Ion channel mechanisms of rat tail artery contraction-relaxation by menthol involving, respectively, TRPM8 activation and L-type Ca\(^{2+}\) channel inhibition

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Melanaphy D, Johnson CD, Kustov MV, Watson CA, Borysova L, Burdyga TV, Zholos AV. Ion channel mechanisms of rat tail artery contraction-relaxation by menthol involving, respectively, TRPM8 activation and L-type Ca\(^{2+}\) channel inhibition. Am J Physiol Heart Circ Physiol 311: H1416–H1430, 2016. First published October 7, 2016; doi:10.1152/ajpheart.00222.2015.—Transient receptor potential melastatin 8 (TRPM8) is the principal cold and menthol receptor channel. Characterized primarily for its cold-sensing role in sensory neurons, it is expressed and functional in several nonneuronal tissues, including vasculature. We previously demonstrated that menthol causes variable mechanical responses (vasoconstriction, vasodilation, or biphasic reactions) in isolated arteries, depending on vascular tone. Here we aimed to dissect the specific ion channel mechanisms and corresponding Ca\(^{2+}\) signaling pathways underlying such complex responses to menthol and other TRPM8 ligands in rat tail artery myocytes using patch-clamp electrophysiology, confocal Ca\(^{2+}\) imaging, and ratiometric Ca\(^{2+}\) recording. Menthol (300 μM, a concentration typically used to induce TRPM8 currents) strongly inhibited L-type Ca\(^{2+}\) channel current (I\(_{\text{L,Ca}}\)) in isolated myocytes, especially its sustained component, most relevant for depolarization-induced vasoconstriction. In contraction studies, with nifedipine present (10 μM) to abolish I\(_{\text{L,Ca}}\) contribution to phenylephrine (PE)-induced vasoconstrictions of vascular rings, a marked increase in tone was observed with menthol, similar to resting (i.e., without α-adrenoceptor stimulation by PE) conditions, when L-type channels were mostly deactivated. Menthol-induced increases in PE-induced vasoconstrictions could be induced both by the TRPM8 antagonist AMTB (thus confirming the specific role of TRPM8) and by cyclopiazonic acid treatment to deplete Ca\(^{2+}\) stores, pointing to a major contribution of Ca\(^{2+}\) release from the sarcoplasmic reticulum in these contractile responses. Immunocytochemical analysis has indeed revealed colocalization of TRPM8 and InsP\(_3\) receptors. Moreover, menthol Ca\(^{2+}\) responses, which were somewhat reduced under Ca\(^{2+}\)-free conditions, were strongly reduced by cyclopiazonic acid treatment to deplete Ca\(^{2+}\) store, whereas caffeine-induced Ca\(^{2+}\) responses were blunted in the presence of menthol. Finally, two other common TRPM8 agonists, WS-12 and icilin, also inhibited I\(_{\text{L,Ca}}\). With respect to L\(_{\text{I,Ca}}\) inhibition, WS-12 is the most selective agonist. It augmented PE-induced contractions, whereas any secondary phase of vasorelaxation (as with menthol) was completely lacking. Thus TRPM8 channels are functionally active in rat tail artery myocytes and play a distinct direct stimulatory role in control of vascular tone. However, indirect effects of TRPM8 agonists, which are unrelated to TRPM8, are mediated by inhibition of L-type Ca\(^{2+}\) channels and largely obscure TRPM8-mediated vasoconstriction. These findings will promote our understanding of the vascular TRPM8 role, especially the well-known hypotensive effect of menthol, and may also have certain translational implications (e.g., in cardiovascular surgery, organ storage, transplantation, and Raynaud’s phenomenon).

transient receptor potential melastatin 8; TRPM8 agonists; voltage-gated Ca\(^{2+}\) channels; vasodilatation; vasoconstriction

NEW & NOTEWORTHY

Although the cold and menthol receptor, calcium-permeable transient receptor potential melastatin 8 (TRPM8), channel is expressed in vascular smooth muscles, surprisingly menthol causes vasorelaxation. Here we dissect the true contractile response of rat tail artery myocytes to TRPM8 activation, which is normally “masked” by the nonspecific inhibition of voltage-activated L-type calcium channels, resulting in vasorelaxation.

TRANSIENT RECEPTOR POTENTIAL MELASTATIN MEMBER 8 (TRPM8) is a cation channel best known for its role in sensory nerve endings, where it is activated by cold temperatures and cooling compounds, such as menthol and icilin (26, 28, 30). In nerve terminals, TRPM8 channel opening initiates membrane depolarization accompanied by an acceleration of action potential discharge that ultimately results in the perception of environmental cold (4, 26). Although the role of TRPM8 is now well established as the primary sensor of thermal stimuli in the innocuous cold range (4, 13, 14), with a temperature threshold of ~26°C (28), the channel was first cloned and identified in prostate epithelia and several types of carcinoma (41), tissues in which temperature would not be expected to deviate substantially from that of the core body temperature. TRPM8 expression has now been found in several nonneuronal tissues, including the bladder and male genital tract (37), lung epithelia (35), and in the vasculature (17, 18, 29, 38, 46, 47). Additionally, numerous endogenous molecules have been proposed to increase [phosphatidylinositol 4,5-biphosphate and lysophospholipids (1, 3, 23, 33)] and decrease [arachidonic acid (3, 6)] TRPM8 activity, and enzymes protein kinase A and protein kinase C have been proposed to regulate TRPM8 by altering its phosphorylated state (5, 6, 32), thus providing putative endogenous biochemical modulatory pathways for TRPM8, distinct from thermal stimuli. These findings raise important questions as to the whole spectrum of expression and functions of this
truly polymodal protein that forms Ca$^{2+}$-permeable cation channels.

There is strong evidence to indicate TRPM8 gene expression in rat and mouse vascular smooth muscle (VSM) (18, 38, 46, 47). As a Ca$^{2+}$-permeable cation channel ($P_{Ca}/P_{Na} \sim 1–3$) (28, 30), activation of TRPM8 located on the plasma membrane and/or the membrane of the sarcoplasmic reticulum (SR) of these VSM cells (VSMC) would be expected to increase intracellular Ca$^{2+}$ ($[Ca^{2+}]_i$). In a study by Yang et al. (47), the application of menthol (300 μM) to transiently cultured rat aortic and pulmonary VSMCs resulted in the expected robust increases in $[Ca^{2+}]_i$, that were dependent on the presence of extracellular Ca$^{2+}$ and could be blocked by the nonselective cation channel inhibitor Ni$^{2+}$ (300 μM), whereas the L-type voltage-gated Ca$^{2+}$ channel (L-VGCC) blocker nifedipine (1 μM) had no effect (47). These findings indicated that menthol-induced responses in rat aortic and pulmonary VSMCs were entirely mediated by Ca$^{2+}$ influx, suggesting TRPM8 expression on the plasma membrane of these cells.

