Nitric oxide suppresses vascular voltage-gated T-type Ca\(^{2+}\) channels through cGMP/PKG signaling

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Harraz OF, Brett SE, Welsh DG. Nitric oxide suppresses vascular voltage-gated T-type Ca\(^{2+}\) channels through cGMP/PKG signaling. Am J Physiol Heart Circ Physiol 306: H279–H285, 2014. First published November 15, 2013; doi:10.1152/ajpheart.00743.2013.—Recent reports have noted that T-type Ca\(^{2+}\) channels (CaV3.x) are expressed in vascular smooth muscle and are potential targets of regulation. In this study, we examined whether and by what mechanism nitric oxide (NO), a key vasodilator, influences this conductance. Using patch-clamp electrophysiology and rat cerebral arterial smooth muscle cells, we monitored an inward Ba\(^{2+}\) current that was divisible into a nifedipine-sensitive and -insensitive component. The latter was abolished by T-type channel blocker and displayed classic T-type properties including faster activation and steady-state inactivation at hyperpolarized potentials. NO donors (sodium nitroprusside, S-nitroso-N-acetyl-dl-penicillamine), along with activators of protein kinase G (PKG) signaling, suppressed T-type currents. Inhibitors of guanylyl cyclase/PKG (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and KT5823, respectively), had no effect on basal currents; KT5823 did, however, mask T-type Ca\(^{2+}\) channel current inhibition by NO/PKG. Functional experiments confirmed an inhibitory effect for NO on the T-type contribution to cerebral arterial myogenic tone. Cumulatively, our findings support the view that T-type Ca\(^{2+}\) channels are a regulatory target of vasodilatory signaling pathways. This targeting will influence Ca\(^{2+}\) dynamics and consequent tone development in the cerebral circulation.

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ARterial Tone is Regulated by multiple stimuli including tissue metabolism (9), humoral/neural agents (4, 10), and intravascular pressure (15). These stimuli influence vasomotor activity in part by altering cytosolic [Ca\(^{2+}\)] in arterial smooth muscle cells (6, 11). Cytosolic [Ca\(^{2+}\)] is primarily set by resting membrane potential and steady-state Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels (16, 27). While L-type Ca\(^{2+}\) channel expression predominates (3, 15), recent studies have noted that T-type Ca\(^{2+}\) channels are additionally present. Of particular note, the T-type conductance is thought to influence arterial diameter when vessels sit at more hyperpolarized potentials (1, 17). There are three subtypes of T-type Ca\(^{2+}\) channels, and in rat cerebral vascular smooth muscle, both CaV3.1 and CaV3.2 are expressed (1). Intriguingly, a recent electrophysiological assessment has noted that vascular T-type Ca\(^{2+}\) channels are a target of regulation, with PKA signaling being specifically shown to suppress this conductance (12). In light of these findings and earlier studies, it is conceived that other vasodilatory pathways including those linked to nitric oxide (NO) may also inhibit vascular T-type Ca\(^{2+}\) channels (14). Such inhibition could, in theory, be mediated through classical cGMP/protein kinase G (PKG) signaling (25), S-nitrosylation (18), or oxidative stress (14).

The goal of this study was to determine whether T-type Ca\(^{2+}\) channels are regulatory targets of NO in cerebral arterial smooth muscle. Using patch-clamp electrophysiology and smooth muscle cells from rat cerebral arteries, we report for the first time that vascular T-type currents are directly targeted and suppressed by NO. Pressure myography confirmed that NO application limits T-type channel contribution to myogenic tone. Subsequent work revealed that NO-induced suppression was mediated through the classical PKG signaling cascade. In closing, this suppressive ability of NO to the T-type conductance is thought to facilitate cerebral vasodilation either directly by restraining Ca\(^{2+}\) entry through the T-type pores or indirectly by altering membrane potential regulation (13).

