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Intracellular Ca²⁺ Silences L-Type Ca²⁺ Channels in Mesenteric Veins: Mechanism of Venous Smooth Muscle Resistance to Calcium Channel Blockers

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Abstract

Rationale—Calcium channel blockers (CCBs) exert their antihypertensive effect by reducing cardiac afterload but not preload, suggesting that Ca^{2+} influx through L-type Ca^{2+} channels (LTCC) mediates arterial but not venous tone.

Objective—The object of this study was to resolve the mechanism of venous resistance to CCBs.

Methods and Results—We compared the sensitivity of depolarization (KCl)-induced constriction of rat small mesenteric arteries (MA) and veins (MV) to the dilator effect of CCBs. Initial findings confirmed that nifedipine progressively dilated depolarization-induced constrictions in MA but not MV. However, Western blots showed a similar expression of the α_{1C} pore-forming subunit of the LTCC in both vessels. Patch-clamp studies revealed a similar density of whole-cell Ca^{2+} channel current between single smooth muscle cells (SMCs) of MA and MV. Based on these findings, we hypothesized that LTCCs are expressed but "silenced" by intracellular Ca^{2+} in venous SMCs. After depletion of intracellular Ca^{2+} stores by the SERCA pump inhibitor thapsigargin, depolarization-induced constrictions in MV were blocked 80% by nifedipine suggesting restoration of Ca^{2+} influx through LTCCs. Similarly, KCl-induced constrictions were sensitive to block by nifedipine after depletion of intracellular Ca^{2+} stores by caffeine, ryanodine, or 2-aminoethoxydiphenyl borate. Cell-attached patch recordings of unitary LTCC currents confirmed rare channel openings during depolarization of venous compared to arterial SMCs, but chelating intracellular Ca^{2+} significantly increased the open-state probability of venous LTCCs.

Conclusions—We report that intracellular Ca^{2+} inactivates LTCCs in venous SMCs to confer venous resistance to CCB-induced dilation, a fundamental drug property that was previously unexplained.

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Disclosures: None

Keywords

arteries; veins; L-type Ca^{2+} channels

INTRODUCTION

The organic calcium channel blocking drugs (CCBs) have been used for three decades as a mainstay of vasodilator therapy to treat hypertension, coronary vasospasm and other diseases of abnormal arterial tone.[1] The three structural families of CCBs share a common mechanism of action to dilate small arteries and arterioles; they bind to the pore-forming α_{1C} subunit of the L-type Ca^{2+} channel (LTCC) in arterial smooth muscle cells (SMCs) to reduce open-state probability.[2] However, the usefulness of the CCBs is limited by their inability to dilate the venous circulation. Ankle edema, a common side effect of CCB therapy, is thought to result partly from CCB-induced dilation of arterioles in the absence of venous dilation; the resulting increase in capillary pressure promotes fluid exudation.[3−6] Additionally, the antihypertensive effect of the CCBs mediated by arterial dilation may be buffered by persistent venoconstriction that sustains preload to maintain blood pressure elevation.[7−10]

Although it is well recognized clinically that the vasodilator effect of CCBs is limited to the arterial circulation, the mechanism that confers venous resistance to this class of drugs *in vivo* is unknown. One possibility is that the LTCCs expressed in arteries and veins are fundamentally different in their biophysical or pharmacological properties. In this regard, only several studies have characterized the biophysical properties and drug profiles of the LTCCs in patch-clamped venous SMCs or even documented their contribution to the contraction of isolated veins. Surprisingly, these studies suggest that similar to arteries, LTCCs in the SMCs of large veins retain sensitivity to the CCBs. For example, depolarization-induced constrictions of porcine femoral and saphenous veins are sensitive to calcium channel blockade.[11] Additionally, voltage-elicited Ca^{2+} currents in patch-clamped SMCs from canine saphenous vein are blocked by nanomolar concentrations of CCBs. The biophysical properties of these channels appear indistinguishable from their arterial counterparts.[12] Finally, the LTCCs in rabbit portal vein myocytes also display a customary profile of voltage-dependent opening, channel kinetics and sensitivity to blockade by nisoldipine, verapamil and diltiazem.[13] Collectively, these findings suggest that the SMCs of conduit veins express prototypic LTCCs, although patch-clamp studies in SMCs isolated from veins or venules that regulate changes in venous capacitance apparently are unavailable.

In contrast, small veins and venules from diverse origins fail to dilate to the CCBs, suggesting a lack of functional LTCCs in these venous SMCs *in situ*. In striated muscle of spontaneously hypertensive rats, the LTCC blockers verapamil, nifedipine and felodipine selectively dilate arterioles but not venules.[14] Similarly nifedipine only dilates the arterioles but not venules in the hamster cheek pouch, although the venules are sensitive to other dilator stimuli.[15] Finally, nifedipine and benipidine dilate the arterioles but not venules of the rat mesenteric circulation. [16,17] Thus, the insensitivity of the venous circulation to CCB-induced dilation, that is clinically recognized also, is observed experimentally in small veins.

Using the second order branches of small mesenteric arteries (MA) and mesenteric veins (MV) of the rat as a model, the present study was designed to resolve the mechanisms that confer venous insensitivity to CCB–induced dilation. We used depolarizing concentrations of high KCl to directly activate voltage-gated LTCCs to induce constriction and thereby circumvent complex receptor-mediated signaling pathways that may differ between arterial and venous SMCs. Complementary vascular reactivity and Ca^{2+} imaging studies were performed in pressurized MA and MV. Additionally, Western blot and patch-clamp studies compared the

expression, properties, and CCB sensitivity of LTCCs between arterial and venous preparations.

METHODS

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Diameter and E_m recording in pressurized vessels

Procedures using animals were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* at the University of Arkansas for Medical Sciences. Second order mesenteric arteries (MA) and mesenteric veins (MV) were dissected from 10-week old male Sprague Dawley rats (Harlan), and cannulated with tapered glass micropipettes. The inflow pipette was connected to a reservoir to control intraluminal pressure.[18] Arteries and veins were pressurized at their native pressures of 80 mmHg and 6 mmHg, respectively. Internal diameters were recorded on-line using an upright microscope/Spot RT camera with images captured by Metavue acquisition software (Metamorph). Intracellular membrane potential (E_m) was recorded using glass microelectrodes.[19]

Ca^{2+} imaging in Fluo-4 loaded vessels

Second order MA and MV were cleaned of adhered fat and connective tissue and loaded with the Ca^{2+} indicator fluo-4 (2 µmol/L) plus pluronic acid (0.01%) in PSS at room temperature for 1.5 hours in the dark. Images were captured using a $10\times$ S Fluor objective (NA = 0.5) on a Nikon TE2000-U microscope with a QImaging Retiga 2000R camera and iVision software (version 4.0.14, Biovision Inc.) that also was used for analysis. For fluorescence quantitation, a region of interest in the lumen of the vessel was chosen, avoiding the vessel walls and sympathetic nerves.

Detection of the α_{1C} subunit

Whole-cell protein homogenates were prepared from MA and MV pooled from 2 rats. The expression level of the pore-forming α_{1C} subunit of the LTCC was assessed by Western blotting using a polyclonal antibody (Alomone Labs, Jerusalem, Israel).[20]

Recording of Ca^{2+} channel currents

SMCs were enzymatically isolated from rat MA and MV.[19–21] Whole-cell Ca^{2+} channel currents were recorded using standard pulse protocols and solutions.[22] Single LTCC current openings in freshly isolated MA and MV SMCs were obtained in the cell-attached mode.

RESULTS

Depolarization-induced constriction in MV is resistant to nifedipine

Direct depolarization of isolated veins by high concentrations of KCl has been used to directly evaluate the contribution of LTCC-mediated Ca^{2+} influx to venoconstriction.[11] We used glass microelectrodes to compare the depolarizing responses of 2nd order branches of the rat MA and MV to high (60 mmol/L) KCl to ensure an equal voltage stimulus for LTCC opening in both preparations. Figures 1A and 1B show that KCl depolarization robustly reduced the membrane potential (E_m) of MA and MV. The average resting E_m in MA and MV were -47 ± 2 mV and -46 ± 3 mV, respectively (n = 6). Figure 1C demonstrates that 60 mmol/L KCl was equally effective in depolarizing the MA ($\Delta 16 \pm 2$ mV) and MV ($\Delta 14 \pm 2$ mV). The same depolarizing stimulus also similarly reduced the diameter of the MA and MV by 70 ± 6 % and 68 ± 6 %, respectively (Fig. 2A).

