

# Ca<sub>v</sub>3.1 T-Type Ca<sup>2+</sup> Channels Contribute to Myogenic Signaling in Rat Retinal Arterioles

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**PURPOSE.** Although L-type Ca<sup>2+</sup> channels are known to play a key role in the myogenic reactivity of retinal arterial vessels, the involvement of other types of voltage-gated Ca<sup>2+</sup> channels in this process remains unknown. In the present study we have investigated the contribution of T-type Ca<sup>2+</sup> channels to myogenic signaling in arterioles of the rat retinal microcirculation.

**METHODS.** Confocal immunolabeling of whole-mount preparations was used to investigate the localization of Ca<sub>v</sub>3.1-3 channels in retinal arteriolar smooth muscle cells. T-type currents and the contribution of T-type channels to myogenic signaling were assessed by whole-cell patch-clamp recording and pressure myography of isolated retinal arteriole segments.

**RESULTS.** Strong immunolabeling for Ca<sub>v</sub>3.1 was observed on the plasma membrane of retinal arteriolar smooth muscle cells. In contrast, no expression of Ca<sub>v</sub>3.2 or Ca<sub>v</sub>3.3 could be detected in retinal arterioles, although these channels were present on glial cell end-feet surrounding the vessels and retinal ganglion cells, respectively. TTA-A2-sensitive T-type currents were recorded in retinal arteriolar myocytes with biophysical properties distinct from those of the L-type currents present in these cells. Inhibition of T-type channels using TTA-A2 or ML-218 diluted isolated, myogenically active, retinal arterioles.

**CONCLUSIONS.** Ca<sub>v</sub>3.1 T-type Ca<sup>2+</sup> channels are functionally expressed on arteriolar smooth muscle cells of retinal arterioles and play an important role in myogenic signaling in these vessels. The work has important implications concerning our understanding of the mechanisms controlling blood flow autoregulation in the retina and its disruption during ocular disease.

Keywords: T-type channels, retinal arterioles, myogenic response

It has long been known that alterations in perfusion pressure generate compensatory changes in the diameters of retinal vessels that result in minor or no effects on overall retinal blood flow.<sup>1</sup> A reduced ability of the retina to autoregulate in this manner has been associated with a number of ocular diseases including diabetes, glaucoma, and age-related macular degeneration (AMD).<sup>2-6</sup> Vascular autoregulation in the retina is driven by a number of different mechanisms, with both myogenic and metabolic components involved.<sup>7</sup> The importance of myogenic mechanisms in the regulation of retinal blood flow has been highlighted by several studies performed on isolated retinal arteries and arterioles, showing vasoconstriction or vasodilation in response to either increases or decreases in intravascular pressure, respectively.<sup>8-11</sup>

According to the classical view, myogenic responses occur when pressure-induced distension of the vessel wall triggers activation of stretch-activated cation channels on the resident vascular smooth muscle cells, resulting in cell membrane potential depolarization and increased Ca<sup>2+</sup> influx through voltage-operated L-type Ca<sup>2+</sup> channels.<sup>12-14</sup> This Ca<sup>2+</sup> influx in turn leads to vascular smooth muscle cell contraction and vessel constriction, both directly by increasing the cytosolic Ca<sup>2+</sup> concentration and indirectly by triggering the release of Ca<sup>2+</sup> from the sarcoplasmic reticulum via ryanodine (RyR) and inositol trisphosphate receptor channels.<sup>12-14</sup> We have previously shown that L-type Ca<sup>2+</sup> channels and RyR receptors play a

key role in myogenic signaling in retinal arterioles.<sup>10,11</sup> These vessels also express voltage-gated K<sub>v</sub>1.5 channels<sup>11,15</sup> and large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels (BK channels),<sup>11,16</sup> which act as a brake on the myogenic response mechanism by limiting the degree of pressure-induced depolarization. Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels have also been found to contribute to the contractile state of these vessels, although their primary role appears to be in the modulation of agonist-induced tone rather than myogenic signaling.<sup>17,18</sup>

Recent studies in other vascular beds have suggested that additional ion channels, such as T-type Ca<sup>2+</sup> channels, might function as alternative voltage-dependent Ca<sup>2+</sup> influx pathways that may be involved in the development and maintenance of myogenic tone.<sup>19,20</sup> Molecular analyses have identified three different isoforms of pore-forming T-type Ca<sup>2+</sup> channels, namely, Ca<sub>v</sub>3.1, Ca<sub>v</sub>3.2, and Ca<sub>v</sub>3.3.<sup>21,22</sup> Several studies have found some of these to be expressed and functional in resistance vessels,<sup>23-25</sup> contributing in some cases to myogenic signaling.<sup>20,23,26-28</sup> T-type Ca<sup>2+</sup> channels appear to be particularly important in mediating myogenic responses in small arterioles where, despite a loss or decrease in L-type Ca<sup>2+</sup> currents, development of myogenic tone is still observed.<sup>26,29,30</sup>

