. 2004) efonidipine as well as its *S*(+)-enantiomer blocked the expressed L-type currents with an IC_{50} of approximately 1 µmol/L. The variability in its reported effects between studies may arise because the potency of this drug against Ca^{2+} channels is affected not only by the type of cell in which the current is expressed (Furukawa et al. 2004), but also because its efficacy increases with the frequency of applied depolarizations (Masumiya et al. 2000). The application of high-K^+ solution to intact arterioles does not constitute high-frequency stimulation; nonetheless, we cannot compare our Ca^{2+} influx data directly with the reported electrophysiologic data.

Nickel has been used to discriminate between L-type and T-type channels owing to a high sensitivity of $Ca_V3.2$ channels towards this divalent metal cation (Table 2). Application of a relatively high concentration of Ni^{2+} (250 µmol/L) inhibited the KCl-induced Ca^{2+} responses by 60% ± 8% (n = 5; $p < 0.01$) (L.J. Jensen, unpublished observations), demonstrating that the mesenteric Ca^{2+} channels are not highly sensitive to Ni^{2+} and arguing against a major role of $Ca_V3.2$ channels. This result is in line with our immunostaining results, which suggests that the most abundantly expressed T-type channel in VSMC of mesenteric arterioles is in fact $Ca_V3.1$ and not $Ca_V3.2$, the latter being most abundant in endothelial cells.

Are mesenteric T-type channels atypical?

There is one important pharmacologic observation that deserves attention. We performed 2 series of experiments to check the effects of L-type and T-type blockers under conditions in which T-type channels would normally be closed due to their strong voltage dependence of inactivation, which is half-maximal at –72 mV (Perez-Reyes 2003). The KCl-induced Ca^{2+} responses were measured under resting conditions in isolated arterioles. Then the arterioles were depolarized by superfusion with PSS containing 25 mmol/L KCl, and the Ca^{2+} responses to micropipette delivery of 155 mmol/L KCl were measured again during this sustained depolarization. The magnitude of the Ca^{2+} increases (measured as ΔR, peak fura ratio – baseline fura ratio) was not significantly different between resting and depolarized arterioles. The equilibrium potential for K^+ (E_K) was estimated to –45 mV with 25 mmol/L KCl, and taking into account the depolarizing influence of E_{Na} and E_{Cl}, we reasoned that

Fig. 3. Effects of various Ca^{2+} channel blockers in arterioles from the transparent part of the rat mesentery evaluated as percentage inhibition of the Ca^{2+} response to 75 mmol/L K^+. The L-type Ca^{2+} channel blocker nifedipine (1 μmol/L) inhibited 40% of the response, whereas there was no effect of the highly specific L-type blocker calciseptine (500 nmol/L). Highly specific neurotoxin blockers of the P/Q-type (100 nmol/L ω-agatoxin IVA), N-type (500 nmol/L ω-conotoxin MVIIA), or R-type Ca^{2+} channels (100 nmol/L SNX-482) did not inhibit the responses to high K^+. There was a minor increase of the Ca^{2+} response in the presence of ω-agatoxin IVA. *, Significant at $p < 0.05$ vs. untreated control experiments (number of arterioles tested) by t test. (From Jensen et al. 2004, reproduced with permission of Br. J. Pharmacol., Vol. 142, p. 713, © 2004 Nature Publishing Group.)