In spite of these similarities, whereas pre-incubation of MA with nifedipine (Nif, 1 µmol/L) for 10 min inhibited KCl constriction by 93%, similar constrictions of the MV were resistant to 1 µmol/L or 10 µmol/L Nif, showing only a 20% and 15% loss of constriction, respectively (Figs. 2A and 2B). Neuronally-released norepinephrine did not contribute to KCl constriction of small MV, since the α-adrenergic receptor antagonist, phentolamine (1 µmol/L), did not blunt this response (Online Figure I). Finally, Nif and a structurally unrelated CCB, diltiazem, failed to reverse established KCl constrictions of rat MV whereas both drugs concentration-dependently reversed arterial constriction (Figs. 2C and 2D).

Studies using fluo-4 loaded MA and MV verified that LTCCs contribute less to depolarization-induced Ca^{2+} mobilization in venous compared to arterial SMCs. In drug-free solution, KCl induced a 3.9-fold and 2.9-fold rise in cytosolic free Ca^{2+} ($[Ca]_i$) that did not differ significantly between MA and MV, respectively (Figs. 3A and 3B). After a 10-minute incubation with 10 µmol/L Nif, KCl failed to increase $[Ca]_i$ in MA (Figs. 3C and 3D). However, KCl still induced a 2.0-fold rise in $[Ca]_i$ in MV implying that the venous SMCs mobilized Ca^{2+} in the absence of functional LTCCs (Figs. 3C and 3D).

Similar expression of α_{1C} in arterial and venous SMCs

Considering the resistance of KCl constrictions in MV to CCBs and the diminished contribution of venous LTCCs to $[Ca]_i$ elevation during depolarization, we explored if MV express fewer LTCC α_{1C} pore-forming subunits compared to MA. Western blots using protein lysates from MA and MV showed that the doublet immunoreactive band corresponding to the long and short forms of α_{1C} was 48% less in MV (Fig. 4A, top blot, n = 4). However, after normalization of α_{1C} to the reduced smooth muscle α-actin signal in venous lysates (Fig. 4A, lower blot, n = 4) to account for the lower SMC content in MV,[23] densitometric analysis suggested that the MA and MV express similar levels of the α_{1C} protein (Fig. 4B).

LTCCs in patch-clamped arterial and venous SMCs have similar properties

Subsequently, we used whole-cell patch-clamp methods to determine if the α_{1C} immunoreactive signal detected in venous lysates corresponded to functional LTCCs. Inward currents of similar amplitudes were observed in venous and arterial SMCs (Figs. 5A, 5C and 5F). Peak Ba^{2+} current elicited at +18 mV was similar between MA and MV SMCs (-6.4 ± 0.7 pA/pF and -6.6 ± 1.1 pA/pF, respectively) (Fig. 5A; n = 14, 7). Membrane capacitance also was similar (MA: 14.8 ± 0.9 pF; MV: 15.7 ± 1.3 pF). Standard pulse protocols were used to obtain the half-maximal activation and inactivation voltages (Fig. 5B).[23] These analyses provided similar values between arterial and venous SMCs for half-maximal ($V_{1/2}$) current activation (MA: 12.2 ± 1.1 mV; MV: 12.0 ± 0.6 mV) and inactivation (MA: -9.3 ± 3.3 mV; MV: -8.6 ± 2.7 mV), revealing no voltage-dependent abnormalities of the venous channels. Interestingly, exposure of the patch-clamped SMCs to Nif at a concentration (100 nmol/L) that failed to dilate the MV but fully dilated the MA (Fig. 2C), was equally effective in blocking inward current in both cell types (Figs. 5C–D, 5F–G). Peak Ba^{2+} current in arterial SMCs was blocked 56% by Nif compared to 55% inhibition in venous SMCs (Figs. 5E and 5H). Thus, although KCl constrictions in isolated, pressurized MV were insensitive to CCBs, the venous SMCs subjected to whole-cell patch-clamp conditions expressed prototypic nifedipine-sensitive LTCCs with properties indistinguishable from the arterial channels.

Depleting intracellular Ca^{2+} restores venous SMC sensitivity to CCBs

The divergent observations that LTCCs in MV did not contribute to KCl constriction (Fig. 2) whereas depolarization elicited whole-cell LTCC currents in dialyzed SMCs of MV (Fig. 5), led us to consider the possibility that venous LTCCs are inactivated by cytosolic factors *in situ*, conferring resistance to CCB-induced dilation. In this regard, a rise in $[Ca]_i$ during cell excitation is recognized as a negative feedback mechanism that inactivates LTCCs in a variety

of cell types.[24–26] Initially we determined if Ca^{2+} influx through store-operated channels (SOCs) inactivated LTCCs during depolarization of MV. Pressurized MV were pre-incubated with gadolinium (Gd^{3+}) or lanthanum (La^{3+}) to non-selectively block SOCs. We observed that neither Gd^{3+} (100 µmol/L) nor La^{3+} (100 µmol/L) reduced the amplitude of KCl constrictions in MV or conferred venous sensitivity to nifedipine (Figs. 6A and 6B).

Since the sarcoplasmic reticulum is in close proximity to the plasma membrane in some venous SMCs,[27] we next explored if depleting intracellular Ca^{2+} stores using the SERCA inhibitor thapsigargin (Thaps, 1 µmol/L)[28] could restore depolarization-induced Ca^{2+} influx through LTCCs and thereby confer sensitivity to CCBs. Since Thaps alone robustly constricted MV (data not shown), vessels were kept in nominally Ca^{2+}-free PSS during exposure to Thaps and then extracellular Ca^{2+} (1.5 mmol/L) was restored concurrently with depolarizing KCl. Depletion of intracellular Ca^{2+} stores with Thaps reduced KCl constriction in MV by 41%. However, after Thaps treatment, Nif blocked KCl constrictions in rat MV by 80% (n = 6) (Fig. 6C). We also pre-incubated the MVs in 10 mmol/L caffeine (Caf) to deplete intracellular Ca^{2+} stores by increasing ryanodine receptor activity.[28] Caf did not alter KCl constrictions, but similar to Thaps, in the presence of Caf, KCl constrictions of MV were blunted 61% by Nif in the presence of Caf (Fig. 6D). Ryanodine (Ryan), which opens ryanodine receptors to a full conductance state also depletes intracellular Ca^{2+} stores[28]. Ryanodine (10 nmol/L) also did not affect KCl constrictions in MV, but in its presence, Nif blunted KCl constrictions by 62% (Fig. 6E). Finally, we used 2-Aminoethoxydiphenyl borate (2-APB), a non-selective IP_3 receptor blocker and SOC channel blocker[28], to deplete intracellular Ca^{2+} stores. We observed that whereas 2-APB (100 µmol/L) did not alter KCl constrictions in MV, Nif reduced KCl constrictions by 86% in the presence of 2-APB (Fig. 6F). Thus, depletion of intracellular Ca^{2+} stores in venous SMCs using four different pharmacological agents apparently restored depolarization-elicited Ca^{2+} influx through LTCCs in MV.

As a final proof-of-principle that the lack of effect of CCBs in MV relates to a "silencing" of LTCCs in venous SMCs by intracellular Ca^{2+} stores, we compared the open-state probability (NP_o) of LTCCs in arterial and venous SMCs using the cell-attached patch configuration that minimally disturbs the intracellular milieu. Reducing the patch potential (PP) from −70 mV to −10 mV resulted in dense and often stacked single-channel currents in cell-attached patches of SMCs of MA (Fig. 7A) but only rare unitary currents in patches of MV (Fig. 7B). The corresponding NP_o values at a PP of −10 mV were 0.26±0.05 (MA, 10 patches) and 0.02 ± 0.01 (MV, 7 patches) (Fig. 7D). However, incubating venous SMCs in the chelator BAPTA-AM (100 µmol/L) to deplete intracellular Ca^{2+} increased the NP_o of venous LTCCs by 6.7–fold (NP_o = 0.13 ± 0.05, 6 patches) (Fig. 7C and 7D). The single-channel conductance was not significantly different between SMCs from MA (22 pS) and MV (21 pS), showing values similar to those reported by others using 50 to 90 mmol/L Ba^{2+} as the charge carrier[29,30] (Fig. 7E).

DISCUSSION

The organic CCBs have been used for several decades to treat hypertension and vasospasm with particular emphasis on the dihydropyridine class that includes nifedipine as a selective vasodilator. It is widely recognized that the CCBs exert their antihypertensive effect by reducing the open-state probability of LTCCs in arterial SMCs to lower afterload, but that they have little suppressant effect on the tone of the venous circulation.[9, 31–33] To our knowledge, the present study provides the first mechanistic explanation for the resistance of veins to CCB therapies, a characteristic that may limit their antihypertensive action and contribute to adverse effects.[3–6] Based on our findings, we propose that the SMCs of small MV express prototypic LTCCs that retain the same sensitivity to CCB–induced blockade of constriction as arterial SMCs. However, during vascular activation, the LTCCs in the SMCs of small veins are

inactivated by Ca^{2+} from intracellular stores. Thus, small veins express phenotypically normal but "silent" LTCCs whose function is only revealed after Ca^{2+} store depletion disinhibits the channels to enable Ca^{2+} influx.