In the present study, we have investigated for the first time the possible involvement of T-type Ca<sup>2+</sup> channels in the myogenic reactivity of rat retinal arterioles. Using immunohis-

**TABLE 1.** Antibodies and Dilutions Used to Investigate Ca<sub>v</sub>3.1-3 Protein Expression

Protein	Antibody (Company)	Epitope (Species)	Dilution
Ca <sub>v</sub> 3.1	ACC-021 (Alomone, Jerusalem, Israel)	AA1-22 (rat)	1:200
Ca <sub>v</sub> 3.2	ACC-025 (Alomone)	AA581-595 (human)	1:200
Ca <sub>v</sub> 3.3	OSC00263W (Osenses, Keswick, Australia)	AA450-500 (human)	1:200

tochemistry, we show that although all three T-type Ca<sup>2+</sup> channel isoforms are expressed in retinal tissue, only Ca<sub>v</sub>3.1 localizes to retinal arteriolar smooth muscle cells. We also demonstrate the functional expression of T-type Ca<sup>2+</sup> channels on the plasma membrane of these cells and characterize their biophysical and pharmacological properties. Finally, we show that two structurally distinct T-type Ca<sup>2+</sup> channel blockers dilate isolated pressurized retinal arterioles under conditions of steady-state myogenic tone, suggesting that these channels make a significant contribution to myogenic signaling in these vessels. These findings have important implications for our understanding of the mechanisms controlling retinal blood flow in both health and disease.

## METHODS

Animal use conformed to the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Sprague-Dawley rats (8–12 weeks of age; 200–250 g; Harlan, Bicester, UK) were euthanized with CO<sub>2</sub> in accordance with guidelines contained within the UK Animals (Scientific Procedures) Act of 1986 and approved by the Queen's University of Belfast Animal Welfare and Ethical Review Body.

## Immunohistochemistry

Immunohistochemistry was carried out on retinal arterioles embedded within retinal flat-mount preparations as described previously.<sup>16</sup> Briefly, freshly enucleated eyes were placed in low-Ca<sup>2+</sup> Hanks' solution (see "Drugs and Solutions") and hemisected along the ora serrata. The vitreous was removed and the posterior eye cup fixed with 4% paraformaldehyde (PFA) for 20 minutes and then washed extensively in phosphate-buffered saline (PBS) for 1 hour. Retinas were subsequently detached and incubated overnight in permeabilization and blocking buffer (0.05% Triton X-100 and 1% donkey serum in PBS; Sigma, Poole, Dorset, UK, and Millipore, Watford, UK) and then incubated in primary antibody in permeabilization and blocking buffer for 3 days at 4°C. Primary antibodies, selected on the basis of their specificity toward rat Ca<sub>v</sub>3.1-3 channels (Table 1), were employed in conjunction with mouse anti- $\alpha$ -smooth muscle actin ( $\alpha$ SMA) antibody (1:200; Sigma) to positively identify vascular smooth muscle cells or isolectin B4 (1:50; Sigma)<sup>31</sup> to label vascular plasma membranes. Following extensive washing (4 hours at 21°C in PBS), donkey anti-rabbit IgG labeled with Alexa-488 (1:200 in permeabilization and blocking buffer; Life Technologies, Paisley UK) or Streptavidin 568 (1:500; Life Technologies) was used for Ca<sub>v</sub>3.1-3 channel and vascular cell membrane detection, respectively (incubated 4°C overnight). The  $\alpha$ SMA primary antibody was conjugated to Cy3, so no secondary antibody was required. Nuclei were labeled with the far-red nuclear stain TOPRO3 (1:1000; Life Technologies; pseudocolored blue in relevant images). The immunohistochemistry for each of the Ca<sub>v</sub>3.1-3 isoforms was repeated using four to eight retinas from at least four different animals. Secondary-only controls and blocking peptide experiments for Ca<sub>v</sub>3.1 (10  $\mu$ g/mL blocking peptide) were also performed. Images were acquired using a Leica SP5 confocal laser scanning microscope

(HCX PL APO 63X oil-immersion lens; Leica Geosystems, Heerbrugg, Switzerland) equipped with argon, HeNe 543, and HeNe 633 lasers. Images were captured in sequential scanning mode with emission wavelengths appropriate for the fluorophores used to reduce overlap (bleed-through) of signals.

## Isolated Arteriolar Preparation

For electrophysiology and pressure myography experiments, isolated retinal arteriole segments were used.<sup>32</sup> Retinas were dissected from freshly enucleated eyes, placed in low-Ca<sup>2+</sup> Hanks' solution, and mechanically triturated using a fire-polished Pasteur pipette. Homogenate was pipetted into a glass-bottomed recording bath mounted on an inverted microscope (Eclipse TE300; Nikon, Tokyo, Japan), and isolated retinal arterioles (length, 100–4000  $\mu$ m; outer diameter, 15–40  $\mu$ m) were anchored down with tungsten wire slips as described previously.<sup>16</sup> Arterioles were continuously superfused with normal Hanks' solution at 37°C during experimentation. Drugs were delivered via a gravity-fed multichannel perfusion manifold connected to a single outlet needle that was positioned adjacent to the vessel of interest.