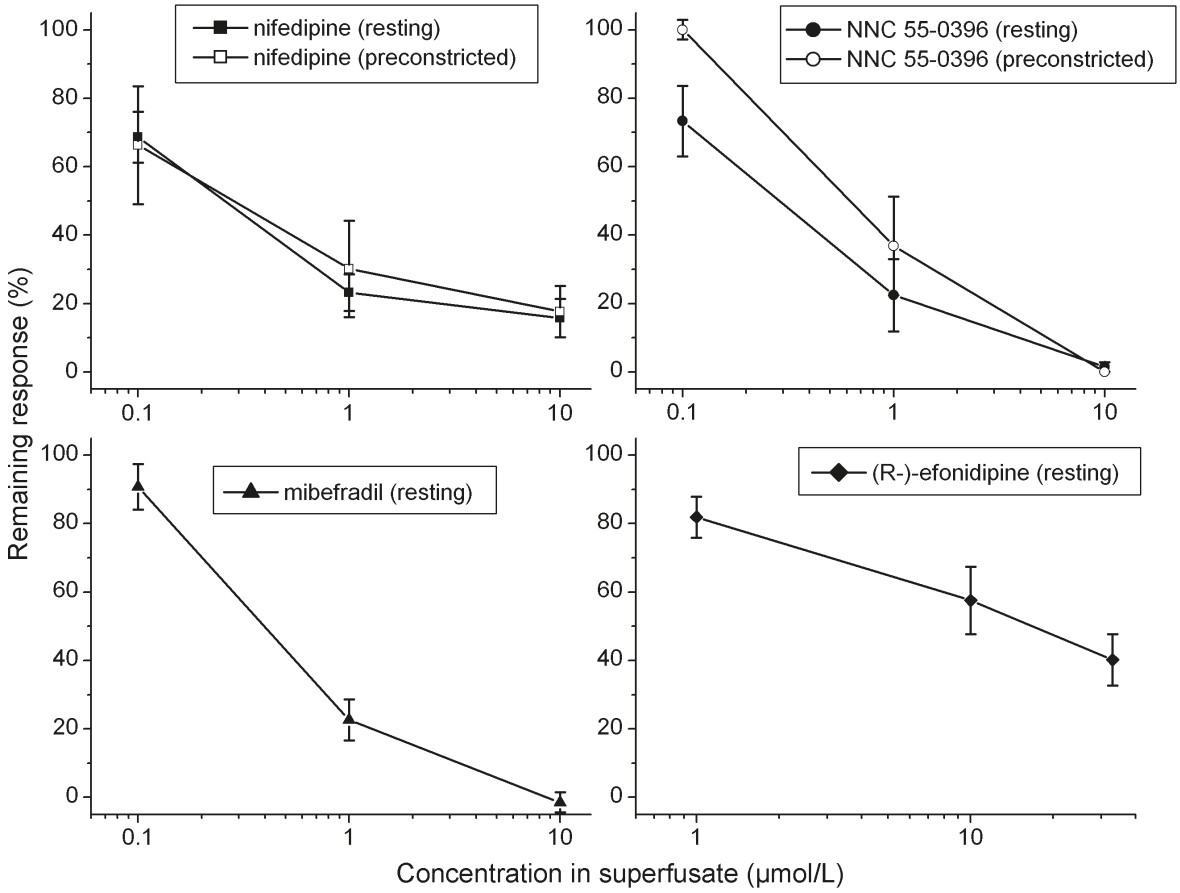
V_m had to be slightly more depolarized, which should leave the majority of 'classical' T-type channels inactivated. As expected, because L-type channels are HVA, there was no change of the nifedipine sensitivity under these circumstances. When this experiment was repeated with the selective T-type blocker NNC 55-0396, we observed a reduction of its effect at low concentration, whereas at medium and high concentrations the effect was identical to that observed in resting arterioles, which presumably had a more hyperpolarized V_m (Braunstein et al. 2009) (Fig. 4). Considering that $Ca_V3.1$ and $Ca_V3.2$ channels are both expressed in mesenteric arteriolar smooth muscle (although to varying degrees) and that the pharmacology strongly suggests a role of T-type channels in electromechanical coupling in mesenteric arterioles, we must assume that these native T-type channels can be activated at more depolarized potentials than their recombinant counterparts. This conclusion is supported by the observation in freshly dispersed mesenteric small artery myocytes of nifedipine-insensitive fast-inactivating HVA Ca^{2+} currents with pharmacologic properties matching those of T-type channels (Inoue and Mori 2003; Itonaga et al. 2002; Morita et al. 1999, 2002). These authors noted that in the most peripheral branches of the guinea pig mesenteric arterial tree, the fraction of the total Ca^{2+} current carried by the NICC channels was close to 100% (Morita et al. 1999), an observation that lends support to our own results, which indicated that the most peripheral arterioles in the transparent part of the mesentery did not express L-, N-, P/Q-, or R-type HVA channels (Gustafsson et al. 2001; Jensen et al. 2004). Nevertheless, it must be emphasized that there are several properties in which the NICC currents do not resemble recombinant $Ca_V3.1$ T-type currents. The thresholds of activation and inactivation are more depolarized, the Ba^{2+} over Ca^{2+} permeability ratio is larger, and the sensitiviy to nimodipine is smaller (Morita et al. 2002). It is also noteworthy that the NICC currents can be distinguished from L-type currents only in the presence of a high concentration of nifedipine, and we cannot exclude the possibility that its remarkable biophysical profile is partly caused by channel interaction with nifedipine.