In this regard, it is well recognized that Ca^{2+} influx through LTCCs primarily mediates arterial but not venous tone *in vivo*[9, 31–33], although there is experimental evidence that some larger veins retain sensitivity to CCBs.[11] The mechanism that confers the resistance of small veins to CCB–induced dilation has been elusive, and it may not be fair to extend our observations to all venous beds, as there are special venous beds, such as the portal veins and pulmonary veins that are exposed to high pO_2 and high pressure, respectively, that may respond differently to CCBs because of their unique environments. Our studies used cannulated second order rat MA and MV, which were pressurized at their high and low *in situ* intraluminal pressures, respectively, as an experimental model to identify these mechanisms. We first considered the possibility that a more negative resting E_m below the voltage required for LTCC activation and/or an inability of KCl to depolarize the venous SMCs may explain the apparent lack of depolarization-induced Ca^{2+} influx through LTCC in MV. The factors that regulate resting E_m in veins are poorly understood and the *in situ* E_m in rat MV is reportedly more negative than in the MA[34] although the *in vitro* values of resting E_m may not differ.[35] Indeed, we observed similar levels of resting E_m between pressurized rat MA and MV and the addition of 60 mmol/L KCl induced a comparable depolarization. Similarly, the lack of LTCC–dependent constriction in MV could not be attributed to fewer channels, since MA and MV expressed similar levels of pore-forming α_{1C} subunits and venous SMCs exhibited whole-cell Ca^{2+} channel currents indistinguishable in properties and densities from arterial SMCs. Thus, the functional LTCCs in SMCs of MV appear to be prototypic channels. However, the paucity of unitary LTCC currents in cell–attached patches of venous SMCs indicated that when the cytosol is minimally disturbed in veins, the LTCCs are either not activated by depolarization or are inactivated by a cytosolic influence that is retained in the cell-attached configuration but lost during cell dialysis in whole-cell recording. Our findings that the Ca^{2+} chelator BAPTA-AM increased unitary LTCC currents in venous SMCs paired with the demonstration that pharmacological depletion of intracellular Ca^{2+} stores confers sensitivity of venous constriction to CCBs provide strong evidence that intracellular Ca^{2+} stores inactivate venous LTCCs during SMC depolarization. However, we cannot rule out the possibility that LTCC-mediated Ca^{2+} entry is partly buffered by SR uptake that acts as a sink for Ca^{2+} entering the cell as postulated by the superficial buffer barrier hypothesis.[36]

Our proposal that the silencing of LTCCs in venous SMCs is mediated by Ca^{2+}-inactivation concurs with the recognition of this process as an important biophysical property of LTCCs that acts as a negative feedback mechanism to limit voltage-gated Ca^{2+} entry during cell excitation.[24–26] The process of Ca^{2+}-dependent inactivation requires the Ca^{2+} binding protein, calmodulin, which binds to the intracellular C-terminus of α_{1C} to cause a conformational change that enables channel inactivation.[37,38] In the heart, Ca^{2+} influx through LTCCs opens ryanodine receptors in the sarcoplasmic reticulum to trigger Ca^{2+}-induced Ca^{2+} release and also mediates Ca^{2+}-dependent inactivation of LTCCs to limit action potential duration.[39] The process of Ca^{2+} inactivation of LTCCs also may assume a physiological role in vascular SMCs, although there are only a few findings in this regard.[40–42] However, we report here that intracellular Ca^{2+} stores in venous SMCs appear to nearly eliminate a contribution of Ca^{2+} influx through LTCCs to constriction, thereby acting as a powerful physiological switch that determines the source of activator Ca^{2+} in this cell type. Additionally, this process also may account for the resistance of venous SMCs to the dilator effect of CCBs, since the inactivation of LTCCs negates a contribution of voltage-gated Ca^{2+} influx to venous contraction. Thus, a physiological event formerly assigned only a modulatory role in SMCs may have a major impact on the fundamental Ca^{2+} handling mechanisms that underlie venous contraction and also determine its pharmacological sensitivity to vasodilator drugs.

Our findings also suggest that venous SMCs rely on other mechanisms for increasing cytosolic Ca^{2+}, which must differ from the LTCCs that predominate in arterial SMCs. Store-operated and receptor-operated Ca^{2+} channels are present on the plasma membrane of SMCs and additional voltage-gated Ca^{2+} channel types (*i.e.* T-type channels) have been proposed.[43–45] Ryanodine receptors and IP_3 receptors located in the membrane of the sarcoplasmic reticulum also have been described.[27] We observed that the rat MV still can contract robustly to KCl-induced depolarization even in the presence of high concentrations of nifedipine or diltiazem, suggesting that depolarization triggers the release of intracellular Ca^{2+} in venous SMCs independently of LTCCs activity under our conditions. Indeed, the SR is present in close proximity to the plasma membrane in the SMCs of many blood vessel types including the small pulmonary arteries, aorta, and small mesenteric arteries and veins,[45] suggesting coupling of intracellular Ca^{2+} stores and plasma membrane ion channels. This interaction has been demonstrated in human cerebral arteries, in which Ca^{2+} release from ryanodine receptors (*i.e.* Ca^{2+} sparks) activates the plasma membrane high-conductance, Ca^{2+}-activated K^+ channel.[46] Thus, we speculate that there may be a difference in sarcoplasmic reticulum-plasma membrane association of ion channels in veins compared to arteries, such that intracellular Ca^{2+} stores in veins can interact with and silence plasma membrane LTCCs.

Limitations

There are several key limitations in our study that should be acknowledged. First, the *in vivo* environment of arteries and veins differs in many respects including oxygen tension, pH, and intraluminal pressure and these factors may alter vascular reactivity. We accounted for the different intraluminal pressures in our experiment by pressurizing arteries and veins near their physiological pressures. However, we exposed both MA and MV to physiological solutions bubbled with a 95% O_2 – 5% CO_2 gas mixture to attain a pH value of 7.4. Thus, differences in pO_2, CO_2 and extracellular pH did not account for venous resistance to CCBs in our study, but conceivably could modulate sensitivity *in vivo*. Second, CCBs including Nif can influence vascular tone aside from their pharmacological block of LTCC that mediates the main dilator effect. The CCBs may induce nitric oxide (NO) release from the endothelium[47] and scavenge superoxide[48]. Since the endothelium was not removed from the rat small MA and MV in our study, it is possible that endothelial-derived NO complemented the dilator effects of Nif attributed to pharmacological block of LTCCs in SMCs. Third, to our knowledge, the types of ion channels and their regulation in venous SMCs have not been rigorously investigated. Thus, it is possible that the populations of ion channels expressed in arteries and veins are different. Although highly speculative, the lack of LTCC activity in veins also could relate to uncharacterized interactions with other cytosolic or membrane proteins. For example, in mouse motor nerve terminals, normally silent LTCCs are revealed by blockade of Ca^{2+}-activated K^+ channels.[49]

Perspectives

Our study has highlighted important differences in mechanisms of vasoconstriction between small MA and MV, and the findings suggest that venous depolarization-induced constriction can occur independently of Ca^{2+} influx via LTCCs. Our findings suggest that although prototypic LTCCs are expressed in the plasma membrane of venous SMCs, these channels are "silenced" *in situ* by intracellular Ca^{2+} stores. To our knowledge, this is the first report providing an explanation for why CCBs are selective arterial dilators in humans and animals *in vivo*, whereas veins fail to respond to these important vasodilator agents. Conversely, defining the unique mechanisms of venoconstriction may present novel targets for new blood pressure lowering drugs that selectively target the venous circulation.

Novelty and Significance

What is known?

- Calcium channel blockers block Ca^{2+} entry through voltage-gated L-type Ca^{2+} channels and are widely prescribed as antihypertensive medications to lower high blood pressure.

- These drugs block Ca^{2+} influx into arteries but not veins to increase vessel diameter and lower blood pressure, but the reason why veins fail to dilate to calcium channel blockers is unclear.

What new information does this article contribute?

- The present study shows that Ca^{2+} channel blockers prevent the constriction of small arteries but not veins from the rat mesenteric circulation; the mesenteric circulation is a key vascular bed that controls blood pressure in animals and humans.

- Next, we show that L-type Ca^{2+} channels are present in similar numbers in mesenteric arteries and veins, but the Ca^{2+} channels in veins are not functional.

- Finally, we provide evidence that in veins, Ca^{2+} from intracellular stores "silences" L-type Ca^{2+} channels.