MATERIALS AND METHODS

Animal procedures and cell isolation. Animal procedures were approved by the Animal Care Committee at the University of Calgary. Briefly, female Sprague-Dawley rats (10–12 wk of age) were euthanized by CO\(_2\) asphyxiation. Brains were removed, placed in cold phosphate-buffered saline (pH 7.4), and the middle/posterior cerebral arteries were dissected free of surrounding tissues. Smooth muscle cells were enzymatically isolated as previously described (2). Briefly, arterial segments (~2 mm in length) were placed in a medium (37°C, 10 min) containing (in mM) 60 NaCl, 80 Na\(^+\) glutamate, 5 KCl, 2 MgCl\(_2\), 10 glucose, and 10 HEPES with 1 mg/ml albumin (pH 7.4). Vessels were then exposed to a two-step enzymatic digestion: (I) 15-min incubation in medium (37°C) containing 0.5 mg/ml papain and 1.5 mg/ml dithioerythritol and (2) 10-min incubation in medium containing 100 \(\mu\)M Ca\(^{2+}\), 0.7 mg/ml type-F collagenase, and 0.4 mg/ml type-H collagenase. Tissues were washed and triturated; liberated cells were stored on ice for use within ~6 h.

Electrophysiology. Conventional patch-clamp electrophysiology was used to monitor whole cell currents in isolated smooth muscle cells as reported earlier (12). Recording electrodes (5–8 M\(\Omega\)) were pulled from borosilicate glass microcapillary tubes (Sutter Instruments, Novato, CA) using a micropipette puller (Narishige PP-830, Tokyo, Japan), and backfilled with pipette solution containing (in mM) 135 CsCl, 5 Mg-ATP, 10 HEPES, and 10 EGTA (pH 7.2). Cells were voltage clamped and equilibrated in bath solution consisting of (in mM) 110 NaCl, 1 CsCl, 10 BaCl\(_2\), 1.2 MgCl\(_2\), 10 glucose, and 10 HEPES (pH 7.4). A NaCl-agar bridge was used between the reference electrode and the bath solution. Whole cell currents were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA), filtered at 1 kHz, digitized at 5 kHz, and were stored on a computer for off-line analysis with Clampfit 10.3 software (Molecular Devices). Whole cell capacitance averaged 13–17 pF, and
all electrophysiological experiments were performed at room temperature (~22°C).

To record whole cell currents, isolated cells held at −60 mV were exposed to a prepulse (~90 mV, 200 ms) and then voltage steps ranging from −50 to +40 mV (10 mV interval, 300 ms). Current-voltage (I–V) relationships were plotted either as peak current (in pA) or current density (in pA/µF) or as normalized currents (I/I_{\text{max}}). Time constants (τ) of activation, inactivation, and deactivation were obtained using Clampfit 10.3 software by standard exponential fitting of the activating, inactivating, and deactivating segments, respectively (12). For activation time constants, the downward segment (from 0 pA to peak at 10 mV) was fitted. To calculate τ_{\text{activation}}, the inactivating segment (50 ms) at 10 mV was exponentially fitted. Deactivating tail currents were elicited by hyperpolarization from +10 to −60 mV to obtain deactivation time constants. Voltage-dependence of steady-state inactivation was assessed by the protocol: 1) prepulse to −90 mV, 2) stepping from −70 to 20 mV (10-mV intervals, 1.5 s each), 3) brief hyperpolarizing step (−90 mV, 10 ms), and 4) test pulse of 10 mV (200 ms). Currents elicited by test pulse were normalized to maximal current (I/I_{\text{max}}). To assess the voltage dependence of activation, isochronal tail currents were monitored. In particular, cells held at −60 mV were prepulsed to −90 mV (300 ms), followed by voltage steps (−80 to 40 mV, 50 ms) and a test pulse (−90 mV, 200 ms) to evoke tail currents.