## Electrophysiological Recordings

Whole-cell membrane currents were recorded from individual arteriolar smooth muscle cells still embedded within their parental arterioles using the perforated patch-clamp technique.<sup>33</sup> Prior to patching, the vessels were superfused with collagenase 1A (0.1 mg/mL, 10 minutes; Sigma), protease type XIV (0.01 mg/mL, 10 minutes; Sigma), and DNase I (0.02 mg/mL, 5 minutes; Millipore) in low-Ca<sup>2+</sup> Hanks' solution to remove surface basal lamina, electrically uncouple endothelial cells from overlying arteriolar smooth muscle cells and individual smooth muscle cells from one another, and remove extraneous DNA.<sup>15,33</sup> Once the whole-cell perforated configuration was acquired, the external Hanks' solution was switched to divalent-free solution (see "Drugs and Solutions") to enhance the magnitude of currents flowing through voltage-gated Ca<sup>2+</sup> channels and to prevent interference by Ca<sup>2+</sup>-activated Cl<sup>-</sup> currents.<sup>34,35</sup> Pipettes pulled from filamented borosilicate glass capillaries (1.5 mm outer diameter w 1.17 mm inner diameter; Harvard Instruments, Kent, UK) were fire polished to resistances of 1 to 2 M $\Omega$ . Membrane currents were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA, USA), low pass filtered at 0.5 kHz, and sampled at 2 kHz. Voltage step protocols were applied using pClamp (version 10.2; Molecular Devices) via a Digidata 1440A interface (Molecular Devices). Leak currents were subtracted offline from the active currents using the response to a hyperpolarizing step from a holding potential of –80 mV to –90 mV as the correction signal. Calculation and plotting of difference curves, offline leak subtraction, and fitting of exponentials were performed using purpose-written software in R.<sup>36</sup>

## Arteriolar Myography

The involvement of T-type Ca<sup>2+</sup> channels in the generation of arteriolar myogenic tone was assessed using pressure myogra-

TABLE 2. Effects of T-Type Blockers on the Diameters (in  $\mu\text{m}$ ) of Pressurized Retinal Arterioles

Vehicle/Inhibitor	After Steady-State Tone Development	In Presence of Vehicle/Inhibitor	Passive Diameter	% Diameter Change
DMSO, $n = 10$	29.5 $\pm$ 1.6	29.4 $\pm$ 1.5	30.6 $\pm$ 1.4	NA
TTA-A2, $n = 16$	29.1 $\pm$ 2.4	29.6 $\pm$ 2.5	30.1 $\pm$ 2.5	45.4 $\pm$ 3.9
ML-218, $n = 13$	31.3 $\pm$ 2.7	31.8 $\pm$ 2.7	32.3 $\pm$ 2.7	54.1 $\pm$ 4.2

phy as previously described.<sup>16</sup> A tungsten wire slip was laid on the arteriole, anchoring and occluding one end. The vessel was then superfused with Ca<sup>2+</sup>-free Hanks' solution. The open end was cannulated using a glass micropipette (1.5 mm o.d., 0.86 i.d. tapered to a tip diameter of 2–5  $\mu\text{m}$ ) filled with Ca<sup>2+</sup>-free Hanks' solution, using a patch-clamp electrode holder and micromanipulator (Molecular Devices). Once the pipette tip had been appropriately positioned, the pipette was advanced so as to wedge it tightly into the lumen of the vessel. To ensure there was no significant flow through the vessel or leakage of fluid around the cannulation site, a small air bubble was introduced into the tubing connecting the cannulating pipette to the manometer. Only vessels in which this bubble remained relatively static following pressurization were used for experimentation. Following introduction of the pipette, the vessel was superfused with normal Hanks' solution for 10 to 15 minutes, allowing the pipette to seal to the inner vessel wall. Vessels were then pressurized to 40 mm Hg and left at that pressure until stable myogenic tone had fully developed (usually  $\sim$ 10 minutes). Intraluminal pressure was regulated by using a manometer connected to the cannulating micropipette (Riester "Big Ben" pressure manometer; Riester, Jungingen, Germany). The maximum passive diameter of the vessels was determined by application of the myosin light chain kinase inhibitor wortmannin (10  $\mu\text{M}$ )<sup>37,38</sup> in the presence of Ca<sup>2+</sup>-free Hanks' solution at the end of the experiment. This value was used to normalize vessel diameter data across individual arterioles. Vessels were viewed under a  $\times 20$ , Numerical Aperture 0.4 objective, and images (saved as BMP images of 1280  $\times$  1024 pixels; 8-bit; 1.2 MB) were captured at a rate of 140 images per minute using a MCN-B013-U USB camera (Mightex, Pleasanton, CA, USA). Acquisition was carried out using custom software implemented in Delphi.<sup>16</sup> The measurement of vessel diameters and tracking of diameter changes were performed using the MyoTracker software package.<sup>39</sup>