Summary of Novelty and Significance

The reason why Ca^{2+} channel blocking drugs lower blood pressure by dilating arteries but not veins is unknown. Our goal was to determine why veins fail to dilate in response to this class of drugs. Using the rat mesenteric circulation as a model, we observed that although L-type Ca^{2+} channels exist in both arteries and veins, Ca^{2+} channel blockers prevent arteries but not veins from constricting. However, depleting intracellular Ca^{2+} stores in the muscle cells of veins restores sensitivity to Ca^{2+} channel blockers. These findings suggest that intracellular Ca^{2+} stores "silence" L-type Ca^{2+} channels in veins, thereby negating their contribution to constriction. Thus, we provide evidence for a novel mechanism of "Ca^{2+} channel silencing" that confers venous insensitivity to Ca^{2+} channel blockers. Our data suggest that Ca^{2+} from intracellular stores may mediate venoconstriction, and these Ca^{2+} mobilizing pathways may represent targets for the development of new venodilator therapies. In the clinical setting, the selective dilation of the venous circulation reduces blood return to the heart to lower its workload, which is beneficial in some cardiovascular diseases such as heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations and Acronyms

2-APB	2-aminoethoxydiphenyl borate
Caf	caffeine
CCB	calcium channel blocker
LTCC	L-type Ca^{2+} channel
MA	mesenteric arteries
MV	mesenteric veins
Nif	nifedipine
PP	patch potential

| PSS | physiological salt solution |
Ryan	ryanodine
SMC	smooth muscle cell
Thaps	thapsigargin

Acknowledgments

References

1. Stern S, Bayes de Luna A. Coronary artery spasm: a 2009 update. Circulation 2009;119:2531–2534. [PubMed: 19433770]

2. Catterall WA, Perez-Reyes E, Snutch TP, Striessnig J. International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. Pharmacol Rev 2005;57:411–425. [PubMed: 16382099]

3. Major TC, Dhamija S, Black N, Liachenko S, Morenko B, Sobocinski G, Okerberg C, Tinholt P, Madore S, Kowala MC. The T- and L-type calcium channel blocker (CCB) mibefradil attenuates leg edema induced by the L-type CCB nifedipine in the spontaneously hypertensive rat: a novel differentiating assay. J Pharmacol Exp Ther 2008;325:723–731. [PubMed: 18326812]

4. Noll G, Lüscher TF. Comparative pharmacological properties among calcium channel blockers: T-channel versus L-channel blockade. Cardiology 1998;89:10–15. [PubMed: 9570424]

5. Opie LH. Calcium channel antagonists. Part IV: Side effects and contraindications drug interactions and combinations. Cardiovasc Drugs Ther 1988;2:177–189. [PubMed: 3154704]

6. Weir MR. Incidence of pedal edema formation with dihydropyridine calcium channel blockers: issues and practical significance. J Clin Hypertens (Greenwich) 2003;5:330–335. [PubMed: 14564133]

7. Johnson RJ, Galligan JJ, Fink GD. Effect of an ET(B)-selective and a mixed ET(A/B) endothelin receptor antagonist on venomotor tone in deoxycorticosterone-salt hypertension. J Hypertens 2001;19:431–440. [PubMed: 11288813]

8. Palacios B, Lim SL, Pang CC. Subtypes of endothelin receptors that mediate venous effects of endothelin-1 in anaesthetized rats. Br J Pharmacol 1997;122:993–998. [PubMed: 9401760]

9. Ricksten SE, Yao T, Thoren P. Peripheral and central vascular compliance in conscious normotensive and spontaneously hypertensive rats. Acta Physiol Scand 1981;112:169–177. [PubMed: 7315410]

10. Safar ME, London GM, Weiss YA, Milliez PL. Altered blood volume regulation in sustained hypertension: A hemodynamic study. Kidney Internat 1975;8:42–47.

11. Magnon M, Gallix P, Cavero I. Intervessel (arteries and veins) and heart/vessel selectivities of therapeutically used calcium entry blockers: variable, vessel-dependent indexes. J Pharmacol Exp Ther 1995;275:1157–1166. [PubMed: 8531077]

12. Yatani A, Seidel CL, Allen J, Brown AM. Whole-cell and single-channel calcium currents of isolated smooth muscle cells from saphenous vein. Circ Res 1987;60:523–533. [PubMed: 2439228]

13. Cox RH, Katzka D, Morad M. Characteristics of calcium currents in rabbit portal vein myocytes. Am J Physiol 1992;263:H453–H463. [PubMed: 1380775]

14. Messing M, Van Essen H, Smith TL, Smits JF, Struyker-Boudier HA. Microvascular actions of calcium channel antagonists. Eur J Pharmacol 1991;198:189–195. [PubMed: 1650702]

15. Kushiro T, Watanabe N, Takahashi A, Koike M, Saito F, Otsuka Y, Kanmatsuse K. Different effects of L-type and L+N-type calcium channel blockers on hamster cheek pouch venules. J Cardiovasc Pharmacol 2004;44(6):672–675. [PubMed: 15550786]

16. Nakayama K, Horikawa N, Ogawa T, Kohno F, Ishii K, Kubo K, Imabeppu S. Arteriolar and venular vasodilating properties of benidipine hydrochloride, a 1,4-dihydropyridine Ca^{2+} antagonist with

long-lasting action, assessed in rat mesenteric microcirculation. J Cardiovasc Pharmacol 1999;33:540–548. [PubMed: 10218723]

17. Araki H, Itoh M, Nishi K. Effects of nipradilol on the microvascular tone of rat mesentery: comparison with other beta-blockers and vasodilators. Arch Int Pharmacodyn Ther 1992;318:47–54. [PubMed: 1361123]

18. Pesic A, Madden JA, Pesic M, Rusch NJ. High blood pressure upregulates arterial L-type Ca^{2+} channels: is membrane depolarization the signal? Circ Res 2004;94:e97–e104. [PubMed: 15131006]

19. Albarwani S, Nemetz LT, Madden JA, Tobin AA, England SK, Pratt PF, Rusch NJ. Voltage-gated K^{+} channels in rat small cerebral arteries: molecular identity of the functional channels. J Physiol 2003;551:751–763. [PubMed: 12815189]

20. Pratt PF, Bonnet S, Ludwig LM, Bonnet P, Rusch NJ. Upregulation of L-type Ca^{2+} channels in mesenteric and skeletal arteries of SHR. Hypertension 2002;40:214–219. [PubMed: 12154116]

21. Jackson WF, Huebner JM, Rusch NJ. Enzymatic isolation and characterization of single vascular smooth muscle cells from cremasteric arterioles. Microcirculation 1997;4:35–50. [PubMed: 9110282]

22. Liu X, Rusch NJ, Striessnig J, Sarna SK. Down-regulation of L-type calcium channels in inflamed circular smooth muscle cells of the canine colon. Gastroenterology 2001;120:480–489. [PubMed: 11159888]

23. Rondelli CM, Szasz IT, Kayal A, Thakali K, Watson RE, Rovner AS, Eddinger TJ, Fink GD, Watts SW. Preferential myosin heavy chain isoform B Expression may contribute to the faster velocity of contraction in veins versus arteries. J Vasc Res 2007;44:264–272. [PubMed: 17377397]

24. Kass RS, Sanguinetti MC. Inactivation of calcium channel current in the calf cardiac Purkinje fiber. Evidence for voltage- and calcium-mediated mechanisms. J Gen Physiol 1984;84:705–726. [PubMed: 6096480]

25. Romanin C, Karlsson JO, Schindler H. Activity of cardiac L-type Ca^{2+} channels is sensitive to cytoplasmic calcium. Pflugers Arch 1992;421:516–518. [PubMed: 1334257]

26. Haack JA, Rosenberg RL. Calcium-dependent inactivation of L-type calcium channels in planar lipid bilayers. Biophys J 1994;66:1051–1060. [PubMed: 8038377]

27. Devine CE, Somlyo AV, Somlyo AP. Sarcoplasmic reticulum and excitation-contraction coupling in mammalian smooth muscles. J Cell Biol 1972;52:690–718. [PubMed: 5061887]

28. Laporte R, Hui A, Laher I. Pharmacological modulation of sarcoplasmic reticulum function in smooth muscle. Pharmacol Rev 2004;56:439–513. [PubMed: 15602008]

29. Liu H, Sperelakis N. Tyrosine kinases modulate the activity of single L-type calcium channels in vascular smooth muscle cells from rat portal vein. Can J Physiol Pharmacol 1997;75:1063–1068. [PubMed: 9365814]

30. Ohya Y, Tsuchihashi T, Kagiyama S, Abe I, Fujishima M. Single L-type calcium channels in smooth muscle cells from resistance arteries of spontaneously hypertensive rats. Hypertension 1998;31:1125–1129. [PubMed: 9576124]

31. Bertolissi M, De Monte A, Giordano F. Comparison of intravenous nifedipine and sodium nitroprusside for treatment of acute hypertension after cardiac surgery. Minerva Anestesiol 1998;64:321–328. [PubMed: 9796241]

32. Kelbaek H, Aldershvile J, Skagen K, Hildebrandt P, Nielsen SL. Pre- and afterload reduction in chronic mitral regurgitation: a double-blind randomized placebo-controlled trial of the acute and 2 weeks effect of nifedipine or isosorbide dinitrate treatment on left ventricular function and the severity of mitral regurgitation. Br J Clin Pharmacol 1996;41:493–497. [PubMed: 8799512]