In our earlier work, we used an organ bath tensiometric preparation along with TRPM8 pharmacological modulators, menthol and icilin, as well as KCl and the α1-adrenoceptor agonist, phenylephrine (PE), to observe the consequences of putative TRPM8 activation on the tone of vascular rings, from several major arteries and during different contractile states (18). From these functional experiments, we observed complex effects of TRPM8 agonists on vascular contractility, causing prominent vasodilatations when applied during PE- and high-K$^+$-induced vasoconstrictions, whereas menthol (300 μM) consistently caused vasoconstrictions when applied to rat tail artery vascular rings at rest. These are disparate effects, as menthol-induced activation of TRPM8 would be expected to invariably induce VSM contraction by its virtue of raising $[Ca^{2+}]_i$ (47). To add to the puzzle, systemic and chronic dietary administration of menthol is known to have a pronounced hypotensive effect (33, 38), and in our earlier study we also observed vasodilatation of cutaneous blood vessels in vivo after topical menthol application (18). Such observations strongly imply that there may be other targets for menthol-induced inhibition of VGCC, activation of which normally initiates and maintains VSM contraction during membrane depolarization, and provided Ca$^{2+}$ imaging and pharmacological evidence for this hypothesis. In this context, it should be noted that 1) smooth muscle and cardiac VGCCs are molecularly similar (the principal transmembrane subunit α1 is CaV1.2 type) (10), and 2) there is direct patch-clamp evidence for menthol-induced inhibition of cardiac L-type VGCC that occurs at concentrations that normally activate TRPM8 (IC$_{50} = 75$ μM) (7). However, the hypothesis does not address the entire complexity of menthol action on VSMs, as it contradicts measurements of $[Ca^{2+}]_i$, rises and vascular contractions of nonstimulated VSM and blood vessels induced by TRPM8 agonists (18, 47). Moreover, even in preconstricted vascular rings, menthol induces notable initial contraction preceding relaxation (18). Thus, in the present study, our aims were 1) to identify the effects of TRPM8 agonists (mainly menthol as the most widely used one, and especially interesting as a common ingredient in many medicines and consumer products) on vascular tissues using a range of physiological and biophysical techniques and a much more rigorous set of experiments than previously used aiming to dissect the specific TRPM8-mediated effects; 2) to further test the hypothesis that TRPM8 is functional in rat tail artery VSM but that the consequences of TRPM8 activation may be masked by nonspecific effects of TRPM8 agonists, most likely on VGCC; and 3) to directly test this latter possibility by patch-clamp measurements of L-type Ca$^{2+}$ channel currents ($I_{Ca},L$). Some of the results have been communicated in an abstract form (27).

**METHODS**

**Ethical approval.** All experimental procedures involving animals were in accordance with the UK Animal Scientific Procedures Act (1986) and were approved by the Queen’s University and University of Liverpool Animal Welfare and Ethics Committees.

**Animal tissue.** Experiments were performed on vessels freshly dissected from 8- to 12-wk-old male Sprague-Dawley rats. Tail arteries were removed from the whole length of the tail. The proximal 2–3 cm of the artery was taken for tensiometric studies, and the rest was used for smooth muscle isolation followed by Ca$^{2+}$ measurement or patch-clamp analysis.

**VSM isolation.** Excised tail arteries were transferred to Ca$^{2+}$-free physiological salt solution (PSS) containing the following (in mM): 120.0 NaCl, 6.0 KCl, 1.0 MgCl$_2$, 10.0 HEPES, and 12.0 glucose, pH 7.4 (adjusted with NaOH). The artery was cleaned free of connective tissue and longitudinally cut. The endothelium was removed by gently rubbing the luminal surface with fine stainless steel wire. The tissue was then sectioned into 5-mm lengths before being transferred to dissociation medium (DM) containing the following (in mM): 110.0 NaCl, 5.0 KCl, 0.5 KH$_2$PO$_4$, 0.5 NaH$_2$PO$_4$, 10.0 NaHCO$_3$, 10.0 HEPES, 1.00 taurine, 0.50 EDTA, 10.00 glucose, 2.00 MgCl$_2$, and 0.16 CaCl$_2$, pH 7.4 (adjusted with NaOH). The tissue was digested at 37°C for 20 min in DM containing collagenase (type XI; 1 mg/ml), papain (1 mg/ml), BSA (0.4 mg/ml), and dithiothreitol (0.8 mM) and was then washed with Ca$^{2+}$-free PSS to stop digestion. Single myocytes were dispersed by trituration with a small-bore glass pipette.

**Electrophysiology.** Isolated vascular myocytes were plated in a CoverWell perfusion chamber (Grace Bio-Labs, Sigma-Aldrich, Dorset, UK) pressed to seal to glass coverslips, and placed on the stage of an inverted microscope (Nikon Eclipse TE2000-S; Nikon, Tokyo, Japan). Ten minutes before commencement of experiments, the cells were superfused with a modified PSS containing the following (in mM): 120.0 NaCl, 6.0 CsCl, 10.0 HEPES, 1.2 MgCl$_2$, 12.0 glucose, and 5.0 CaCl$_2$, pH 7.4 at room temperature. Borosilicate glass pipettes (Harvard Apparatus, Cambridge, UK) were fabricated and fire polished to resistances of 3–4 MΩ and were back filled with pipette solution containing the following (in mM): 80.0 CsCl, 2.0 MgCl$_2$, 11.0 EGTA, 7.0 Na$_2$ATP, 0.5 Na$_2$GTP, 10.0 HEPES, and 5.0 creatine (pH 7.3 adjusted with CsOH). Pipette electrodes were mounted on a CV203BU head stage connected to a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Union City, CA). Whole cell currents were filtered at 2 kHz (8-pole low-pass Bessel) and sampled at 5 kHz.

To determine the presence of a persistent Ca$^{2+}$ current ($I_{Ca,P}$) through VGCCs (i.e., the so-called nonactivating “window current” that occurs in a specific range of potentials as an important determinant of vascular tone) in the tail artery VSMCs, a double-pulse voltage
protocol was employed with a constant depolarizing test potential to 10 mV, preceded by a variable amplitude prepulse ranging from −100 to 40 mV with a 10-mV increment (Fig. 1A, top).

A custom-made solution delivery system consisting of eight presurized reservoirs attached to an eight-channel solution delivery pen (AutoMate Scientific, Berkeley, CA) with a single-outlet needle (250-μm internal diameter; AutoMate Scientific) was used for fast solution application. The needle outlet was positioned in close proximity (0.1–0.5 mm) to the recorded cell and was used to superfuse the cell with the test compound (bath solution, vehicle, or drug) until a steady-state response had been attained. In addition, a gravity-driven solution exchange attached to one side of the bath and vacuum to the other ensured fast and complete removal of test compounds from the perfusion chamber.