**Experimental protocols.** Voltage protocols were run on isolated cells bathed in solutions containing 10 mM Ba^{2+}. Consistent with past investigations, nifedipine (200 nM) was used to fully abolish L-type Ca^{2+} channel activity (12, 20). The residual T-type currents were subsequently monitored in the absence (control) and presence of 1) NNC 55-0396 (T-type blocker, 1 µM), 2) sodium nitroprusside (SNP, 10 µM), 3) S-nitroso-N-acetyl-dl-penicillamine (SNAP, 10 µM), 4) dibutyryl cyclic-GMP (db-cGMP, 100 µM), 5) KT5823 (PKG inhibitor, 1 µM), or 5) 1H-[1,2,4]oxadiazolo[4,3-d]quinazolin-1-one [ODQ; guanylyl cyclase (GC) inhibitor, 30 µM]. The SNAP- and db-cGMP-mediated modulation of T-type Ca^{2+} channels was also assessed in cells pretreated with KT5823.

Pressurized vessel myography. Endothelially denuded cerebral arteries were mounted in a customized arteriograph and superfused with warm (37°C) physiological saline solution as previously described (1). External diameter was measured using an automated edge detection system (IonOptix, Milton, MA). Following equilibration, intra-vascular pressure was increased incrementally from 15 to 50 mmHg, and arteries were then superfused with 200 nM nifedipine. The dilatory response to SNP 55-0396 was then monitored in the absence or presence of SNAP (10 µM). Each protocol was followed by the addition of a Ca^{2+} free physiological saline solution (0 externally added Ca^{2+} + 2 mM EGTA).

**Statistical analysis.** Data are expressed as means ± SE, and n indicates the number of cells or vessels. Paired or unpaired t-tests were performed, where appropriate, to compare the effects of treatment on whole cell current. P values < 0.05 were considered statistically significant. Averaged current-voltage relationships were fitted to the following peak Gaussian function:

$$I(V) = I_{\text{max}} \exp\left\{-0.5\left[(V - V_{\text{max}})/b\right]^2\right\}$$  \hspace{1cm} (1)

where $$I_{\text{max}}$$ is peak current (I), $$V_{\text{max}}$$ is V at $$I_{\text{max}}$$, and b is the slope of the distribution. The voltage dependencies of activation and steady-state inactivation were described with single Boltzmann distributions of the following forms:

**Activation:**

$$I(V) = I_{\text{max}}/\left\{1 + \exp[-(V - V_{50})/k]\right\}$$  \hspace{1cm} (2)

**Inactivation:**

$$I(V) = I_{\text{max}}/\left\{1 + \exp[(V - V_{50})/k]\right\}$$  \hspace{1cm} (3)

where $$I_{\text{max}}$$ is the maximal activatable current, $$V_{50}$$ is the voltage at which the current is 50% activated or inactivated, and k is the slope (voltage dependence) of the distribution.

**Chemicals and drugs.** Nifedipine, NNC 55-0396, SNP, EGTA, and buffer reagents were purchased from Sigma-Aldrich (St. Louis, MO). Di-butyl cyclic-GMP and SNAP were obtained from Enzo Life Sciences (Farmingdale, NY) and Invitrogen (Eugene, OR), respectively. ODQ and KT5823 were acquired from Tocris Bioscience (Minneapolis, MN) and Calbiochem (Gibbstown, NJ), respectively.

**RESULTS AND DISCUSSION**

**Isolation of the T-type conductance in cerebral arterial smooth muscle.** Using arterial smooth muscle cells isolated from rat cerebral arteries, we started with monitoring whole cell currents. To augment charge flow through L-type (Ca_{V1.2}) and T-type (Ca_{V3.1}/Ca_{V3.2}) channels, this study monitored whole cell currents in 10 mM Ba^{2+} (12). In the presence of this charge carrier, a robust voltage-dependent inward current was observed (Fig. 1A). Subsequent addition of 200 nM nifedipine, a dihydropyridine that blocks the vascular Ca_{V1.2} splice variant with an IC_{50} of ~10–15 nM (12, 20), reduced the whole cell conductance by 75%. This dihydropyridine-mediated reduction has been shown earlier to arise from full abolishment of L-type current while exerting negligible effect, if any, on T-type channels (12). Three lines of evidence indicated that T-type channels dominate the residual nifedipine-insensitive current. First, the putative T-type blocker NNC 55-0396 fully inhibited this remaining component (Fig. 1A). Second, the voltage dependence of normalized plots of whole cell currents along with steady-state inactivation/activation showed that the NNC-sensitive component of the whole cell current was hyperpolarized shifted (~15 mV) compared with the nifedipine-sensitive component (Fig. 1, B and C). Lastly, kinetic analysis revealed that nifedipine quickened the rates of inactivation and activation while slowing that of deactivation (Fig. 1, D and E). Note that while the properties of this isolated T-type current are comparable with other native vascular cells (17, 21), they are rightward shifted compared with T-type conductances monitored in other cell types or expression systems (22). As shown earlier, these shifts are primarily attributable to the use of 10 mM Ba^{2+} as the charge carrier and the subsequent screening effect and preferential augmentation of L-type (over T-type) channel activity (12). While Ba^{2+} (10 mM) should trigger a T-type current similar to that obtained in 10 mM Ca^{2+} solutions, the latter would induce smooth muscle cell contraction, making stable electrical recordings difficult. To summarize, experiments outlined in Fig. 1 confirmed the successful isolation of a current component predominantly carried by T-type channels.