33. Klugmann S, Salvi A, Camerini F. Haemodynamic effects of nifedipine in heart failure. Br Heart J 1980;43:440–446. [PubMed: 7397044]

34. Harder DR, Contney SJ, Willems WJ, Stekiel WJ. Norepinephrine effect on in situ venous membrane potential in spontaneously hypertensive rats. Am J Physiol 1981;240:H837–H842. [PubMed: 7246748]

35. Stekiel WJ, Contney SJ, Lombard JH. Small vessel membrane potential, sympathetic input, and electrogenic pump rate in SHR. Am J Physiol 1986;250:C547–C556. [PubMed: 3008565]

36. Chen Q, Cannell M, van Breemen C. The superficial buffer barrier in vascular smooth muscle. Can J Physiol Pharmacol 1992;70:509–514. [PubMed: 1386774]

37. Erickson MG, Alseikhan BA, Peterson BZ, Yue DT. Preassociation of calmodulin with voltage-gated Ca^{2+} channels revealed by FRET in single living cells. Neuron 2001;31:973–985. [PubMed: 11580897]

38. Pitt GS, Zühlke RD, Hudmon A, Schulman H, Reuter H, Tsien RW. Molecular basis of calmodulin tethering and Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels. J Biol Chem 2001;276:30794–30802. [PubMed: 11408490]

39. Takamatsu H, Nagao T, Ichijo H, Adachi-Akahane S. L-type Ca^{2+} channels serve as a sensor of the SR Ca^{2+} for tuning the efficacy of Ca^{2+}-induced Ca^{2+} release in rat ventricular myocytes. J Physiol 2003;552:415–424. [PubMed: 14561825]

40. Heaps CL, Bowles DK, Sturek M, Laughlin MH, Parker JL. Enhanced L-type Ca^{2+} channel current density in coronary smooth muscle of exercise-trained pigs is compensated to limit myoplasmic free Ca^{2+} accumulation. J Physiol 2000;528:435–445. [PubMed: 11060122]

41. Ganitkevich, VYa; Shuba, MF.; Smirnov, SV. Inactivation of calcium channels in single vascular and visceral smooth muscle cells of the guinea-pig. Gen Physiol Biophys 1991;10:137–161. [PubMed: 1650728]

42. Petkov GV, Fusi F, Saponara S, Gagov HS, Sgaragli GP, Boev KK. Characterization of voltage-gated calcium currents in freshly isolated smooth muscle cells from rat tail main artery. Acta Physiol Scand 2001;173:257–265. [PubMed: 11736688]

43. Chalmers S, Olson ML, MacMillan D, Rainbow RD, McCarron JG. Ion channels in smooth muscle: regulation by the sarcoplasmic reticulum and mitochondria. Cell Calcium 2007;42(4–5):447–466. [PubMed: 17629940]

44. Thorneloe KS, Nelson MT. Ion channels in smooth muscle: regulators of intracellular calcium and contractility. Can J Physiol Pharmacol 2005;83(3):215–242. [PubMed: 15870837]

45. Ureña J, del Valle-Rodríguez A, López-Barneo J. Metabotropic Ca^{2+} channel-induced calcium release in vascular smooth muscle. Cell Calcium 2007;42(4–5):513–520. [PubMed: 17559931]

46. Wellman GC, Nathan DJ, Saundry CM, Perez G, Bonev AD, Penar PL, Tranmer BI, Nelson MT. Ca^{2+} sparks and their function in human cerebral arteries. Stroke 2002;33:802–808. [PubMed: 11872907]

47. Berkels R, Egink G, Marsen TA, Bartels H, Roesen R, Klaus W. Nifedipine increases endothelial nitric oxide bioavailability by antioxidative mechanisms. Hypertension 2001;37:240–245. [PubMed: 11230278]

48. Brovkovych VV, Kalinowski L, Muller-Peddinghaus R, Malinski T. Synergistic antihypertensive effects of nifedipine on endothelium: Concurrent release of NO and scavenging of superoxide. Hypertension 2001;37:34–39. [PubMed: 11208753]