To investigate the direct effects of TRPM8 agonists on calcium currents, L(ICa) was activated by applying 400-ms depolarizing pulses to a test potential of 10 mV from a holding potential of −60 mV at 10-s intervals. L(ICa) parameters were measured as the peak inward current (L-ICa-Peak) achieved soon after the start of the depolarizing pulse and as the late noninactivating current (L-ICa-Late) measured as the mean current during the final 10 ms of the 400-ms pulse. To allow quantitative comparison of experimental conditions, all currents were

Fig. 1. Investigation of L-type Ca2+-channel current (L-ICa) in rat tail artery vascular smooth muscle cells (VSMCs) indicates the existence of noninactivating window current in these cells and L-ICa inhibition by transient receptor potential melastatin 8 (TRPM8) agonist, menthol. A: representative recording of L-ICa in a rat tail artery VSMC, elicited by stepping the membrane potential to a range of prepulses (−100 to +40 mV with a 10-mV increment, 500-ms duration) from a holding potential of −60 mV to determine channel steady-state activation properties before stepping to +10 mV for 50 ms to determine the steady-state inactivation. Top: voltage protocol. B: plotting activation and inactivation curves for the L-ICa demonstrated an overlap region whereby a small but persistent current exists, known as a window current. Inset: data from 8 cells (Cm = 42.2 ± 4.0 pF; n = 8; N = 4) were used to calculate mean window currents in tail artery VSMCs. C: representative current traces showing the effects of menthol (300 μM) and the specific L-type voltage-gated Ca2+ channel (VGCC) blocker nifedipine (10 μM) on L-ICa in the same rat tail artery VSMC. Currents were evoked by step depolarization from −60 mV to +10 mV for 400 ms, applied every 10 s. D: example time course showing the inhibitory effects of vehicle-only control (0.3% ethanol) and menthol (300 μM) application on mean peak and late L-ICa in rat tail artery VSMCs (33.1 ± 1.8 pF; n = 8; N = 5). E: peak and late L-ICa were compared in individual cells in control conditions and in the presence of vehicle-only control (V; 0.3% ethanol) or menthol (M; 300 μM). Both peak and late L-ICa, in the presence of menthol were significantly reduced compared with their vehicle-only condition (**p < 0.001; n = 8; N = 3). F: representative organ bath tensiometric trace showing that nifedipine, applied at the same concentration (10 μM), strongly suppresses the sustained component of the phenylephrine (2 μM) (PE)-evoked contraction, which is most likely mediated by the window L-ICa in rat tail artery VSMCs.
normalized such that \( I_{\text{Ca,late}} \) measured following 30-s incubation in nifedipine (10 \( \mu \)M) was set as the baseline current for each cell studied.

**Ratiometric \( \text{Ca}^{2+} \) recording.** Isolated cells in low-\( \text{Ca}^{2+} \) PSS were loaded with fura-2 AM (final concentration: 5 \( \mu \)M; Molecular Probes, Invitrogen, Carlsbad, CA) and pluronic acid (final concentration: 2.5 mg/ml; Sigma-Aldrich) dissolved in DMSO for 25–30 min in a glass-bottomed recording chamber. Cells were washed with low-\( \text{Ca}^{2+} \) PSS after the loading period for up to an hour at room temperature to allow deesterification of the dye. A gravity-driven continuous flow of PSS (\( \text{Ca}^{2+} \) = 2 mM) was used to superfuse the preparation for 10 min before experiment commencement, with excess solution removed by a suction line.

A photometric system consisting of an inverted microscope (Olympus IX50; Olympus UK, London, UK) and a Cairn monochromator (Cairn Research, Faversham, UK) with dual excitation (340 and 380 nm) were used to study \( \text{Ca}^{2+} \) responses and their temporal characteristics in rat tail artery VSMCs upon the application of various pharmacological agents. Signals were recorded using a \( \times 60 \) oil immersion objective (N.A. 1.3), and emitted fluorescence was measured at 510 nm from the side port of the microscope, via an adjustable rectangular window that allowed isolation of a region of interest around a selected cell. Acquisition software (pClamp 9; Molecular Devices) was used for analysis and online computation of the \( F_{\text{340}}/F_{\text{380}} \) Ratio.

**Laser confocal \( \text{Ca}^{2+} \) imaging.** Similarly to fura-2, the AM ester derivative of fluo-3 (fluor-3 AM, Invitrogen), with the addition of pluronic acid, was used to monitor \( \text{Ca}^{2+} \) signals in isolated myocytes using a laser-scanning confocal microscope (MR-A1; Bio-Rad Laboratories, Hercules, CA) coupled to an inverted microscope (Nikon Eclipse TE300). On this system, images were collected at one frame per second via oil immersion objectives (N.A. 1.4), and emitted light was filtered through a 530–560-nm band-pass filter and detected using a photomultiplier tube. Data acquisition was controlled with Lasersharp software (Bio-Rad), and analysis was performed using EZ-C1 FreeViewer software (Nikon). In addition, fast \( \text{Ca}^{2+} \) imaging was performed using an Ultraview LCI spinning Nipkow disc, wide-field Olympus IX70 inverted microscope (Perkin-Elmer Confocal Microscope System, Cambridge, UK), with a high-sensitivity, OraC-ER-cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) to capture emitted fluorescence. Images were collected at a rate of 33 frames per second via oil-immersion objectives (\( \times 40 \), N.A. 1.4; \( \times 60 \), N.A. 1.4). The fluo-3 dye was excited by an argon laser at 488 nm, and emitted light was filtered through a 530–560-nm band-pass filter and detected using a photomultiplier tube. Data acquisition was controlled with Lasersharp software (Bio-Rad), and analysis was performed using EZ-C1 FreeViewer software (Nikon). In addition, fast \( \text{Ca}^{2+} \) imaging was performed using an Ultraview LCI spinning Nipkow disc, wide-field Olympus IX70 inverted microscope (Perkin-Elmer Confocal Microscope System, Cambridge, UK), with a high-sensitivity, OraC-ER-cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) to capture emitted fluorescence. Images were collected at a rate of 33 frames per second via oil-immersion objectives (N.A. 1.2). The system was excited by a \( \times 60 \) water immersion objective (NA 1.2) with fluo-4-loaded cells excited by an argon laser at 488 nm, and emitted fluorescence was measured at 510 nm. Data acquisition and analysis were performed using Perkin-Elmer Microscope System software.

**Contraction studies.** Tail artery sections with endothelium removed had mechanical responses recorded as previously described (18). The condition of the tissue was initially tested by adding KCl (60 mM) and rejecting tissue that did not respond with a robust contraction (>0.5 g tension). One of three basic protocols was then conducted. 1) Vessels were examined for a contractile response to menthol alone (0.1–1.0 mM; experiment performed at room temperature). 2) Vessels were contrated with PE (2 \( \mu \)M), and nifedipine (300 \( \mu \)M) was added during contraction (having allowed 10 min for contraction to stabilize). 3) To eliminate the involvement of L-type VGCC in TRPM8 agonist-induced responses, vessels were incubated with the \( \text{Ca}^{2+} \) antagonist nifedipine (10 \( \mu \)M) for 10 min before repetition of the second protocol. Vascular responses to TRPM8 agonist application were measured as the peak constriction induced (peak response) and the tone remaining 10 min after application (late response) relative to PE-induced tone immediately prior, as illustrated in Fig. 4B. Experiments examining the effects of WS-12 (50 \( \mu \)M) were performed, and data was analyzed in the same manner. All summary data presented have been normalized to the PE-vasoconstriction amplitude 10 min after PE application.
and those achieved during PE action, with peak at about -10 mV (Fig. 1B, inset).

After the examination of the activation/inactivation characteristics of the persistent L-\(I_{\text{Ca}}\), a different protocol was used to investigate the direct effects of TRPM8 agonists on L-\(I_{\text{Ca}}\). Menthol has been shown previously to inhibit L-\(I_{\text{Ca}}\) in other cell types, namely neurons and cardiac myocytes (7, 39), suggesting, based on molecular similarities of channels, the presence of such inhibition in vascular myocytes as well. In the present study, using the same menthol concentration (300 \(\mu\)M) as used in our previous tensiometric experiments (18), we observed strong inhibitory effects of menthol on L-\(I_{\text{Ca}}\) in tail artery VSMCs (Fig. 1C). Following subsequent nifedipine application for several minutes, a residual transient current was often observed. This current likely represented Ca\(^{2+}\) influx through T-type VGCCs, which are coexpressed with L-type channels in a subpopulation of rat tail artery VSMCs (31).