### Drugs and Solutions

The composition of the solutions used was as follows (in mM): normal Hanks' solution—140, NaCl; 6, KCl; 5, D-glucose; 2, CaCl<sub>2</sub>; 1.3, MgCl<sub>2</sub>; 10, HEPES; pH set to 7.4 with NaOH; divalent-free solution—120, NaCl; 5, KCl; 5, D-glucose; 10, HEPES; 5, EGTA; 20, tetraethylammonium chloride (TEA); K<sup>+</sup>-based internal pipette solution; 52, KCl; 80, gluconic acid; 80, KOH; 1, MgCl<sub>2</sub>; 0.5, EGTA; 10, HEPES; pH set to 7.2 with KOH. The low-Ca<sup>2+</sup> Hanks' solution was of Hanks' composition but contained only 0.1 mM Ca<sup>2+</sup>. For the Ca<sup>2+</sup>-free Hanks' solution, Ca<sup>2+</sup> was omitted. Amphotericin B (final concentration of 0.39 mM) was dissolved in the pipette solutions as the pore-forming agent.

Unless otherwise stated, stock solutions of drugs were initially prepared in DMSO and then diluted to the final concentration. The final bath concentration of DMSO was  $\leq$ 0.1%. In vehicle control experiments, application of DMSO, at the maximal concentration used in these studies, had no effect on arteriolar diameter (Table 2). Amphotericin B, nimodipine, TEA, and EGTA were obtained from Sigma; TTA-

A2, ML-218, and wortmannin were purchased from Alomone Labs (Jerusalem, Israel). HEPES was obtained from Melford Laboratories (Ipswich, UK).

### Statistical Analysis

Data are presented as the mean  $\pm$  SEM. Statistical significance was determined by using either paired *t*-tests (when data conformed to Kolmogorov-Smirnov normality tests) or the Wilcoxon matched-pairs signed rank test for which no assumptions about normality were made. In all comparisons, the 95% level was accepted as statistically significant. Analysis was carried out using Prism 5 for Windows (version 5.03; GraphPad Software, Inc., La Jolla, CA, USA). In all graphical representations of the data, statistical significance is indicated as follows: NS,  $P > 0.05$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

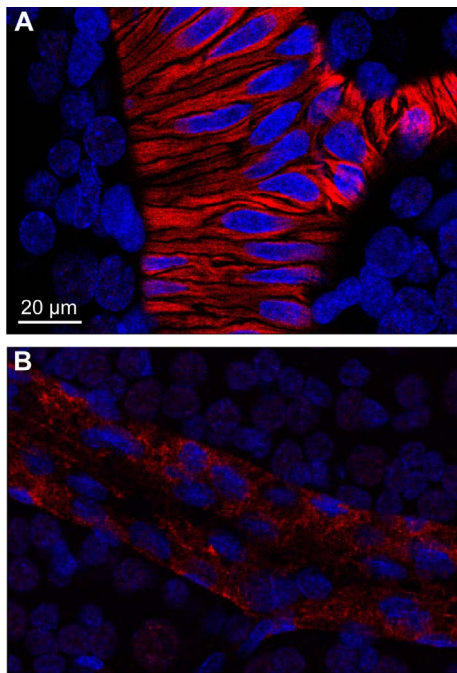
## RESULTS

### Immunolocalization of T-Type Ca<sup>2+</sup> Channels in Retinal Arteriolar Smooth Muscle

The expression of the three T-type channel isoforms, Ca<sub>v</sub>3.1, Ca<sub>v</sub>3.2, and Ca<sub>v</sub>3.3, was examined in rat retinal whole-mount preparations using immunohistochemistry. Retinal arterioles were identified by positive staining for  $\alpha$ SMA with a single monolayer of vascular smooth muscle cells running transversely to the long axis of the vessels (Fig. 1A). In contrast, venules showed much weaker  $\alpha$ SMA staining of pericyte-like mural cells (Fig. 1B). As shown in Figure 2, strong immunolabeling for Ca<sub>v</sub>3.1 proteins was detected on the surface of the retinal arteriolar smooth muscle cells, along with weaker, more diffuse, cytosolic staining (Fig. 2A). Plasma membrane expression of Ca<sub>v</sub>3.1 channels was confirmed by colocalization with isolectin B4 (Fig. 2B). In the surrounding retinal neuropile, Ca<sub>v</sub>3.1 expression was also localized to retinal ganglion cells (RGCs; Fig. 2A). Control experiments performed with secondary antibodies only or primary anti-Ca<sub>v</sub>3.1 antibodies preabsorbed with a specific blocking peptide were negative (Fig. 2C). Expression of Ca<sub>v</sub>3.2 was detected in close proximity to the retinal arterioles (Fig. 2D). However, on closer inspection, it was evident that this expression was limited to the perivascular end-feet of glial cells surrounding the vessels. Ca<sub>v</sub>3.3 expression was absent from retinal arterioles, but strong immunolabeling was present in the surrounding retinal neuropile primarily associated with RGCs (Fig. 2E).