49. Flink MT, Atchison WD. Iberiotoxin-induced block of Ca^{2+}-activated K^+ channels induces dihydropyridine sensitivity of ACh release from mammalian motor nerve terminals. J Pharmacol Exp Ther 2003;305:646–652. [PubMed: 12606686]
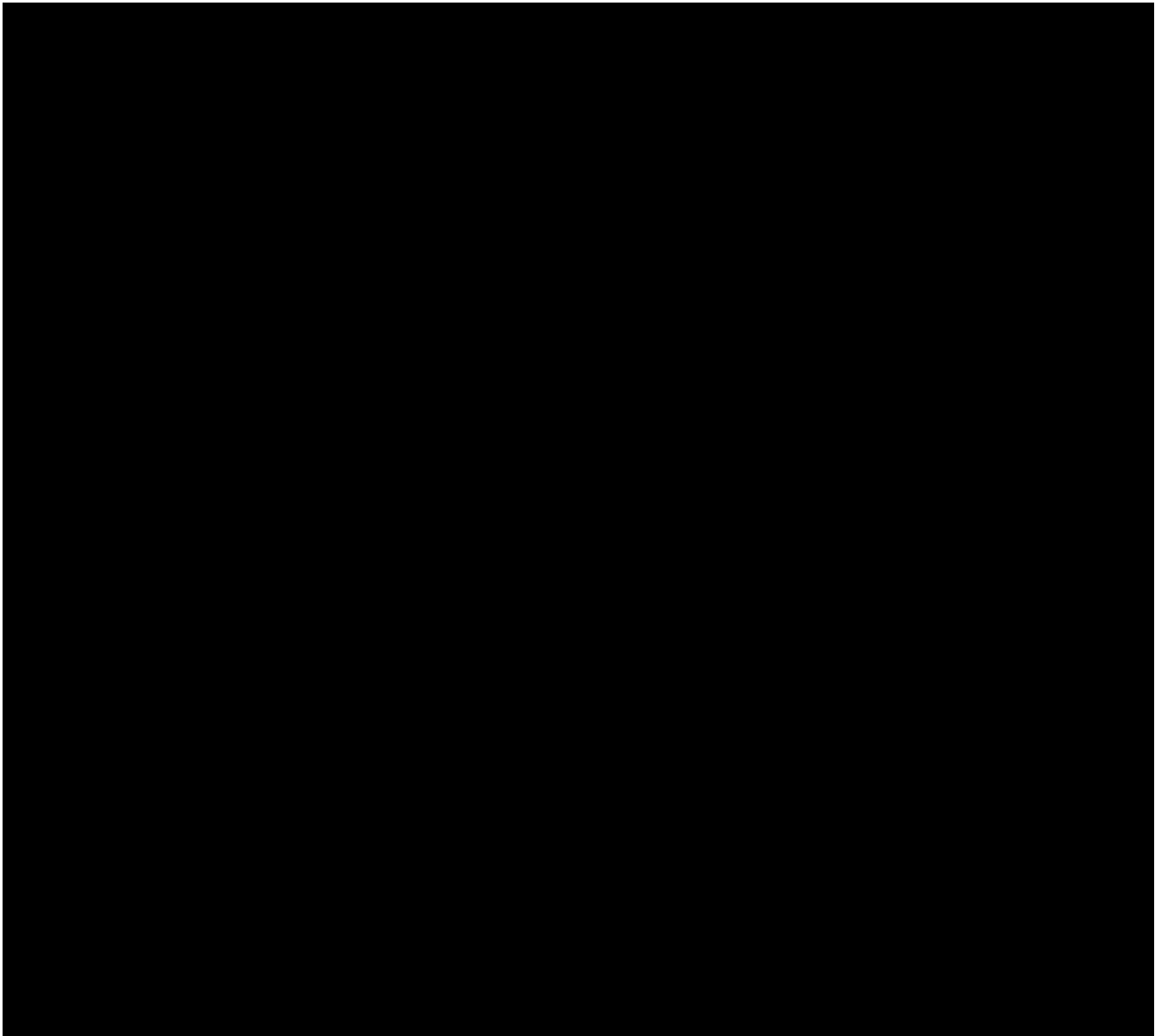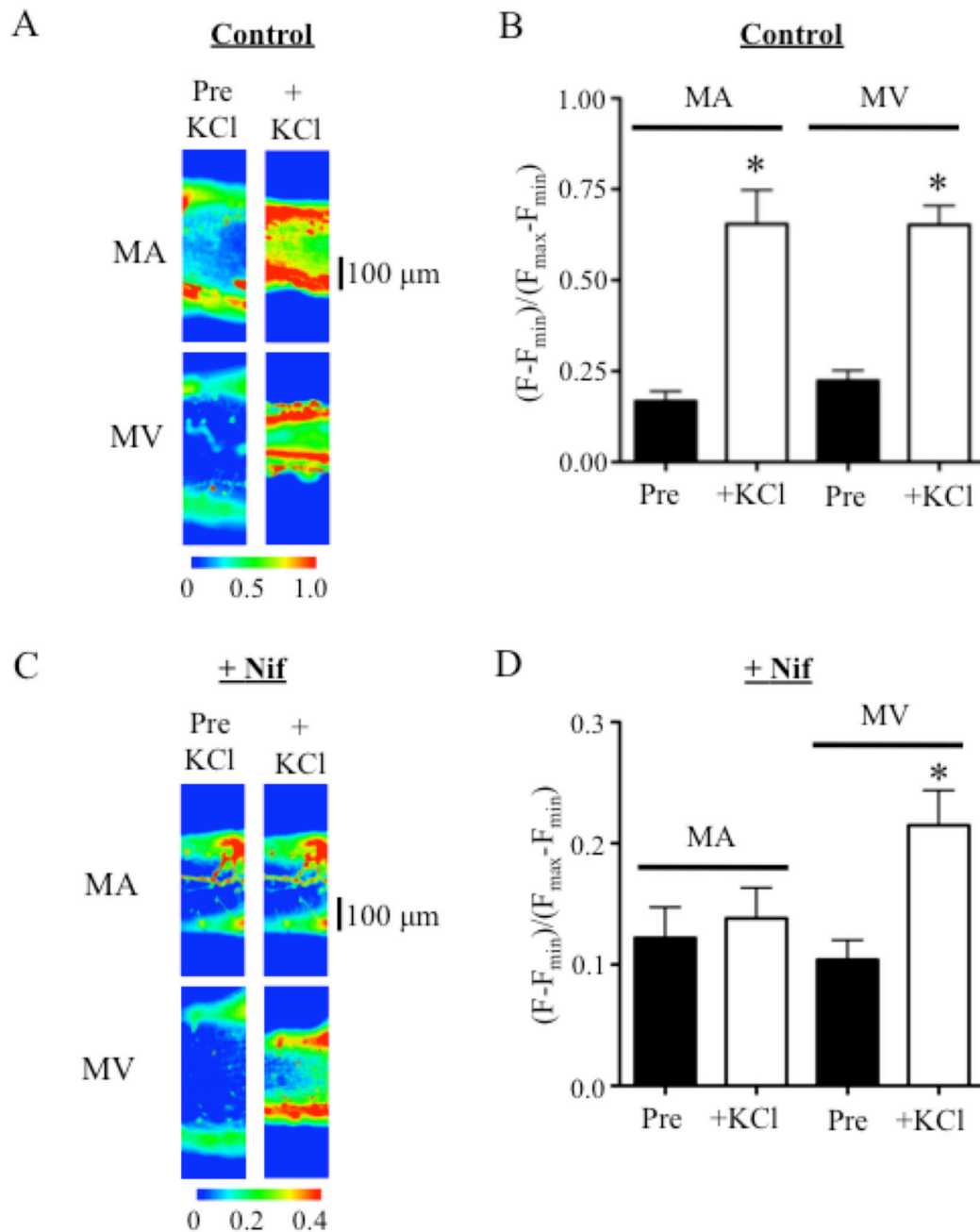
Figure 1.
Depolarization of rat mesenteric arteries (MA) and veins (MV) by 60 mmol/L KCl. (**A**, **B**)
Membrane potential tracings in isolated, pressurized mesenteric arteries (MA) and mesenteric
veins (MV), respectively, under control (Con) drug-free conditions and after incubation with
KCl. (**C**) Resting membrane potential and amplitude of KCl-induced depolarization was not
significantly different between MA and MV (n = 4–6). * = significant difference between Con
and KCl (MA), † = significant difference between Con and KCl (MV) ($p<0.05$).
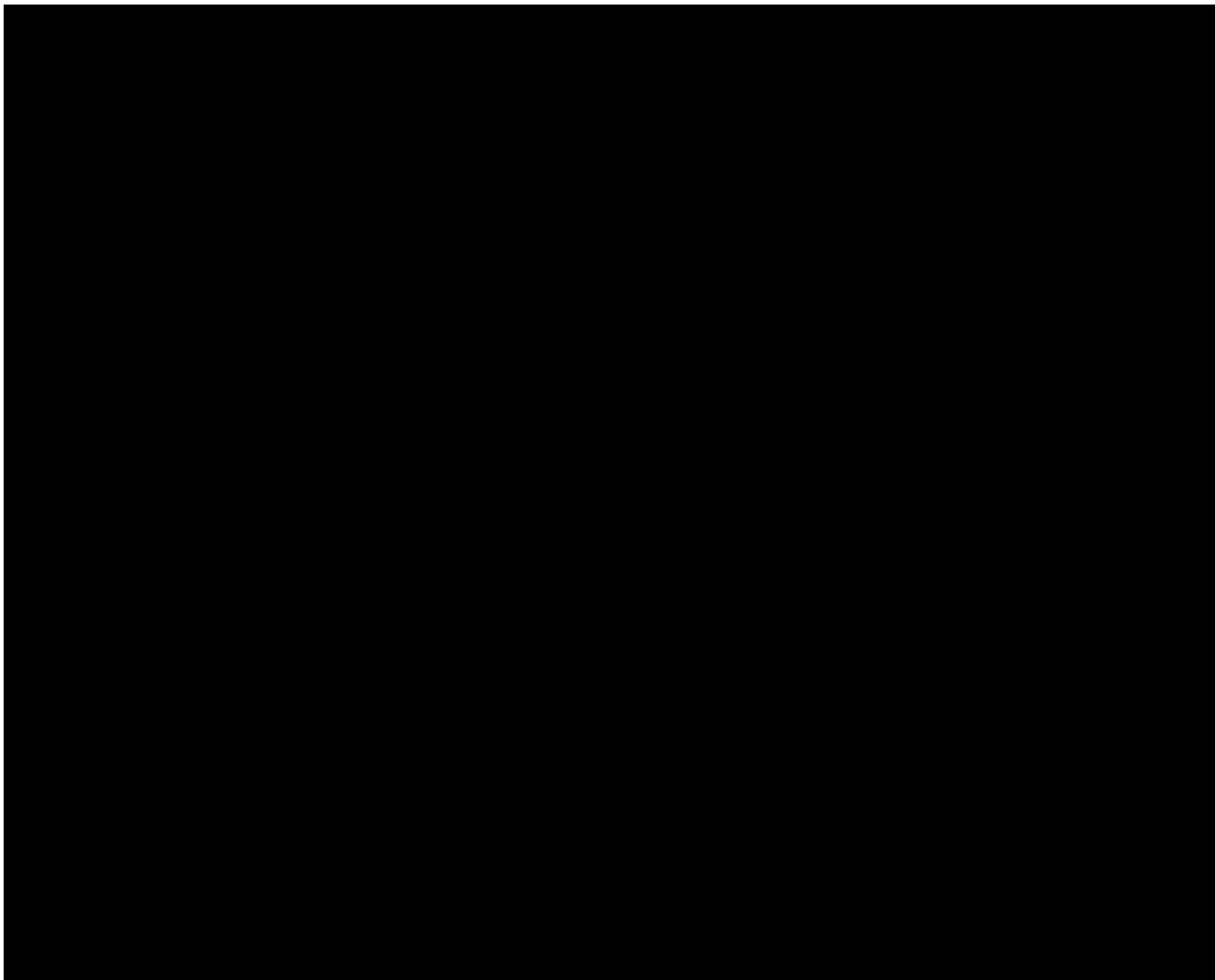
Figure 2.
KCl -induced constrictions in the mesenteric vein (MV) but not artery (MA) are resistant to the LTCC blockers, nifedipine and diltiazem. (**A**) Nifedipine (Nif) fully blunts depolarization-induced constriction in MA whereas the constriction in MV was only mildly attenuated. Panels (left to right): resting diameters (Control, Con), KCl (60 mmol/L)-induced constriction, and KCl–induced constriction in the presence of 1 µmol/L Nif. (**B**) Averaged values of 5 experiments as in **Fig. 2A**. * = significant difference between Control and KCl in MA, † = significant difference between Control and KCl in MV (p<0.05). (**C**) Nifedipine (1 nmol/L – 1 µmol/L) and (**D**) diltiazem (10 nmol/L – 10 µmol/L) concentration-dependently dilates KCl (60 mmol/L)-induced constrictions of MA but not MV (n = 4 – 5). * = significant dilation of MA (p<0.05).