Inactivation kinetics of L-\(I_{\text{Ca}}\) could be well approximated by the sum of two exponentials, and in the presence of menthol it was clearly accelerated (e.g., in Fig. 1C, \(\tau_{\text{fast}}\) was reduced by menthol from 26.6 to 21.0 ms). Thus menthol is likely to be a gating modifier, rather than a channel blocker that simply hinders the channel pore.

A time-course plot showing mean peak and late L-\(I_{\text{Ca}}\) for eight cells tested (\(C_m = 33.1 \pm 1.8\) pF; \(n = 8; N = 3\)), under control conditions and in the presence of vehicle-only or menthol application, is illustrated in Fig. 1D. Menthol was found to significantly reduce peak L-\(I_{\text{Ca}}\) by 32.9 \(\pm\) 2.7% compared with a 7.6 \(\pm\) 1.6% reduction induced by the vehicle-only control (0.3% ethanol; \(P < 0.001; n = 8; N = 3\)), whereas the late L-\(I_{\text{Ca}}\) was significantly reduced by 67.0 \(\pm\) 7.0% compared with a 18.8 \(\pm\) 5.5% reduction induced by the vehicle-only control (\(P < 0.001; n = 8; N = 3\)). These findings are summarized in Fig. 1E.

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**Fig. 2.** TRPM8 agonists induced intracellular Ca\(^{2+}\) responses in rat tail artery VSMCs. A: ratiometric Ca\(^{2+}\) recording of representative responses to caffeine (10 \(\mu\)M), PE (10 \(\mu\)M), and menthol (300 \(\mu\)M) with increases in \(F_{340}/F_{380}\) ratio in a rat tail artery VSMC. B: ratiometric Ca\(^{2+}\) recording of a representative rat tail artery VSMC responding to repeated applications of menthol (300 \(\mu\)M), followed by the application of caffeine (1 mM), with increases in \(F_{340}/F_{380}\) ratio. C: summary data of the peak increases in \(F_{340}/F_{380}\) in the presence of caffeine (1 and 10 mM), PE (2 and 10 \(\mu\)M), and menthol (300 \(\mu\)M) relative to baseline ratio before applications. Student’s paired \(t\)-test was used to compare peak increases in the ratio for each compound to mean baseline in the 30–60 s before application. \(**P < 0.05, \***P < 0.001\). D: intracellular calcium responses recorded in the same myocytes were normalized to baseline and superimposed on the same time scale to illustrate the differences in their 1–90% rise time, as illustrated for the menthol trace. E: summary data of mean rise times of \([\text{Ca}^{2+}]_i\) responses to caffeine (10 mM, \(n = 11\)), PE (10 \(\mu\)M, \(n = 4\)), and menthol (300 \(\mu\)M, \(n = 11\)). The Kruskal-Wallis nonparametric ANOVA with Dunn’s post hoc test was used to compare these values. \(\*P < 0.05\).
These findings strongly suggest that the vasodilatory effects of menthol on preconstricted vascular rings, as documented previously by Johnson et al. (18) and Cheang et al. (11), may largely be accounted for by a direct inhibitory action on VGCCs opened by either membrane depolarization induced by KCl or PE (hypothesis 1). Figure 1F shows that, indeed, a substantial part of PE-induced contraction is underlined by VGCC activity.

However, if a major indirect effect of menthol (e.g., non-TRPM8 related) on vascular tone is vasorelaxation via the inhibition of VGCCs, it is likely that TRPM8 channels present in vessel are also activated but that their role in vascular tone is masked by the more robust action of L-ICa inhibition. Therefore, to minimize this secondary effect, one method would be to apply menthol to unstimulated VSM, when VGCCs are largely closed at resting potential and L-ICa contribution is minimal (see window current-voltage relation in Fig. 1B, inset). As such, the application of activators of a Ca2+-permeable ion channel in VSM would be expected to induce an increase in [Ca2+], according to Yang et al. (46), and vasoconstriction (hypothesis 2). Indeed, such vasoconstrictions have been observed previously when menthol was applied to pretensed rat tail artery sections under resting conditions (18).

Effect of TRPM8 agonists on VSM [Ca2+], and basal vascular tone. In rat tail artery VSMCs, menthol induced elevations in [Ca2+]i, and caused concentration-dependent vasoconstrictions in tail artery vascular rings (Figs. 2 and 3). Enzymatically isolated VSMCs, loaded with fura-2 AM, responded to menthol (300 μM) application with robust and reproducible F340/F380 ratio increases, responses that were comparable in size to the responses induced by the application of sub-/ supramaximal concentrations of the ryanodine receptor (RyR) channel activator caffeine (1–10 mM) and α1-adrenoceptor agonist PE (2–10 μM) when tested in the same cell (Fig. 2, A and B). The summary bar chart shows the peak increases in F340/F380 (means ± SE) induced by caffeine (1 mM: P < 0.05, Student’s paired t-test, n = 5, N = 4; 10 mM: P < 0.001, n = 14, N = 5), PE (2 and 10 μM), and menthol (300 μM), relative to basal F340/F380 before each compound application (Fig. 2C).

However, there was one notable difference in the rate of rise of these various Ca2+ signals (quantified as time from 10–90% of its maximal size, or t10–90), as menthol-induced responses were significantly slower compared with caffeine- or PE-induced responses (Fig. 1, D and E). This observation suggests that the density of TRPM8-mediated Ca2+ influx into the cytosol should be considerably lower compared with the pathways that involve α1-adrenoceptor and RyR activation. Consistent with these findings at the cellular level, application of incremental concentrations of menthol to pretensed, nonconstricted tail artery vascular rings at room temperature (20–22°C) induced vasoconstrictions that were concentration dependent (100 μM: 0.020 ± 0.003 g or 4 ± 1% of KCl vasoconstriction; n = 29, N = 14; 300 μM: 0.062 ± 0.010 g or 10 ± 1% of KCl; n = 29, N = 14; NS; 500 μM: 0.115 ± 0.013 g or 17 ± 2% of KCl; n = 29, N = 14; 1 mM: 0.339 ± 0.032 g or 52 ± 5% of KCl; n = 29, N = 14) and were significantly greater than baseline (300 μM: P < 0.01; 500 μM: P < 0.001; 1 mM: P < 0.001; peak amplitudes significantly different from 0, 1-way ANOVA; Fig. 3). When menthol (300 μM) was applied to pretensed, relaxed tail artery vascular rings at 37°C, vasoconstrictions were significantly smaller (0.025 ± 0.004 g or 3 ± 1% of KCl; n = 8, N = 3; P < 0.01; unpaired t-test with Welch’s correction) than those observed at room temperature and were more transient in appearance than those observed at room temperature: B: bar chart summarizing the peak amplitudes of menthol-induced vasoconstrictions relative to 60 mM KCl-induced vasoconstriction. Menthol concentrations of 0.3–1.0 mM were sufficient to induce vasoconstrictions that were consistently and significantly greater than baseline tone (1-way ANOVA, n = 29; N = 14). *P < 0.05, ***P < 0.001.