### Electrical Characterization of the T-Type Currents

Whole-cell patch-clamp experiments were conducted to examine the functional expression of T-type Ca<sup>2+</sup> channels on the plasma membrane of retinal arteriolar smooth muscle cells. Figure 3 shows voltage-dependent inward T-type currents sensitive to external application of the selective T-type Ca<sup>2+</sup> channel inhibitor, TTA-A2 (1  $\mu\text{M}$ ; Fig. 3A, left),<sup>40,41</sup> and L-type currents sensitive to nimodipine (1  $\mu\text{M}$ ; Fig. 3A, right).<sup>10</sup> Representative and average current-voltage (I-V) relationships for both TTA-A2- and nimodipine-sensitive currents are also



**FIGURE 1.** (A) Arteriole and (B) venule within a retinal whole-mount preparation immunolabeled for  $\alpha$ -SMA (red channel). Nuclei were labeled with the far-red nuclear stain TOPRO3 (pseudocolored blue).

shown (Fig. 3B, left and right, respectively). As can be seen, the TTA-A2-sensitive I-V curve activates at a more negative membrane potential than the nimodipine-sensitive current ( $-70$  mV for the T-type current compared to  $-50$  mV for the L-type current; Fig. 3B). This is also the case for the peak currents, with T-type current peaking at approximately  $-30$  to  $-20$  mV compared to  $-10$  to  $0$  mV for the L-type current (Fig.

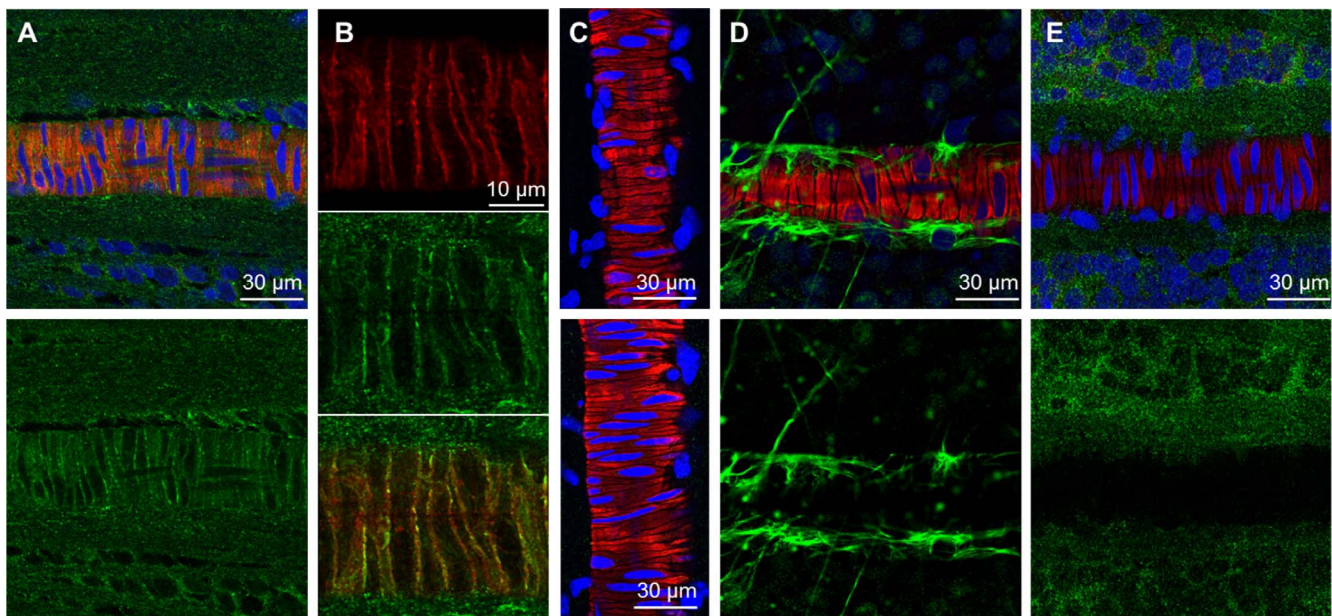
3B). These currents also displayed significant differences in their rates of activation and inactivation, with the T-type currents having a faster rate of both activation ( $T_{ac}$   $4.3 \pm 0.7$  vs.  $8.2 \pm 1.3$  ms) and inactivation ( $T_{inac}$   $18.2 \pm 1$  vs.  $24.1 \pm 1.5$  ms) compared with the L-type currents ( $P < 0.01$  in both cases,  $n = 8$ ; Figs. 3C, 3D). Thus, these results confirm that T-type Ca<sup>2+</sup> channels are functionally expressed in retinal arteriolar smooth muscle cells; and in the final experiments, analyses were performed to examine their possible role in the myogenic reactivity of retinal arterioles.