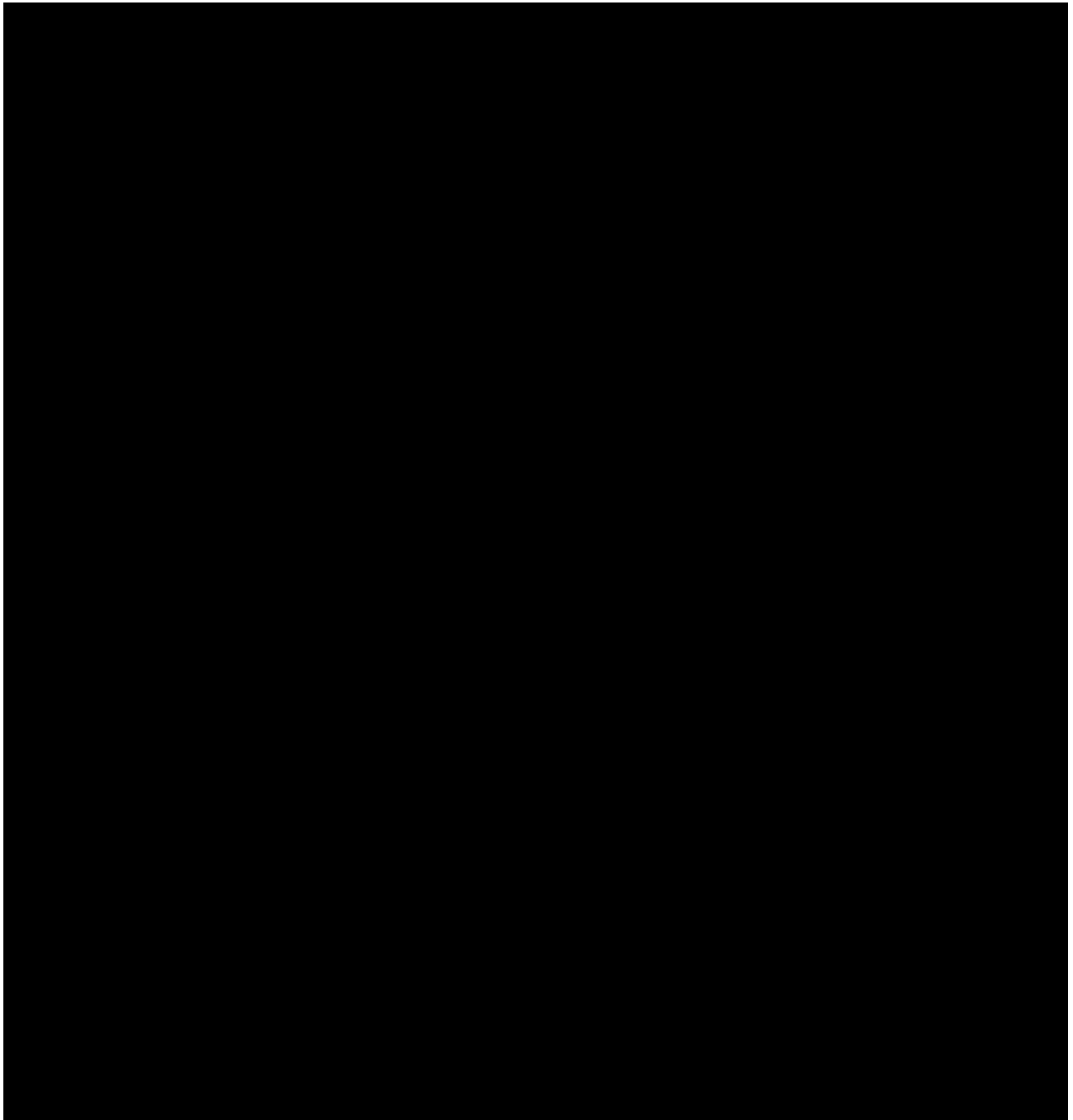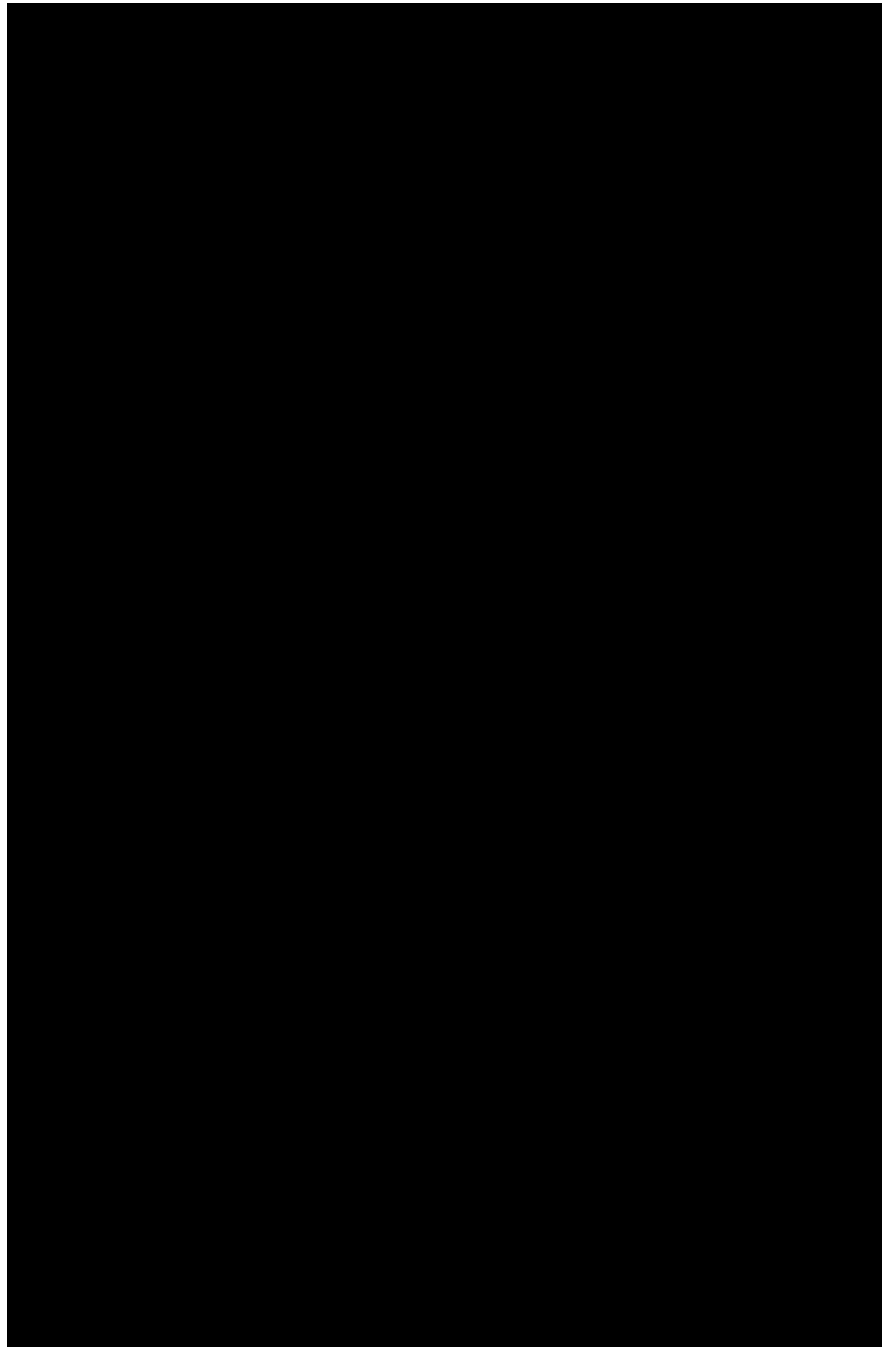
Figure 3.
Nifedipine (Nif) blocks KCl-induced elevations of $[Ca]_i$ in pressurized MA but not MV. (**A, B**) KCl (60 mmol/L) stimulates a similar rise of $[Ca]_i$ in pressurized MA and MV loaded with fluo-4 (2 µmol/L) (n = 5–7). (**C, D**) Nif (10 µmol/L, +Nif) prevents KCl-induced increases in $[Ca]_i$ in MA, while $[Ca]_i$ still rises 2.0-fold in MV. (n = 5) * = significant difference from pre-KCl levels (Pre) compared to the maximum response after KCl (+KCl) in the same preparation (p<0.05).
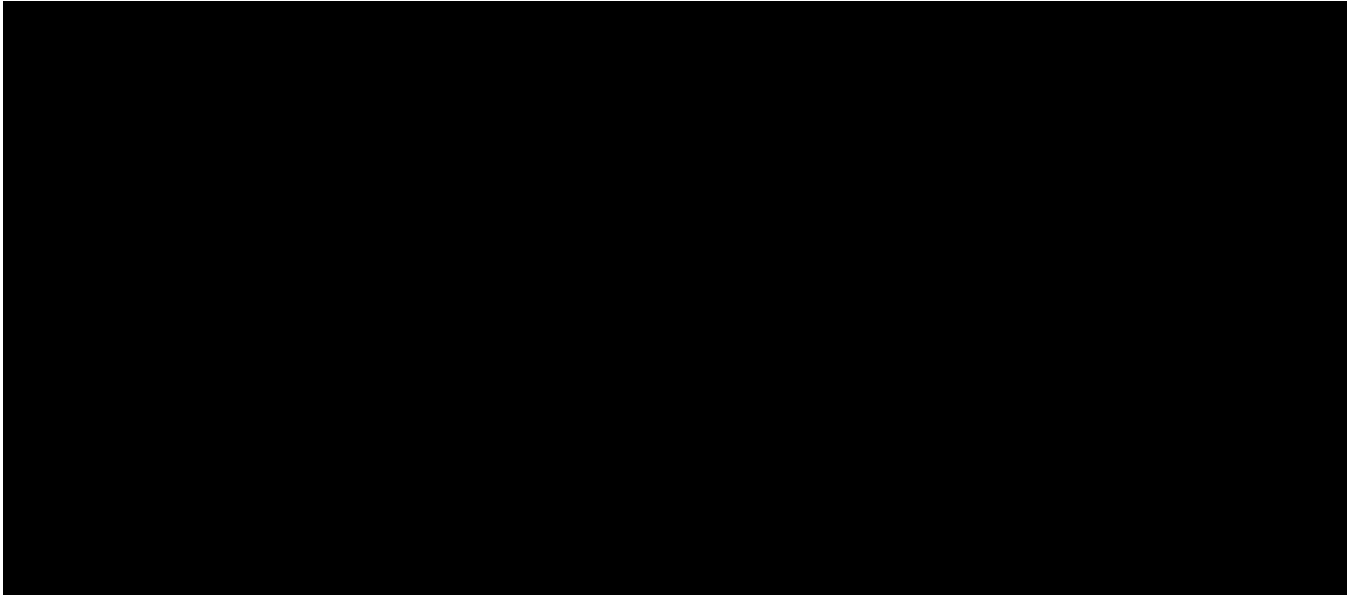
Figure 4.
(**A**) Western blot analysis of the α_{1C} pore-forming subunit of the LTCC (top panel) and smooth muscle α-actin (lower panel) in protein lysates from rat mesenteric artery (MA) and vein (MV). Forty (40) µg of MA or MV lysate (pooled from 2 rats) was added to each lane. (**B**) After normalization to smooth muscle-specific α-actin, α_{1C} expression was not significantly different between MA and MV (n = 4).
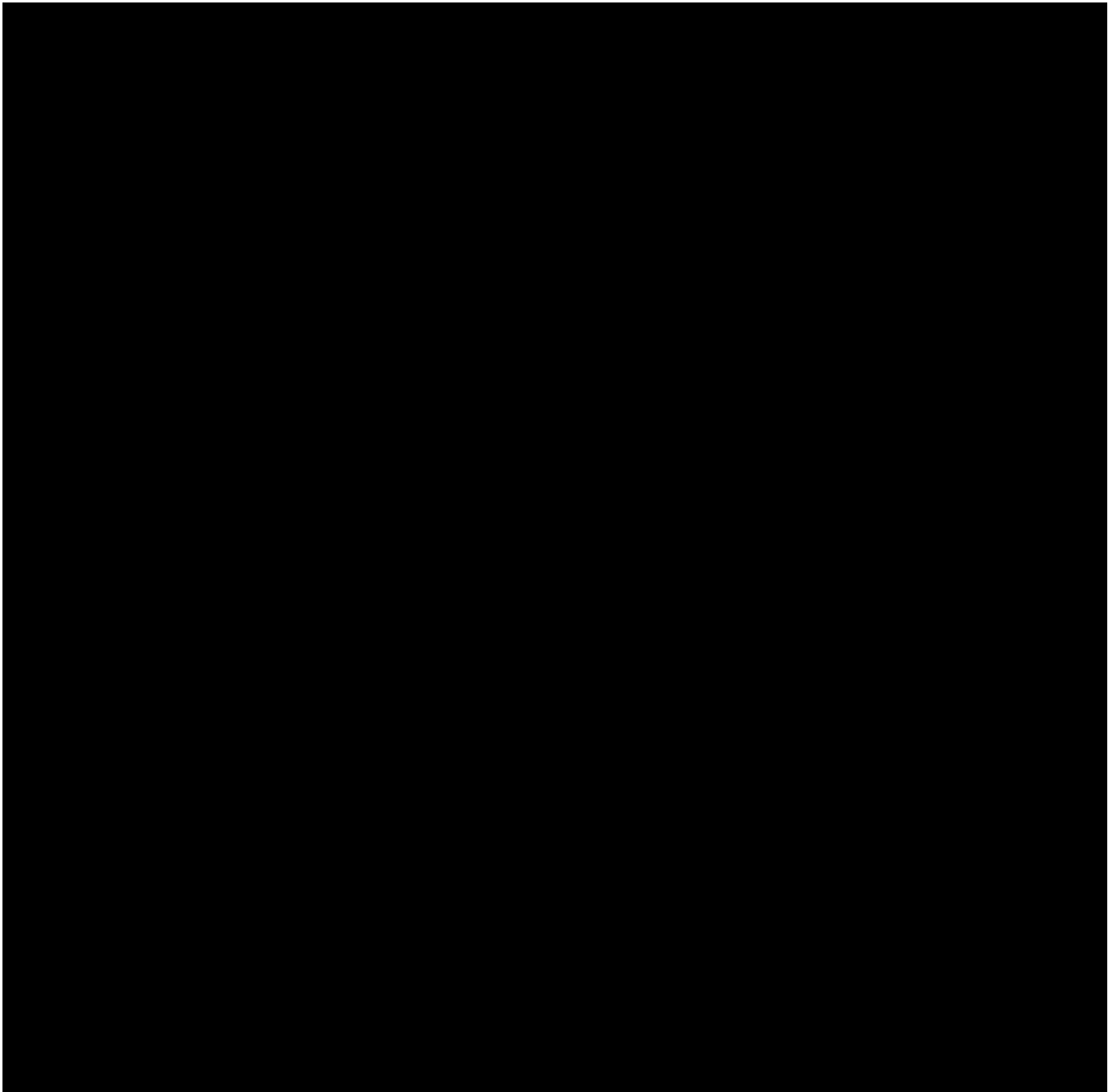
Figure 5.
Similar properties and densities of whole-cell LTCC currents are observed in SMCs of rat MA and MV. (**A**) Current-voltage (I–V) relationships for Ca^{2+} channel currents in MA and MV reveal similar current densities (n = 14, 7). (**B**) Voltage-dependent activation and inactivation were not significantly different between SMCs of MA (n = 9, 14) and MV (n = 5, 9). (**C–E**) Arterial SMCs treated with nifedipine (Nif, 100 nmol/L) showed a 56% reduction in LTCC channel current density (n = 9–14). (**F–H**) Venous SMCs showed a similar level of nifedipine–induced block of 55% (n = 4–7). * = significant difference from control (Con) in the same preparation (p<0.05).