As mentioned above, the membrane potential in VSMC when measured in situ in pressurized arterioles is relatively depolarized, reported values ranging from –45 to –35 mV (Knot and Nelson 1998; Loutzenhiser et al. 1997; Stekiel et al. 1986; Welsh and Segal 1998). It is tempting to speculate that a T-type channel variant has evolved, which can be activated at depolarized membrane potentials to serve a functional role in arteriolar smooth muscle cells. There might be several ways in which original T-type channels have evolved as an HVA variant. One is by alternative splicing of the gene(s). Expression of 5 different variants of the human $Ca_V3.1$ T-type channel in HEK293 cells revealed that compared with the other splice variants, the $\alpha_{1G\text{-}b}$ variant in particular shifted the steady-state activation and inactivation curves to more depolarized potentials (Chemin et al. 2001). The midpoint voltage of Ca^{2+} channel window currents varied over 11–12 mV in 9 different splice variants of the human $Ca_V3.1$ T-type channel expressed in HEK cells, variant No. 153 exhibiting the most depolarized midpoint voltage at –46 mV (Emerick et al. 2006). To date, T-type splice variants have not been detected in vascular smooth muscle, but it would be relevant to search for such molecular variants in mesenteric arterioles.

Another possible explanation for an atypical activation threshold of T-type channels is by an unusual subunit composition whereby subunits modify the voltage dependence of activation and inactivation by shifting the window currents to depolarized potentials. In support of this hypothesis, overexpression of β_{2a}- or $\alpha_2\delta$-subunits induced sustained Ca^{2+} currents at potentials above –30 mV in undifferentiated NG108–15 cells endogenously expressing only T-type Ca^{2+} currents (Wyatt et al. 1998). Furthermore, in HEK293 cells transiently expressing the $Ca_V3.1$ α_1-subunit together with 2 different $\alpha_2\delta$-subunits ($\alpha_2\delta$-2a or $\alpha_2\delta$-2b), both of these shifted the steady-state inactivation curve of the Ca^{2+} channel currents about 5 mV in the depolarized direction (Hobom et al. 2000), suggesting that the window currents would be closer to the physiologic range of membrane potentials. In rat mesenteric terminal arterioles we did not find expression of the 3 most common Ca^{2+} channel β-subunits (Jensen et al. 2004), but it would be important to investigate the expression pattern and physiologic role of different $\alpha_2\delta$-variants in these vessels.

What would be the physiologic advantage for the mesenteric terminal arterioles to let the role of L-type channels be replaced by atypical HVA T-type channels with window

Fig. 4. Effects of the L-type blocker nifedipine (n = 5) and the T-type blockers mibefradil (n = 5), NNC 55-0396 (n = 5), and R(–)-efonidipine (n = 5) on local Ca^{2+} responses to local KCl application in arterioles from the rat mesenteric fat pad. Between stimulations, arterioles were maintained under control ('resting') or depolarized ('preconstricted') conditions. (From Braunstein et al. 2009, reproduced with permission of J. Vasc. Res., Vol. 46, p. 148, © 2008 Karger Publishers.)