Effect of TRPM8 agonists on vascular tone independent of VGCC activity. Consistent with the above results, menthol had an initial rapid vasoconstrictive effect of variable amplitude when applied during PE-induced vasoconstrictions under control conditions (compare Fig. 4A, left, Fig. 4B, and Fig. 1A, control trace, average menthol peak amplitude was 108.4 ± 7.3% of PE control, P < 0.01, 1-way ANOVA, n = 26, N = 19) but caused a significant slower vasodilatory effect (Fig. 4, A and B). The steady-state inhibition of contraction was observed after 10-min exposure (menthol late) amounted to 73.5 ± 2.4% of PE control (P < 0.001), as illustrated in Fig. 4A, left, and Fig. 4B. We reasoned that, if the above-described VGCC inhibition by menthol (Fig. 1) was indeed involved in the vasorelaxation, prior inhibition of VGCC activity by a saturating nifedipine dose (10 μM) should reveal the specific TRPM8-mediated component in vascular response. Application of nifedipine 10 min before PE resulted in a 46.5 ± 3.0%
Fig. 4. In the absence of VGCC activity, menthol induces vasoconstrictions in rat tail artery vascular rings. A: representative tensiometric trace showing the predominant vasoconstrictive effect of menthol (300 µM) on PE-constricted rat tail artery vascular rings, which was suppressed while the constrictive effect was unmasked in the presence of the L-type VGCC blocker, nifedipine (10 µM; n = 22, N = 13). B: bar chart summarizing the effects of menthol on PE vasoconstriction, causing an additional vasoconstriction (denoted Peak) followed by a later vasodilatory effect measured 10 min after menthol application (denoted Late), under control conditions and when nifedipine was applied 10 min before the application of PE. Summary data have been normalized to the PE vasoconstriction amplitude 10 min after PE application, as illustrated in the inset, namely: Peak = B/A; Late = C/A and expressed as % of PE response before menthol application. Values for each condition, i.e., PE after 10 min, menthol peak, and after 10 min (Late), were then compared by 1-way ANOVA for repeated measures followed by Tukey’s post hoc test. Peak and late values in control conditions and in the presence of nifedipine were compared by Student’s t-test. ***p < 0.01, **p < 0.001.

These findings indicate that the most likely mechanism of menthol-induced vasodilatation of preconstricted vessels [Fig. 4A, left (18)] was a direct effect on VSM VGCCs because this effect was reversed in the absence of L-type VGCC contribution to PE vasoconstrictions (Fig. 4A, right). Importantly, these experiments indicate that the inhibition by menthol of L-ICa, which is more pronounced for its late component (Fig. 1E), also likely masks a substantial part of the vasoconstrictive effects of the compound even in nonstimulated tissue because, even at the resting membrane potential, considerable window Ca2+ current (up to 40% of maximum) flows (Fig. 1B, inset) contributing to resting vascular tone.

Ca2+ store contribution to menthol-induced vasoconstrictions.

In different cell types, TRPM8 channels can be expressed, not only in the plasma membrane, where they mediate Ca2+ influx, but also in the endoplasmic reticulum, where they promote Ca2+ release from the intracellular stores (2, 8, 9, 35, 39, 46). In this context, it should be noted that menthol is a membrane-permeable molecule. In addition, it is possible that TRPM8-mediated Ca2+ influx can trigger Ca2+ -induced Ca2+ release. To determine whether intracellular Ca2+ stores contributed to menthol-induced vasoconstrictions, we inhibited reuptake of Ca2+ into the SR using the SR/endoplasmic reticulum calcium ATPase pump inhibitor cyclopiazonic acid (CPA). When vessels were preincubated with CPA (10 µM) for 30 min before a PE-induced vasoconstriction, application of menthol (300 µM) 10 min later did not induce any increase in tone (peak), as was seen in control conditions, but instead resulted in a more pronounced vasodilatation that was significantly greater than seen in the absence of CPA (12.2 ± 9.0% relaxation of PE vasoconstriction in control conditions compared with 60.6 ± 6.8% relaxation in the presence of CPA; P < 0.01; Student’s paired t-test; n = 8, N = 8; Fig. 5, A and B).

To strengthen the theory that menthol activation of TRPM8 could result in Ca2+ release in tail artery VSMCs to contribute to vasoconstriction, immunocytochemistry was performed on isolated tail artery myocytes using a specific TRPM8 antibody. A thin, intense immunofluorescence signal on the perimeter of the cell was identified, as well as a pronounced signal within the deeper cytosol (Fig. 5C, left). These signals could be attributed to TRPM8 presence at plasmalemma and or subplasmalemmal SR in addition to a large intracellular structure surrounding the nucleus, most likely the SR. This hypothesis was confirmed by the parallel labeling of type I InsP3 receptors (Fig. 5C, middle) followed by examination of merged images, which revealed such colocalization of TRPM8 and InsP3R, as evident by yellow color in Fig. 5C, right. These results are consistent with the view that TRPM8 can be localized both on the plasma membrane and/or the SR membrane and mediates Ca2+ influx and/or Ca2+ release correspondingly, as in other cell types (2, 8, 9, 35, 38, 40, 48).
VGCC activity to PE-induced vasoconstrictions when SR was dysfunctional. To distinguish between these possibilities, we next focused on the role of SR in menthol-evoked Ca\textsuperscript{2+} responses. To evaluate the relative contribution of VGCC-mediated Ca\textsuperscript{2+} influx vs. Ca\textsuperscript{2+} store release to menthol-evoked intracellular Ca\textsuperscript{2+} rise, ratiometric fura-2 measurements were performed on single myocytes, whereas VGCCs were inhibited by nicardipine (5 μM) or Ca\textsuperscript{2+} store was depleted with CPA (10 μM), respectively. Whereas nicardipine had no statistically significant effect, the Ca\textsuperscript{2+} transients in response to menthol were inhibited by 48.9 ± 1.2% (n = 3) after Ca\textsuperscript{2+} store depletion (Fig. 6). It should be noted that this effect of Ca\textsuperscript{2+} store depletion on the amplitude of menthol-induced Ca\textsuperscript{2+} response is even underestimated because the measured F_{340}\text{/}F_{380} signal includes, in addition to the menthol-induced signal, also a more sustained store-operated Ca\textsuperscript{2+} entry component, as can be seen in Fig. 6C.

Consistent with this, in confocal Ca\textsuperscript{2+} imaging experiments performed on single fluo-3 AM-loaded myocytes with the use of the MR-A1 system, we observed significant intracellular Ca\textsuperscript{2+} rises both with and without external Ca\textsuperscript{2+}, with indications of a propagation through the cytosol Ca\textsuperscript{2+} wave, a phenomenon typically attributed to the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release process (Fig. 7, A–C). On average, in Ca\textsuperscript{2+}-free PSS, the F/F₀ signal was reduced from 1.92 ± 0.10 to 1.39 ± 0.03 (n = 4; P = 0.002), indicating that menthol-induced [Ca\textsuperscript{2+}], rises were due to both Ca\textsuperscript{2+} entry and Ca\textsuperscript{2+} release. Moreover, Ca\textsuperscript{2+} responses to subsequent caffeine application were relatively smaller if caffeine was applied in the presence of CPA (10 μM) (100 ± 60% vs. 78 ± 50% (n = 36)) and then incubated in Alomone anti-TRPM8 antibody (1:200) followed by Alexa 488 secondary antibody (1:200) and mounted in DAPI-containing medium. Left: TRPM8 staining only. Middle: composite of TRPM8 and DAPI staining. Right: representative permeabilized cell, which was incubated only in the Alexa 488 secondary antibody (white lines = 20 μm).