### Blocking T-Type Ca<sup>2+</sup> Channels Causes Dilation of Pressurized Retinal Arterioles

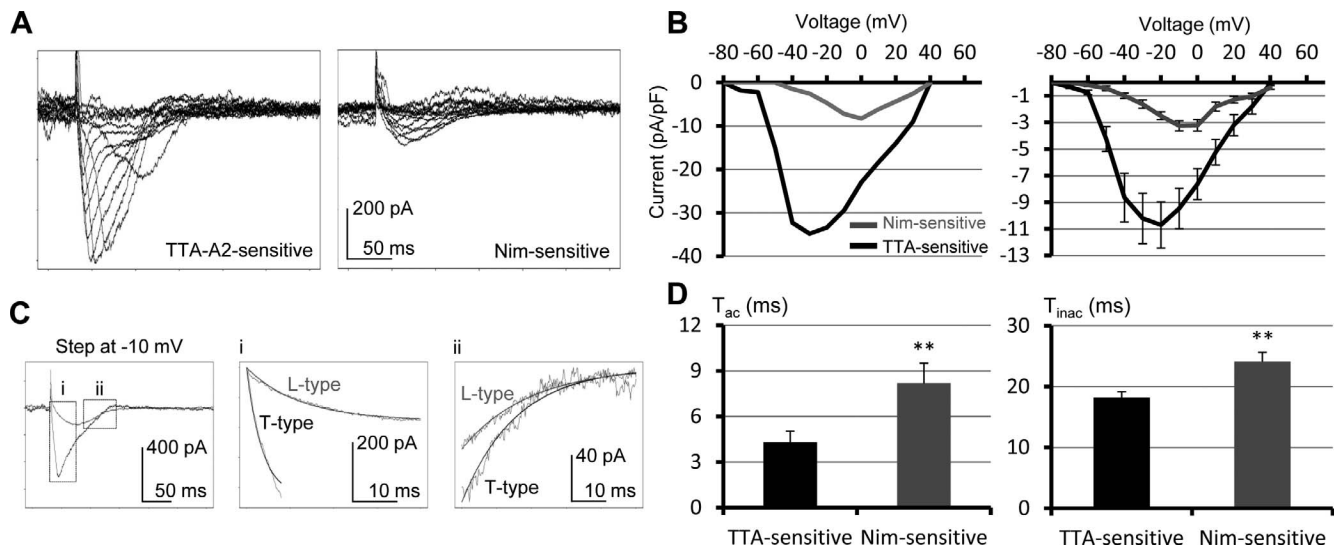
To investigate the possible role of T-type channels in myogenic signaling, we carried out pressure myography experiments on isolated retinal arterioles. Vessels were cannulated and pressurized to 40 mm Hg until myogenic tone had fully developed and stabilized. Arterioles were then exposed to TTA-A2 (1  $\mu$ M) or another selective T-type Ca<sup>2+</sup> channel blocker, ML-218 (1  $\mu$ M),<sup>42</sup> and changes in vessel diameter recorded. At the end of the experiment, wortmannin (10  $\mu$ M) was applied in Ca<sup>2+</sup>-free Hanks' solution in order to obtain the maximal passive diameter of the vessels. Both TTA-A2 and ML-218 caused a significant dilation of myogenically active rat retinal arterioles by  $\sim 50\%$  of the maximal passive diameter ( $P < 0.001$ , Wilcoxon matched-pairs signed rank test; Figs. 4A, 4B; Table 2). These findings are consistent with the view that T-type Ca<sup>2+</sup> channels contribute to the generation of myogenic tone in these vessels.

### DISCUSSION

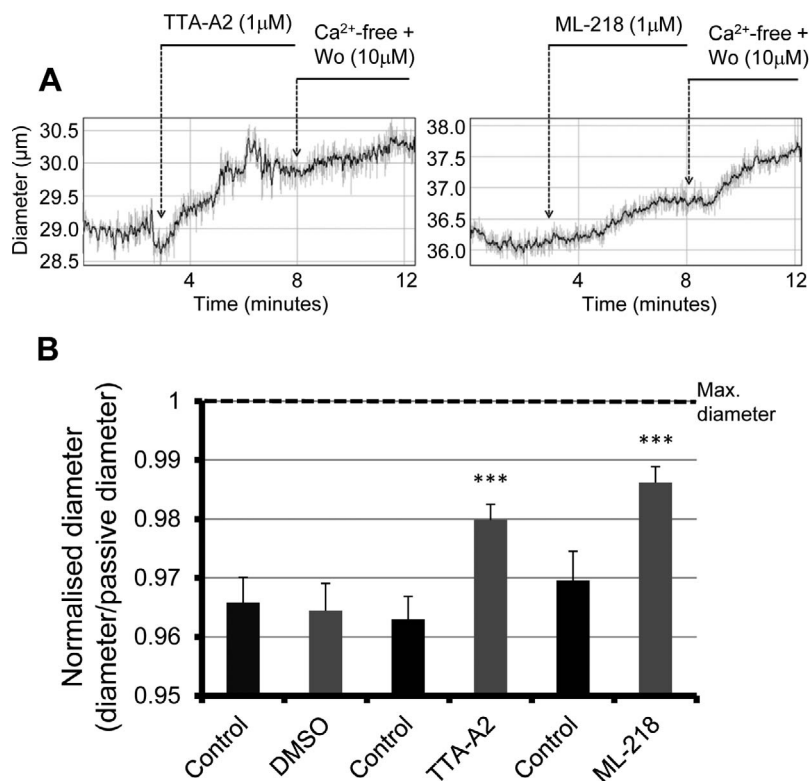
This study shows for the first time that the T-type Ca<sup>2+</sup> channel isoform, Ca<sub>v</sub>3.1, is functionally expressed in vascular smooth muscle cells of rat retinal arterioles and plays an important role