**NO suppresses T-type Ca^{2+} channels.** T-type Ca^{2+} channels represent key regulatory targets for a range of hormones and neurotransmitters in different tissues (19, 26). These modulatory effects are typically, although not exclusively, mediated through G protein signaling linked to protein kinases A, G, C, and calmodulin-dependent kinase II (28). With that said, electrophysiological regulatory observations in native vascular smooth muscle remain limited, with one exception being recent work documenting a suppressive effect of β-adrenergic/PKA signaling on T-type channel (12). These observations intriguingly raise the possibility that other vasodilatory pathways, for example, NO signaling, could also inhibit T-type channels.

Functional vascular studies have recently alluded to a role for NO modulation of T-type Ca^{2+} channels (8, 24, 14). In the cerebral circulation, this view arose from observations showing
Fig. 1. Delineating T-type currents in cerebral arterial smooth muscle. A: representative traces and averaged current-voltage (I–V) plots (n = 10) of whole cell Ba2+ currents before (control) and after the application of 200 nM nifedipine (Nif) and 1 μM NNC 55-0396 (NNC). Traces display channel activity at voltage steps (−40 to +30 mV). Inset: I–V plots of the nifedipine-sensitive (L-type) and NNC-sensitive (T-type) currents, as obtained as difference currents. B: normalized I–V plots of currents (I/I_max) obtained in A. Average data were fit with Eq. 1 to generate the curves (V_max): 1.4 ± 0.5 mV (NNC sensitive) and 17.1 ± 1.3 mV (nifedipine sensitive). C: averaged effect of nifedipine (200 nM) on voltage dependence of activation and steady-state inactivation. Activation curves were obtained using isochronal tail currents (see MATERIALS AND METHODS). Average normalized data (%I/I_max) were fit with Eq. 2: V_50 act/16.8 ± 1.2 mV (control), 32.4 ± 1.5 mV (nifedipine, n = 11). For steady-state inactivation, average normalized data were fit with Eq. 3: V_50 inact/17.5 ± 0.3 mV (control), 35.5 ± 0.3 mV (nifedipine, n = 11). D: kinetic analysis of voltage-dependent activation and inactivation of control and nifedipine-insensitive currents. Top traces: absolute (in pA) and normalized (I/I_max) peak current amplitudes obtained at +10 mV before and after the application of 200 nM nifedipine. Bottom traces (magnified): differential kinetics of activation (left) and inactivation (right). The time interval to I_max was shorter in the presence of nifedipine. The extent of voltage-dependent inactivation, over similar time intervals, was greater after nifedipine. E: Time constants (τ) of activation, inactivation, and deactivation before and after nifedipine application. *P < 0.05, significant difference from control values (n = 11, paired t-test).
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that the T-type contribution of the myogenic response was enhanced in a rat model of acute NO inhibition (14). In line with these vasomotor observations, the overall contribution of T-type Ca\(^{2+}\)/H\(_{\text{11001}}\) channels to cerebral arterial tone development was suppressed by the preapplication of NO donors (Fig. 2, A and B). The T-type component, defined by sensitivity to NNC 55-0396, was attenuated from 12% (control) to 5% (SNAP-pretreated) of the maximal response. We hypothesize based on this change and the documented Ca\(^{2+}\)/H\(_{\text{11001}}\) response to intravascular pressure (15) that NO suppression of the T-type conductance Fig. 3. Basal cGMP/PKG activity does not alter T-type currents. A: traces and averaged I-V plots (n = 7) of whole cell T-type currents before and after the application of KT5823 (PKG inhibitor, 1 \(\mu\)M). B: peak T-type current (in pA/pF) at different time points under control conditions (n = 7) or in cells treated with KT5823 (1 \(\mu\)M, n = 7) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 30 \(\mu\)M, n = 6). Inset: traces represent peak T-type currents before (control) and 15 min after treatment. NS, not significantly different from the 0-min time point (paired t-test).