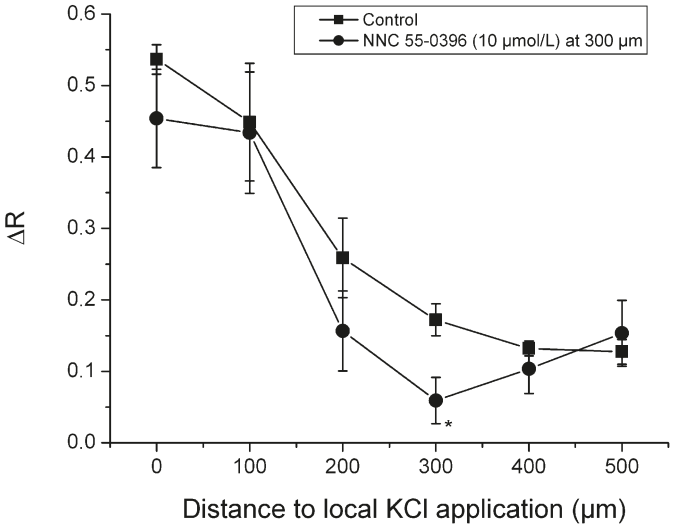
currents in the physiologic range of membrane potentials? It might be that L-type channels simply conduct too much Ca^{2+} owing to their long openings and large conductance compared with the T-type channels. Arteriolar smooth muscle cells are small with a large surface-to-volume ratio, which means that they are exposed to a relatively large Ca^{2+} leak across the plasmalemma against a relatively small volume of the sarcoplasmic reticulum (SR). It appears that the role of the SR in agonist-induced contraction of resistance vessels becomes smaller with vessel size and conversely that the Ca^{2+} fluxes across the plasmalemma predominate (Low et al. 1996). Thus, if either the superficial barrier function against Ca^{2+} entry (van Breemen et al. 1995) or the Ca^{2+} extrusion mechanisms are limited in mesenteric terminal arterioles, then $[Ca^{2+}]_i$ could quickly rise to deleterious values during continuous L-type channel activities.

Another fact to consider is that to control their surface expression and activity, L-type α_1-subunits require expression of both β- and $\alpha_2\delta$-subunits (Dalton et al. 2005; Murakami et al. 2003; Walker and De Waard 1998), whereas the essential biophysical properties of T-type currents are reproduced by expressing only the α_1-subunits (Perez-Reyes 2003, 2006). At present we do not know whether native T-type channels form complexes with auxiliary subunits (Perez-Reyes 2003); however, it is a possibility that the cost of expressing the L-type channel complexes in arteriolar SMC simply outweighs the benefits. Instead, it may be beneficial to the VSMC to express T-type channels, which may have a much simpler subunit composition.

Role of T-type channels in conducted vasomotor responses

The coordination of local hemodynamic resistances and blood flow distribution within tissues is believed to depend on conducted vasomotor responses, whereby local application of an agonist can induce a local vasoconstriction or vasodilatation that propagates up to several millimetres along an arteriole, independently of peripheral nerve activity, blood flow, or diffusible factors (Gustafsson and Holstein-Rathlou 1999; Segal et al. 1989; Segal 2005). In many cases, the propagation of vasomotor responses appears to rely on electrotonic conduction of an electrical signal along the vessel wall. Upon depolarization of the membrane, VDCCs will conduct an inward current, which, depending on the current density, may contribute to the depolarization, thereby augmenting activation of voltage-dependent ion channels. We previously investigated the possibility that voltage-dependent Ca^{2+} entry through T-type channels participates in the conduction mechanism behind conducted

Fig. 5. Ca^{2+} responses (ΔR = peak fura ratio – baseline fura ratio) obtained before and after micropipette delivery of the T-type blocker NNC 55-0396 (10 μmol/L) to a narrow region of the arteriole at ~300 μm from the site of local KCl application (*, Significant at $p < 0.05$; $n = 5$). (From Braunstein et al. 2009, reproduced with permission of J. Vasc. Res., Vol. 46, p. 149, © 2008 Karger Publishers.)