**FIGURE 2.** Distribution patterns of Ca<sub>v</sub>3.1-3 T-type channel isoforms in retinal arterioles and the surrounding retinal parenchyma. (A) *Top*: rat retinal whole-mount preparation immunolabeled for Ca<sub>v</sub>3.1 (green channel),  $\alpha$ -SMA (red channel), and cell nuclei (blue channel; TOPRO3). *Bottom*: Ca<sub>v</sub>3.1 staining segmented from the same image. (B) Individual and merged images showing Ca<sub>v</sub>3.1 (green channel) colocalization with the plasma membrane marker, isolectin B4 (red channel), in retinal arteriolar myocytes. (C) *Top*: secondary-only control image. *Bottom*: blocking peptide experiment for the anti-Ca<sub>v</sub>3.1 primary antibody. (D, E) Immunolabeling equivalent to that shown in (A), but for Ca<sub>v</sub>3.2 and Ca<sub>v</sub>3.3 isoforms, respectively.



**FIGURE 3.** (A) TTA-A2-sensitive T-type currents (*left*) and nimodipine-sensitive L-type currents (*right*) elicited in response to voltage steps from  $-80$  to  $+40$  mV in  $10$ -mV increments from a holding potential of  $-80$  mV. Current traces are presented as difference currents. (B) Representative (*left*) and mean (*right*) I-V relationships ( $n = 8$  cells) for the TTA-A2 and nimodipine-sensitive currents. (C) Kinetic rate constants of activation and inactivation for the currents obtained in (A). Analysis was carried out on currents obtained at a voltage step to  $-10$  mV (*left*). Dashed boxes indicate both the activation (magnified in *middle*) and inactivation (magnified at *right*) of the currents at that voltage. Activating and inactivating currents were fitted using single exponentials. (D) Mean time constants of activation (*left*) and inactivation (*right*) ( $n = 8$ ).



**FIGURE 4.** Effects of T-type blockers on the diameter of isolated retinal arterioles under myogenic tone. (A) Representative traces showing the diameters of vessels under steady-state myogenic tone (*gray line*, actual diameter; *black line*, smoothed trace showing the moving average of 11 points superimposed on top of the raw diameters) at an intraluminal pressure of  $40$  mm Hg (first 2–3 minutes), dilation of the vessel after application of the T-type blocker (TTA-A2,  $1$   $\mu$ M, *left*; ML-218,  $1$   $\mu$ M, *right*), and subsequent further dilation to the maximal passive diameter after application of wortmannin in Ca<sup>2+</sup>-free Hanks' solution. (B) Mean arteriole diameters normalized to the maximal diameters following application of DMSO, TTA-A2, or ML-218.

in myogenic signaling in these vessels. Although we found no evidence for expression of Ca<sub>v</sub>3.2 or Ca<sub>v</sub>3.3 channels in these vessels, these isoforms were identified in glial cell end-feet surrounding the vessels (Ca<sub>v</sub>3.2; Fig. 2D) and RGCs (Ca<sub>v</sub>3.3; Fig. 2E). Our results are consistent with previous studies reporting Ca<sub>v</sub>3.1 expression in vascular smooth muscle cells of other vascular beds, including those of the renal, skeletal muscle, mesenteric, brain, and pulmonary circulations.<sup>23,24,27,30,43-45</sup> Although expression of Ca<sub>v</sub>3.3 has not been widely reported in vascular smooth muscle cells, Ca<sub>v</sub>3.2 has been detected in cerebral and pulmonary artery myocytes.<sup>20,45</sup> Recent work has established that Ca<sup>2+</sup> influx through vascular smooth muscle Ca<sub>v</sub>3.2 channels is principally involved in mediating vasodilatory responses via activation of RyR-dependent Ca<sup>2+</sup> sparks and BK channels.<sup>28</sup> It would appear unlikely, however, that such a mechanism is active in retinal arterioles, given that Ca<sub>v</sub>3.2 channels could not be detected in these vessels.