To investigate the spatial dynamics of menthol-induced [Ca\textsuperscript{2+}], rises at a higher temporal resolution, we employed the spinning Nipkow disc system. With the improved temporal resolution, we observed that, indeed, menthol clearly induced a propagation through the cytosol Ca\textsuperscript{2+} wave (Fig. 7D, top set of confocal frames). In Ca\textsuperscript{2+}-free PSS, not only was the amplitude of the Ca\textsuperscript{2+} signal reduced, but also the rate of the spread...
Fig. 6. Ratiometric [Ca\(^{2+}\)]\(_i\) measurements in fura-2 AM-loaded rat tail artery myocytes indicate that inhibition of VGCC has no effect, whereas Ca\(^{2+}\) store depletion strongly reduces the amplitude of menthol-induced [Ca\(^{2+}\)]\(_i\) transients in resting cells. A: in control, 2 consecutive menthol (300 μM) applications evoked reproducible [Ca\(^{2+}\)]\(_i\) rises. B: in cells treated for 5 min with the selective L-type Ca\(^{2+}\) channel blocker nicardipine (5 μM), the Ca\(^{2+}\) response to menthol was only marginally reduced. C: after calcium store depletion induced by CPA (10 μM) treatment for 5 min, the menthol-induced Ca\(^{2+}\) transient was decreased by about 50%, suggesting that Ca\(^{2+}\) release from the SR plays a significant role in the intracellular Ca\(^{2+}\) rise attributable to TRPM8 activation by menthol. D: mean normalized to control response F\(_{340/F380}\) values summarizing the effects of VGCC inhibition (P = 0.006, n = 3) and Ca\(^{2+}\) store depletion (P = 0.002, n = 3) on menthol-evoked [Ca\(^{2+}\)]\(_i\) transients. **P < 0.01.

of the signal became slower, as can be seen by the increased time lag between the fluo-4 signal measured at the opposite sides of the myocyte (Fig. 7D, bottom set of frames and black and blue lines in Fig. 7E).

Effect of TRPM8 antagonist (AMTB) on menthol-induced vasoconstrictions in the presence of nifedipine. We next examined the selectivity of menthol action with regard to TRPM8 activation, expecting loss of its effect following TRPM8 blockade by AMTB. In the presence of AMTB (10 μM), menthol-induced vasoconstrictions in the absence of VGCC contribution (Fig. 8A, left) were greatly inhibited (right). On average, in the presence of nifedipine, menthol-induced peak and late vasoconstriction amplitudes that were 173.3 ± 10.3% and 145.9 ± 9.0% of the PE amplitude, respectively, became significantly reduced to 135.1 ± 6.4% and 79.5 ± 8.7% of the PE control, respectively (peak: P < 0.01; late: P < 0.001; 1-way ANOVA; n = 9, N = 9) when preparations were preincubated in nifedipine plus AMTB (Fig. 8B).

Effects of other TRPM8 agonists on L-ICa in rat tail artery VSMCs. In addition to menthol, we examined the effects of two other, more recently introduced TRPM8 agonists (icilin and WS-12) on L-ICa in tail artery VSMCs (Fig. 9). The concentration of icilin tested was the same as used in our previous tensiometric experiments (17) and as deemed sufficient for supramaximal activation of TRPM8 (50 μM), WS-12 was used at its supramaximal concentration of 50 μM (36). As shown in Fig. 9A, both agonists had strong inhibitory effects on L-ICa in these cells. Similar to menthol, icilin accelerates current inactivation of L-ICa (e.g., in Fig. 9A \(\tau_{fast}\) was reduced by icilin from 32.4 to 25.5 ms). Thus icilin is also likely to be a VGCC gating modifier.

A time course showing the effects of vehicle-only and icilin application on peak and late L-ICa is illustrated in Fig. 9B. Icilin significantly reduced peak ICa by 58.5 ± 2.6%, compared with a 11.0 ± 1.8% reduction induced by the vehicle-only control (0.5% DMSO; P < 0.001; n = 8; N = 3), and caused a 78.5 ± 5.7% inhibition of late ICa compared with 17.2 ± 3.0% by its vehicle-only control (P < 0.001; Fig. 9C). WS-12 induced a 30.0 ± 2.2% reduction in peak L-ICa compared with 16.3 ± 1.7% by its vehicle-only control (0.5% ethanol; P < 0.001), but there was no significant difference between the effects of drug and vehicle-only control on late L-ICa (55.3 ± 4.8% reduction vs. 40.5 ± 4.1%; Fig. 9D). These observations in vascular cells contrast to those in rabbit cardiac myocytes in which icilin appeared to have no effect on L-ICa (7).

The latter observation that WS-12 had little or no effect on the sustained L-ICa suggested that this menthol derivative is a more selective TRPM8 agonist compared with menthol. We have thus performed similarly to the above-described Ca\(^{2+}\) ratiometric measurements using fura-2 AM-loaded myocytes (Fig. 10) and contractile recordings (Fig. 10B, data summarized in Fig. 10C). WS-12 (10 μM) induced robust [Ca\(^{2+}\)]\(_i\) rises comparable to those induced by caffeine, PE, or menthol (Fig. 10A). However, similar to menthol, the rise phase of these responses was much slower compared with caffeine- and PE-induced responses, with \(t_{10–90} = 6.16 ± 1.97 s (n = 4)\) (compared with Fig. 2E). When applied in the presence of PE, WS-12 invariably induced an additional contraction, which was augmented in the presence of nifedipine (Fig. 10, B and C). This indicates that, although some L-ICa occurred (i.e., vehicle-only effect in the case of late L-ICa), the associated relaxation component was masked by a more pronounced contractile component attributable to TRPM8 activation.

DISCUSSION

The present study confirms that vasodilatory effects of menthol on preconstricted vascular rings (11, 18) are mediated via a direct inhibitory action on L-type VGCCs. We thus refer to this effect as nonspecific if menthol is employed as a pharmacological tool for the investigation of physiological effects of TRPM8 activation. When organ bath tensiometric experiments were performed on tail artery rings in the presence of nifedipine, TRPM8 agonist-induced vasodilations were abolished, indicating that these were a consequence of a direct inhibition of L-type VGCC activity. However, it should be noted that moderate contribution of endothelium to these re-
laxations was observed (18), and this likely holds true concerning VGCCs are lacking in endothelial cells although further tests of endothelial involvement are now needed using experimental protocols we developed here for testing TRPM8-specific roles. However, in the present study, the endothelium was removed to avoid this additional complexity.