vasoconstriction. We investigated the local and conducted Ca^{2+} or diameter responses to local KCl or current application in rat mesenteric terminal arterioles, in vitro or in vivo, and monitored the effects of localized pharmacologic blockade of T-type channels at an intermediate position between the local and conducted sites. In isolated terminal arterioles from the mesenteric fat pad, microapplication of NNC 55-0396 (10 μmol/L) almost abolished Ca^{2+} entry at an intermediate site along the arteriole (Braunstein et al. 2009) (Fig. 5), whereas both local and remote Ca^{2+} responses to local KCl application were unchanged. Because arterioles from this region of the mesentery also express the L-type channel, we repeated this experiment using microapplication of nifedipine. This experiment showed a similar effect of nifedipine (Braunstein et al. 2009). In terminal arterioles from the transparent part of the mesentery, expressing only non-L-type Ca^{2+} channels, microapplication of mibefradil (10 μmol/L) abolished the vasoconstriction at the site of drug application, whereas both local and conducted vasoconstriction to local current application were unchanged (Gustafsson et al. 2001). Thus both studies suggest that T-type channels are not involved in the conduction mechanism per se, but they appear to be important for local electromechanical coupling at all sites along the vessel whereto the conducted electrical signal arrives.

A recent study has proposed a new role for endothelial $Ca_V3.2$ channels in mediating conducted vasodilatation to local current application in mouse cremaster arterioles (Figueroa et al. 2007). The evidence for involvement of endothelial T-type channels in the vasodilatation was that buffering of changes to endothelial [Ca^{2+}] delayed and diminished the dilatations, which were highly sensitive to Ni^{2+} (10 μmol/L) (Table 2) and were significantly reduced in $Ca_V3.2$ knockout mice (Figueroa et al. 2007). Local current application led to a local vasoconstriction and a conducted vasodilatation. The mechanism proposed by the authors for the involvement of T-type channels in reversing the vasoconstrictor signal to a conducted vasodilatation was as follows: the conducted depolarization to local current application activates Ca^{2+} entry via endothelial $Ca_V3.2$ channels, thereby activating "Ca^{2+}-sensitive vasodilator signals", possibly involving NO and endothelium-derived hyperpolarizing factor (EDHF). Although these data are interesting with respect to defining the role of T-type channels in vasodilatation, it is not clear how opening of T-type channels would persist to sustain endothelial Ca^{2+} influx during prolonged vasodilatation. In rat mesenteric arterioles expressing endothelial $Ca_V3.2$ T-type channels (Braunstein et al. 2009), we have observed only local and conducted vasoconstriction to local current application, in other words, no biphasic diameter changes (Gustafsson et al. 2001).

Expression and possible function(s) of T-type channels in microvascular endothelium

In our recent study, we reported for the first time the localization of T-type channel protein in mesenteric arterioles. We showed that the $Ca_V3.2$ channel was predominantly expressed in endothelial cells, as evidenced by a strong immunohistochemical labeling of the intima using a newly available polyclonal antibody directed against an intracellular epitope of rat $Ca_V3.2$ (Braunstein et al. 2009). This direct demonstration of T-type channel expression in microvascular endothelium may have an impact on future investigations into endothelium-dependent vascular responses. Coronary arteries isolated from $Ca_V3.2$ knockout mice showed normal contractile responses, but reduced NO-mediated relaxation, whereas in wild-type mice, the normal relaxation was prevented by an intermediate concentration of Ni^{2+} (Chen et al. 2003) (Table 2). The authors suggested that $Ca_V3.2$ currents in coronary artery smooth muscle cells were required for normal relaxation, an effect that was not dependent on activation of BK_{Ca} (large-conductance calcium-activated K^+) channels. However, it cannot be excluded that endothelial expression of $Ca_V3.2$ channels may control NO-mediated relaxation, either at the level of nitric oxide synthase (NOS) activity, or at a process downstream of NO production.

Although endothelial cells are considered non-excitable cells, emerging evidence suggests that depolarization of endothelial cells occurs under physiologic adaptation to changes in transmural pressure or flow. In isolated arterioles from hamster gracilis muscle, a physiologically relevant increase in transmural pressure from 40 to 100 mm Hg induced a significant depolarization of the endothelial cells (Krötz et al. 2002). The authors suggested that this effect was due to spread of the pressure-induced depolarization from VSMC to endothelial cells via myoendothelial (gap) junctions. Endothelial $Ca_V3.2$ channels would then be able to function in negative feedback of myogenic constriction via voltage-dependent Ca^{2+} entry in endothelial cells followed by NOS activation and NO-mediated relaxation.