C: time-course changes in peak current (I\(_{\text{max}}\), in pA) in a cerebral arterial myocyte treated with 1 \(\mu\)M KT5823. D: voltage-dependence of activation and steady-state inactivation of T-type conductance in the absence (control) and presence of KT5823 (n = 6). No significant shifts were observed.

Fig. 2. Nitric oxide (NO)/protein kinase G (PKG) signaling inhibits T-type channels. A: effects of NO on T-type channel contribution to myogenic tone. Cerebral arteries were pressurized to 50 mmHg, while diameter changes were monitored in response to 1 \(\mu\)M NNC 55-0396 in absence or presence of NO. Representative traces reveal the diameter responses to nifedipine (200 nM) followed by NNC 55-0396 (1 \(\mu\)M) in presence or absence of S-nitroso-N-acetyl-dl-penicillamine (SNAP; 10 \(\mu\)M). Ultimately, Ca\(^{2+}\) free solution (2 mM EGTA) was applied to maximally dilate arteries and reveal the residual myogenic tone. Inset: bars represent 5 min.

B: NNC-sensitive and residual (EGTA) vasomotor responses are plotted as absolute changes (\(\Delta\)diameter, in \(\mu\)m) or as percentages of maximal response. *P < 0.05, significant difference from matching values in absence of SNAP (n = 4, unpaired t-test).

C: representative traces and averaged I-V plots of nifedipine-insensitive T-type currents before and after the application of SNAP (10 \(\mu\)M). Average data were fit with Eq.1 (n = 6).

D: peak T-type currents (in pA/pF) at different time points under control conditions (n = 7) or in the presence of sodium nitroprusside (SNP; 10 \(\mu\)M, n = 5), SNAP (10 \(\mu\)M, n = 8), or dibutyryl cyclic-GMP (db-cGMP; 100 \(\mu\)M, n = 5). Bottom traces: peak T-type currents pre- and posttreatment. NS, not significant. *P < 0.05, significant difference (paired t-test). E: time-course changes in I\(_{\text{max}}\) in a cell treated with SNAP. F: voltage dependence of activation and steady-state inactivation of T-type conductance in the absence (control) and presence of 10 \(\mu\)M SNAP (n = 7).
would reduce the global rise in cytosolic \([\text{Ca}^{2+}]\) by ~10–15 nM. While the \([\text{Ca}^{2+}]\) and vasomotor changes are modest, previous computational modeling indicates that they are sufficient to reduce network blood flow by 20–35% (1). Although the functional data suggest that NO could conceivably modulate T-type \(\text{Ca}^{2+}\) channels, direct electrical evidence remains lacking.