It is now well documented that menthol interacts with proteins other than TRPM8. It has been shown that the compound can potently inhibit skeletal muscle and neuronal voltage-operated Na⁺ channels (16), neuronal and cardiac VGCCs (7, 39), and now smooth muscle VSMCs (present study and Ref. 11). These findings could be expected considering the similarity of vascular to cardiac muscle VGCC molecular composition (10). In addition, menthol has also been shown to activate TRPV3 at millimolar concentrations and bimodally activate and inhibit TRPA1 in a concentration-dependent manner (21, 24). Millimolar concentrations have also been shown to induce Ca²⁺ release from the internal stores in non-TRPM8-expressing HEK293 cells (25). Thus, in the present study, a combination of a number of different TRPM8 agonists and selective TRPM8 antagonist AMTB were used to determine whether a specific and physiologically relevant TRPM8-mediated effect existed in tail artery VSMCs.

Despite its robust inhibitory effects on L- Ca, we show that menthol induces robust Ca²⁺-mobilizing effects in tail artery VSMCs that were comparable with caffeine and PE. In addition, at room temperature, menthol induced concentration-dependent vasoconstrictions in the organ bath preparation. Cheang et al. (11) noted that they did not observe vasoconstrictive effects of menthol in rat aorta, mesenteric artery, or coronary artery at 37°C. This is in agreement with our own observations (D. Melanaphy, C. Johnson, and A. Zholos; unpublished observations). Menthol vasoconstrictions were only investigated in tail artery vascular rings in the present study and, moreover were larger and observed more consistently at room temperature. Clearly, the very complex interactions between voltage, menthol, and temperature in TRPM8 activation (2, 15, 44) may explain these differences depending on temperature at which experiments are performed, as there is strong synergy between cooling and menthol (2, 16, 47).
Indeed, according to Peier et al. (30), menthol was rather an inefficient activator of TRPM8 at 35°C compared with 22°C. Interestingly, when experiments were performed in the presence of nifedipine, we were able to observe prominent menthol-induced vasoconstrictions in PE preconstricted arteries obviously masked by the inhibitory action of menthol on L-type Ca\(^{2+}\) currents described in previous studies (11, 18). In addition, menthol application to the organ bath while vascular

![Image](https://example.com/image1.png)

Fig. 8. TRPM8 antagonist N-(3-aminopropyl)-2-[(3-methylphenyl)methyl]oxy]-N-(2-thienylmethyl)-benzamide hydrochloride salt (AMTB) strongly inhibits menthol-induced vasoconstrictions of rat tail artery vascular rings, which were unmasked in the presence of the L-type VGCC blocker nifedipine. A: representative tensiometric traces showing the effects of menthol (300 μM) on a PE-constricted rat tail artery vascular ring in the presence of nifedipine (10 μM) and in the presence of both nifedipine (10 μM) and the TRPM8 antagonist AMTB (10 μM). B: summary data quantifying the effects of menthol when applied 10 min into a PE vasoconstriction in control conditions, in the presence of nifedipine, and in the presence of nifedipine plus AMTB. Summary data presented were normalized to the PE vasoconstriction amplitude 10 min after PE application for each experimental condition (% control) as was already illustrated in Fig. 4B, left. Menthol peak and late (after 10 min) percentage control values in the presence of nifedipine were significantly higher than those in the presence of nifedipine and AMTB (peak: **P < 0.01; late: ***P < 0.001; 1-way ANOVA for repeated measures followed by Tukey’s post hoc test; n = 9, N = 9).

![Image](https://example.com/image2.png)

Fig. 9. Investigation of L-I\(_{\text{Ca}}\) inhibition by icilin and WS-12 in rat tail artery VSMCs. A: representative current traces showing the effects of icilin (50 μM) and WS-12 (50 μM) on the L-I\(_{\text{Ca}}\) in the same rat tail artery VSMC (same cell as in Fig. 1C). Currents were evoked by step depolarization from −60 mV to +10 mV for 400 ms, applied every 10 s. Example time course showing the inhibitory effects of vehicle-only control (0.5% DMSO) and icilin (50 μM) application on mean peak and late L-I\(_{\text{Ca}}\) in rat tail artery VSMCs. C: peak and late L-I\(_{\text{Ca}}\) were compared in individual cells in control conditions and in the presence of vehicle-only control (0.5% DMSO; V) or icilin (50 μM; I). Both peak and late L-I\(_{\text{Ca}}\) in the presence of icilin were significantly reduced compared with the vehicle-only condition (**P < 0.001; n = 8; N = 3). D: peak and late L-I\(_{\text{Ca}}\) were compared in individual cells in control conditions and in the presence of vehicle-only control (0.5% ethanol; V) or WS-12 (50 μM; W). Peak L-I\(_{\text{Ca}}\) was significantly reduced in the presence of WS-12 compared with the vehicle-only condition (**P < 0.001; n = 8; N = 3).
The importance of Na influx could be explained by several factors. First, we found that InsP3R activity is potentiated by Ca2+ directly, and thus it can synergize with PE-induced Ca2+ entry via the NCX. The observation that menthol-induced vasoconstrictions became more pronounced at 37°C in the presence of PE (compare Fig. 8A and 3A) could be explained by several factors. First, we found significant colocalization between TRPM8 and InsP3R1 (Fig. 5C). Because InsP3R activity is potentiated by [Ca2+]i rise, it is possible that Ca2+ efflux via TRPM8 in its close vicinity potentiates PE-induced Ca2+ signaling via InsP3R. Second, TRPM8 has been recently shown to activate Gq proteins directly, and thus it can synergize with α1-adrenoceptor in this same action (20). Third, although Ca2+ influx via TRPM8 makes a prominent contribution to the menthol-induced Ca2+ responses (Fig. 7, A, B, and E), one should not overlook the importance of Na+ influx, as TRPM8 is a nonselective cation channel primarily admitting Na+ under physiological gradients. Indeed, Na+ entry via the Na+-Ca2+ exchanger (NCX) facilitates Ca2+ entry via the NCX operating in the reverse mode, and this way it contributes to PE-induced [Ca2+]i oscillations and vasoconstriction. This mechanism becomes even more important in the presence of nifedipine (24), which is consistent with our observations assuming that TRPM8-mediated Na+ entry facilitates Ca2+ entry via the reverse mode of NCX. Mahieu et al. (25) described a TRPM8-independent menthol-mediated Ca2+ release observed in four different non-TRPM8-expressing cell lines. In contrast to our observations, however, the menthol responses observed by Mahieu et al. (25), were temperature dependent with large responses measured at 33°C but only minor responses at room temperature, the opposite to our observations in rat tail artery smooth muscle. If the Ca2+-mobilizing and vasoconstrictive effects of menthol in the present study were indeed a nonspecific effect of the compound, it is reasonable to suggest that they would induce similar nonspecific effects in other vascular beds. Menthol was applied to organ bath preparations of rat aorta, mesenteric artery, and femoral artery vascular rings at both room temperature and 37°C but had no effect on basal tone (data not shown).