Another proposed role of endothelial depolarization has been demonstrated in flow-adapted endothelial cells. In subpleural microvascular endothelial cells in the isolated rat lung, flow cessation was followed by rapid endothelial depolarization, the magnitude of which (approximately 20 mV)

was maintained during the progress of ischemia (Song et al. 2001). The endothelial depolarization was rapidly followed by the formation of reactive oxygen species (ROS), then an ensuing increase in Ca^{2+}, and finally a detectable increase in NO production. In a separate study, these authors showed that $Ca_V3.1$ T-type channels were upregulated during increased shear stress and flow adaptation in cultured endothelial cells (Wei et al. 2004). Thus, the proposed signaling events resulting from loss of endothelial shear stress and onset of ischemia involve endothelial depolarization and activation of T-type channels leading to Ca^{2+} entry and NOS activation; for review see Fisher et al. 2002.

T-type channels may have additional roles in pulmonary vascular endothelium (Zhou and Wu 2006). In cultured rat lung, microvascular endothelial cells expressing mRNA of the $Ca_V3.1$ channels had a T-type window current that was detected in the voltage range from –60 to –30 mV, and activation of this current coincided with procoagulant phenotypic changes of endothelial cells (Wu et al. 2003). The physiologic role of this T-type channel in lung microvascular endothelium appeared to be the control of von Willebrand factor (vWF) secretion, as shown by a decrease in the thrombin-induced release of vWF-containing vesicles after shRNA-mediated $Ca_V3.1$ gene silencing (Zhou et al. 2007). Expression of T-type channels has been linked to proliferation and cell cycle regulation of VSMC (Cribbs 2006; Lory et al. 2006). One previous study reported that mibefradil blocked proliferation of cultured pulmonary artery endothelial cells; however this effect was attributed to inhibition of volume- and Ca^{2+}-activated Cl^- channels, and not T-type channels (Nilius et al. 1997). At the systemic level, inhibition of T-type channels with efonidipine (but not L-type inhibition with nifedipine) improved endothelial dysfunction in 40 patients who had mild to moderate essential hypertension (Oshima et al. 2005). Although it was not shown directly that this effect of efonidipine was caused by an action on endothelial T-type channels, this would be an important subject for future investigation.

To summarize this section, we do not have data to suggest the physiologic role of endothelial $Ca_V3.2$ channels in rat mesenteric terminal arterioles, but it is possible that they serve as a Ca^{2+} influx pathway necessary for (*i*) negative feedback regulation of myogenic constriction, (*ii*) physiologic adaptation (vasodilatation) to flow-cessation and ischemia, and (*iii*) participation in normal coagulation by control of vWF secretion from endothelial cells.

Concluding remarks

Our studies have been carried out mainly in freshly dissected mesenteric arterioles by utilizing molecular biology tools, Ca^{2+} imaging, and pharmacologic intervention to dissect the role of T-type channels. The data suggest that T-type channels, most likely $Ca_V3.1$ channels shown to be abundantly expressed in VSMC, are important for electromechanical coupling in mesenteric arterioles. Progress in our understanding of the role of T-type channels has suffered from the lack of electrophysiologic data from these tiny vessels, and until recently mibefradil was the most specific pharmacologic tool available. However, future studies using transgenic mice or RNA interference for specific downregulation of L- and T-type channels, in combination with the new specific T-type channel inhibitor NNC 55-0396, may provide further clues to the relative importance of T-type channels in electromechanical coupling in rat mesenteric terminal arterioles. The subject of the functional role of non-L-type Ca^{2+} channels in the microcirculation is a new and exciting area, and important questions still remain to be answered regarding the role of T-type channels in vasoconstriction. Our demonstration of $Ca_V3.2$ channel expression in endothelial cells should inspire future investigations of their role in the physiology and cell biology of mesenteric arterioles.

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