Figure 6.
Pharmacological depletion of intracellular Ca^{2+} stores restores nifedipine (Nif) sensitivity to depolarization-induced contractions in rat MV. Blocking store-operated Ca^{2+} release with Gd^{3+} (**A**, 100 µmol/L) or La^{3+} (**B**, 100 µmol/L) did not reduce venous KCl-induced constriction or restore sensitivity to Nif in veins. (n = 4–5) (**C**) KCl-induced constrictions after Thaps were blocked 80% by Nif (10 µmol/L). (n = 6) (**D**) Store depletion by caffeine (Caf, 10 mmol/L) also resulted in sensitivity to block by Nif. (**E**) Using ryanodine (Ryan, 10 nmol/L) to deplete intracellular Ca^{2+} stores also resulted in MV sensitivity to nifedipine, (n = 5–6), as did incubating with 2-APB (100 µmol/L) (**F**) (n = 6). * = significant difference from Control (Con),

$p < 0.05$; † = significant difference between Thaps, Caf, Ryan, or 2-APB in the presence and absence of Nif.

Figure 7.
Ca^{2+} inactivation may silence LTCCs during depolarization of venous smooth muscle (**A**) Tracings of single LTCC currents in freshly isolated SMCs of rat MA revealed frequent openings in the cell-attached mode. (**B**) Under the same conditions, venous SMCs showed only rare openings, but activity was greatly increased after chelating intracellular Ca^{2+} with BAPTA-AM (**C**, 100 µmol/L). (c = closed state). (**D**) The open-state probability (NP_O) was significantly higher in control MA compared to control MV and chelating intracellular Ca^{2+} with BAPTA-AM significantly increased MV NP_O (n = 6–10). (**E**) The single channel conductance was not significantly different between MA and MV (n = 30–40 openings from

3 different cell isolations). * = significant difference from MA Control ($p<0.05$) (n = 30–40 openings from 3 different cell isolations). † = significantly different from MV Control ($p<0.05$).