We found that menthol-induced vasoconstrictions in the presence of PE were mediated, at least in part, by Ca2+ release from the internal stores because they were abolished by CPA pretreatment, which is known to deplete the Ca2+ store. Moreover, the absence of the vasoconstrictive component of the action of menthol after Ca2+ store depletion, the vasodilatory effect of the compound was significantly greater. The involvement of TRPM8 in Ca2+ release is further supported by the localization of anti-TRPM8 immunofluorescence signals in close proximity to type 1 InsP3 receptors on intracellular structures analogous to SR (45) in immunocytochemistry experiments performed on isolated rat tail artery VSMCs. In mouse mesenteric artery myocytes, TRPM8 is also mainly localized in the cytosolic region (38). In menthol-induced [Ca2+]i transients, the large initial transient component was also suggestive of predominant Ca2+ release contribution.

Fig. 10. The more selective TRPM agonist, menthol-derivative WS-12, induces [Ca2+]i rises in single fura-2 AM-loaded rat tail artery myocytes and, in tensiometric recordings, alters the balance between vasoconstriction and vasorelaxation toward predominant contraction. A: ratiometric [Ca2+]i, recording of a representative fura-2-loaded VSMC that responded to caffeine (10 μM), PE (10 μM), WS-12 (10 μM), and menthol (300 μM) with increases in F340/F380 ratio. B: WS-12 induces vasoconstrictions in rat tail artery vascular rings both with and without VGCC activity. Representative tensiometric trace shows that WS-12 (50 μM) had a predominantly vasoconstrictive effect (i.e., no net vasorelaxation phase was evident) on PE-constricted rat tail artery vascular rings that were potentiated in the presence of nifedipine (n = 12, N = 7). C: bar chart on the left shows summary data quantifying the effects of WS-12 when applied 10 min into a PE vasoconstriction, causing a significant vasoconstriction (Peak) followed by a prolonged elevated tone measured 10 min after WS-12 application (Late, compared with menthol, no relaxation was evident). The bar chart on the right shows summary data of similar measurements performed during incubation with nifedipine applied 10 min before the application of PE. Both Peak and Late responses were increased, suggesting that some unmasking of the contractile response in the absence of VGCC activity still takes place. *P < 0.05, **P < 0.01, ***P < 0.001.
Furthermore, a significant population of these intracellular TRPM8 channels showed colocalization with type 1 InsP₃ receptors although it is possible that another part of intracellular TRPM8 staining simply represents synthesis and transport of TRPM8 proteins. Despite this uncertainty, in our Ca²⁺ confocal imaging experiments, we obtained strong evidence for the contribution of both Ca²⁺ influx and Ca²⁺ release (the latter was evident after external Ca²⁺ removal) to [Ca²⁺]ᵢ rises induced by menthol, and we also found that menthol partially reduces the caffeine-releasable Ca²⁺ pool. In addition, ratiometric Ca²⁺ measurements showed significant reduction of menthol-induced Ca²⁺ transients following Ca²⁺ store depletion by CPA treatment. Taken together, these observations provide strong structural and functional evidence for the role of TRPM8 channels expressed in the SR. Notably, however, menthol (and WS-12) [Ca²⁺]ᵢ rises developed much more slowly compared with those initiated by PE (mainly via activation of InsP₃ receptors) and caffeine (via activation of RyR receptors). This difference can in part be explained by slower diffusion of TRPM8 agonists into the cytosol, but it is more likely that lower expression of TRPM8 channels and/or the lack of their positive feedback activation by Ca²⁺, as is the case for both InsP₃ and RyR receptors, can account for such differences in kinetics.

Furthermore, menthol-induced vasoconstrictions were significantly reduced in the presence of the selective TRPM8 antagonist AMTB as an additional independent test of specificity. Notably, the physiologically relevant involvement of TRPM8 in Ca²⁺ release mediated by the intracellular (ER) TRPM8 channels has also been documented in other cell types, such as DRG neurons, normal prostate secretory epithelial cells, and in LNCaP cells, a prostate cancer cell line (8, 9, 40, 42).

Although menthol has previously been shown to inhibit VGCCs, the present study is the first that we know of that describes inhibitory effects of icilin and WS-12 on L-IC₅₀. Baylie et al. (7) did not observe any inhibitory effect of icilin on L-IC₅₀ at concentrations up to 100 μM in rabbit cardiac myocytes. The reason for this difference in L-VGCC inhibition by icilin may be any combination of differences between the experimental conditions employed and species (e.g., rabbit vs. rat) or tissues studied. In the present study, electrophysiologically performed experiments were performed at room temperature, whereas Baylie et al. (7) recorded the effects of icilin on L-IC₅₀ at 35°C. In addition to potential species- and tissue-specific (vascular vs. cardiac) differences in L-VGCC subunit composition (e.g., in their α₂δ- and β-subunits) and sequence variations, there is further possibility for variation of responses to pharmacological reagents attributable to tissue-specific alternative splicing resulting in altered channel structures (12, 22). Among the three TRPM8 agonists, WS-12 appears to be the most selective; at least it did not affect the late component of L-IC₅₀ (although this was still inhibited to some extent by vehicle). Correspondingly, the vasorelaxation phase was absent when WS-12 was applied in the presence of PE.

In conclusion, the TRPM8 agonist menthol has dual effects on vascular tone, normally resulting in a biphasic response (trace 1 in Fig. 11A). Menthol causes an endothelium-independent nonspecific vasodilatory effect as a result of L-IC₅₀ inhibition, which could be “unmasked” by interrupting normal Ca²⁺ release (trace 2 in Fig. 11A), and a TRPM8-mediated vasoconstriction effect that was at least partly dependent on normal Ca²⁺ release, which could be unmasked under conditions in which VGCC activity was minimized (trace 3 in Fig. 11A). We thus propose that TRPM8 is expressed and functional in rat tail artery VSM and that it is potentially localized both on the plasma membrane and the SR, the latter predominantly functionally relevant, as illustrated in our summary schematic model (Fig. 11B). The role for TRPM8 channels in vasculature is, as yet, unclear. Specifically for vessels involved in thermoregulation, it is possible that the constrictor effect of TRPM8 activation by cold may maintain a degree of constriction and limit heat loss if sympathetic vasoconstriction fails, as may occur at more extreme cold temperatures (43). Sun et al. (38) have recently provided evidence for beneficial effects of TRPM8 activation by menthol in hypertension treatment. Although much remains to be learned about the pathophysiology of TRPM8 activation by cold, environmental cold is a well-known risk factor for hypertension. Other conditions in which the present findings may have translational implications include cardiovascular surgery, which is often performed at temperatures below 37°C, in which TRPM8 can be activated, organ storage and transplantation, and Raynaud’s phenomenon.

![Fig. 11. Normalized superimposed tensiometric traces demonstrating the effects of menthol on vascular tone under different experimental conditions and a schematic model of the probable underlying mechanisms. A: demonstrative tensiometric traces showing representative effects of menthol in control conditions (1) and in the absence of either VGCC (2) or SR Ca²⁺ (3) involvement, as indicated. Responses have been normalized to the tension observed 10 min after PE (2 μM) application in each experimental condition. In other words, traces were scaled such that, at the moment of menthol application, all 3 had the same amplitude, i.e., the level of tension after 10 min, just before the application of menthol, represents 100% PE-induced contraction. B: schematic mechanistic model showing the potential pathways by which the TRPM8 agonist menthol induces its multiple effects on vascular tone through the activation of the intracellular (major) and plasma membrane TRPM8 channels to cause vasoconstriction and through the inhibition of VGCC to cause vasorelaxation.](http://ajpheart.physiology.org/Downloadedfrom)
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