In this context, we isolated cerebral arterial smooth muscle cells and sought to test our hypothesis that NO acutely modulates the T-type electrical conductance through \(\text{cGMP/PKG}\) signaling. We present two sets of electrophysiological observations supporting the view that this vasoactive molecule elicits an inhibitory effect on T-type channels through classical PKG signaling. First, bath application of different NO donors (SNP or SNAP) suppressed the nifedipine-insensitive T-type inward current (Fig. 2, C and D). Interestingly, a cell-permeable PKG activator (db-cGMP) evoked comparable suppression (Fig. 2D), suggesting a direct modulatory role for NO/cGMP/PKG cascade. This suppression peaked ~15 min after incubation and coincided with a slight but significant hyperpolarized shift in steady-state inactivation (Fig. 2, A and F). Matching time controls revealed no significant changes in T-type current amplitude, a finding consistent with past reports showing that the T-type conductance, unlike the L-type, is rundown resistant (12). Interestingly, blockade of PKG signaling using the kinase inhibitor KT5823 or the GC inhibitor ODQ had no direct effect on the amplitude of the basal T-type current or its voltage dependence (Fig. 3, A–D). Even though and as a second line of evidence, preincubating smooth muscle cells with KT5832 attenuated the ability of SNAP (Fig. 4, A and B) and db-cGMP (Fig. 4, C and D) to suppress T-type channel activity. The sensitivity of SNAP (NO)-mediated inhibition to PKG blockade reemphasized a direct modulatory role for NO through cGMP/PKG pathway. In summary, our electrophysiological observations are the first to demonstrate that vascular T-type channels are directly targeted by NO signaling through GC/cGMP/PKG pathway.

**Physiological/pathological implications.** Previous reports including our own have reported a role of T-type \(\text{Ca}^{2+}\) channels in setting cerebral arterial tone (1, 17). Of particular note is the purported ability of the T-type conductance to drive myogenic tone when vessels sit at more hyperpolarized potentials around ~50 mV (1). In theory, \(\text{Ca}^{2+}\) entry through T-type \(\text{Ca}^{2+}\) channels could set arterial tone through one of two general mechanisms (13). First, it could directly contribute to the rise in cytosolic \([\text{Ca}^{2+}]\), a process that would enhance myosin light chain phosphorylation via the activation of myosin light chain kinase (6, 11). On the other hand, \(\text{Ca}^{2+}\) influx through the T-type pores could indirectly elevate cytosolic \([\text{Ca}^{2+}]\) by locally modulating \(\text{Ca}^{2+}\)-activated channels that set resting membrane potential (13). Potential targets include the \(\text{Ca}^{2+}\)-activated transient receptor potential 4 or chloride (TMEM16A) channels; both conductances have been recently identified in cerebral arterial smooth muscle cells (5, 7). Irrespective of which mechanism dominates, NO/PKG-mediated inhibition of the T-type conductance would suppress arterial...
tone development in the cerebral circulation. Such regulation may be compromised under conditions of endothelial dysfunction where NO bioavailability is decreased. This loss of NO-induced suppression may interestingly be one of the underlying mechanisms of cerebral vasospasm and could also contribute to impaired tissue perfusion observed during hypertension and diabetes (23).

Summary. In conclusion, the present study delineated T-type Ca\textsuperscript{2+} channels and tested whether this conductance is modulated by NO/PKG signaling in rat cerebral arteries. We employed patch-clamp electrophysiology to isolate a non-L-type whole cell current that was sensitive to T-type blockers and exhibited electrical characteristics of T-type Ca\textsuperscript{2+} channels. We subsequently showed for the first time that vascular T-type channels represent a direct regulatory target of the putative vasodilator NO through classical cGMP/PKG signaling. The latter was confirmed using both electrophysiological and pressurized vessel myography experiments. This selective targeting may implicate NO/PKG/Ca\textsubscript{V3.x} signaling in controlling smooth muscle function through direct control of cytosolic [Ca\textsuperscript{2+}] or indirect modulation of membrane potential.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

O.F.H. and D.G.W. conception and design of research; O.F.H. and S.E.B. data collection; O.F.H. and D.G.W. drafting manuscript; O.F.H. and D.G.W. acquisition of funding; O.F.H., S.E.B., and D.G.W. analysis and interpretation of data; O.F.H. and D.G.W. editing and revising manuscript; O.F.H., S.E.B., and D.G.W. critical revision of the manuscript for important intellectual content; O.F.H. prepared figures; O.F.H. and D.G.W. drafting manuscript; O.F.H. and D.G.W. drafted manuscript; O.F.H. and D.G.W. edited and revised manuscript; O.F.H., S.E.B., and D.G.W. granted approval of final version of manuscript.

REFERENCES