Consistent with the presence of Ca<sub>v</sub>3.1 channels on the plasma membrane of retinal arteriolar myocytes, we identified T-type currents with pharmacological and kinetic features similar to those produced by Ca<sub>v</sub>3.1 subunits in other systems (Fig. 3).<sup>21,46</sup> These currents were different from the L-type voltage-dependent Ca<sup>2+</sup> currents in these cells in that they were activated at lower membrane potentials and had faster kinetic rates of activation and inactivation (Fig. 3). Similar kinetic rates have been reported for T-type channels in other vascular tissues.<sup>21,28,47</sup> Some studies carried out on mesenteric and cerebral arteries, however, have reported an unexpected depolarizing shift in the activation and inactivation profiles of these channels compared to traditional T-type channels, leading to the suggestion that in vascular myocytes the T-type channels may be formed from splice variants.<sup>48</sup> In our study, however, we could detect two clearly distinguishable peaks in the I-V curves for T-type and L-type currents, closer to traditional T-type values; and the peak I-V values shown here (−30 to −20 mV) closely match those reported for T-type currents in other cells, including neurons, cardiomyocytes, and coronary and aortic vascular smooth muscle cells.<sup>21</sup>

Although L-type Ca<sup>2+</sup> channels are generally believed to play a central role in the development of myogenic tone,<sup>13</sup> it has been noted in several studies that L-type Ca<sup>2+</sup> channel inhibition does not completely abolish the myogenic response.<sup>19,20,49-51</sup> In the present study, we have demonstrated that two different T-type blockers, TTA-A2 and ML-218, are capable of partially inhibiting myogenic signaling in isolated rat retinal arterioles (Fig. 4; Table 2). While the absolute changes in vessel diameter that we have recorded following T-type Ca<sup>2+</sup> channel blockade are relatively small, we have calculated for individual arterioles that these would be expected to decrease vascular resistance in the range of ~3% to 15% (mean of ~7%; based on  $R \propto 1/d^4$ ).<sup>10</sup> Thus it seems likely that these channels will make at least some contribution to the modulation of retinal blood flow autoregulation in vivo. Our data concur with other studies showing that Ca<sub>v</sub>3.1 T-type channels play an important role in the development and maintenance of myogenic tone in vessels from several vascular beds. Navarro-Gonzalez et al.,<sup>43</sup> for example, showed that myogenic tone in the rat basilar artery results from Ca<sup>2+</sup> influx through nifedipine-insensitive voltage-dependent Ca<sup>2+</sup> channels with characteristics similar to the T-type channel isoform Ca<sub>v</sub>3.1. Björling et al.,<sup>52</sup> working with mice deficient in the Ca<sub>v</sub>3.1 T-type isoform, showed that T-type channels are crucial for myogenic tone in mesenteric arteries at low arterial pressure (<80 mm Hg), but are inactivated at high arterial pressure where L-type Ca<sup>2+</sup> channels predominate in the myogenic response.<sup>20,53,54</sup> Since the arterial input pressure in the retina is thought to be ~40 mm Hg<sup>55,56</sup> and retinal arterioles exhibit

myogenic tone between 10 and 70 mm Hg,<sup>9,10,57</sup> these observations would support the view that Ca<sub>v</sub>3.1 T-type channels are likely to play an important role in both setting basal vascular tone and modulating vascular tone in response to changes in systemic blood pressure (i.e., in mediating blood flow autoregulation) in the retinal microcirculation in vivo.

In summary, this study is the first to identify Ca<sub>v</sub>3.1 T-type currents in vascular smooth muscle cells of retinal arterioles and to characterize their contribution to myogenic signaling in these vessels. The work provides an important foundation for better understanding of the mechanisms regulating retinal perfusion in both health and disease. It seems likely, for example, that in addition to contributing to the myogenic reactivity of retinal arterioles, endothelial and glial cell mediators known to modulate retinal vascular tone and blood flow may, at least in part, act by targeting the activity of these channels. In support of this idea, previous studies have shown that vasodilator molecules such as nitric oxide and 5,6-epoxyeicosatrienoic acid are capable of inhibiting T-type Ca<sup>2+</sup> channel activity,<sup>58,59</sup> while the vasoconstrictor peptide, endothelin-1, has been shown to enhance currents through these channels.<sup>60</sup> From a pathophysiological perspective, we currently have only a limited knowledge of the role of T-type Ca<sup>2+</sup> currents in the development of vascular disease, although Ca<sub>v</sub>3.1 channels have been identified as potential therapeutic targets for the prevention of restenosis following angioplasty.<sup>61</sup> Our current work opens up several new avenues for further research. The possibility that alterations in Ca<sub>v</sub>3.1 channel expression and activity contribute to the loss of retinal vascular autoregulation and blood flow disturbances during diseases such as diabetes, glaucoma, and AMD now merits further investigation.

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