Am J Physiol Heart Circ Physiol 296: H1868–H1877, 2009.
First published April 10, 2009; doi:10.1152/ajpheart.01112.2008.

Transient receptor potential melastatin 8 channel involvement in the regulation of vascular tone

Christopher D. Johnson, Donal Melanaphy, Andrew Purse, Susan A. Stokesberry, Paula Dickson, and Alexander V. Zholos

Cardiovascular Biomedical Research Centre, School of Medicine, Dentistry and Biomedical Sciences, Medical Biology Centre, Queen's University, Belfast, United Kingdom

Submitted 21 October 2008; accepted in final form 5 April 2009

Johnson CD, Melanaphy D, Purse A, Stokesberry SA, Dickson P, Zholos AV. Transient receptor potential melastatin 8 channel involvement in the regulation of vascular tone. *Am J Physiol Heart Circ Physiol* 296: H1868–H1877, 2009. First published April 10, 2009; doi:10.1152/ajpheart.01112.2008.—The transient receptor potential melastatin 8 (TRPM8) channel has been characterized as a cold and menthol receptor expressed in a subpopulation of sensory neurons but was recently identified in other tissues, including the respiratory tract, urinary system, and vasculature. Thus TRPM8 may play multiple functional roles, likely to be in a tissue- and activation state-dependent manner. We examined the TRPM8 channel presence in large arteries from rats and the functional consequences of their activation. We also aimed to examine whether these channels contribute to control of conscious human skin blood flow. TRPM8 mRNA and protein were detected in rat tail, femoral and mesenteric arteries, and thoracic aorta. This was confirmed in single isolated vascular myocytes by immunocytochemistry. Isometric contraction studies on endothelium-denuded relaxed rat vessels found small contractions on application of the TRPM8-specific agonist menthol (300 μM). However, both menthol and another agonist icilin (50 μM) caused relaxation of vessels precontracted with KCl (60 mM) or the α-adrenoceptor agonist phenylephrine (2 μM) and a reduction in sympathetic nerve-mediated contraction. These effects were antagonized by bromoenol lactone treatment, suggesting the involvement of Ca^{2+}-independent phospholipase A_2 activation in TRPM8-mediated vasodilatation. In thoracic aorta with intact endothelium, menthol-induced inhibition of KCl-induced contraction was enhanced. This was unaltered by preincubation with either N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 nM), a nitric oxide synthase inhibitor, or the ACh receptor antagonist atropine (1 μM). Application of menthol (3% solution, topical application) to skin caused increased blood flow in conscious humans, as measured by laser Doppler fluximetry. Vasodilatation was markedly reduced or abolished by prior application of L-NAME (passive application, 10 mM) or atropine (iontophoretic application, 100 nM, 30 s at 70 μA). We conclude that TRPM8 channels are present in rat artery vascular smooth muscle and on activation cause vasoconstriction or vasodilatation, dependent on previous vasomotor tone. TRPM8 channels may also contribute to human cutaneous vasculature control, likely with the involvement of additional neuronal mechanisms.

human; rat; artery

ION CHANNELS, ESPECIALLY Ca^{2+}-permeable channels, are of central importance to the control of vascular tone as well as long-term phenotypic remodeling. In recent years, members of the transient receptor potential (TRP) superfamily of cation channels attracted considerable interest as novel nonvoltage gated Ca^{2+} permeable cation channels, important determinants of vascular function and disease (2, 4, 5, 13). These channels can be constitutively active or activated by various physiological stimuli, including receptor agonists, Ca^{2+} store depletion, and membrane stretch (2).

Expression patterns and numerous key vascular functions of several members of the family of classical or canonical TRP channels (TRPC) are particularly well characterized (9, 13). There is comparatively little knowledge of the expression profiles and physiological roles of vascular TRPs belonging to the other two major families, vanilloid-related (TRPV) and melastatin-related (TRPM) channel proteins. In nonvascular tissues, members of the TRPV and TRPM families play prominent roles in sensory physiology by allowing cells to sense their local environment by responding to diverse stimuli, such as changes in acidity, osmolarity, mechanical forces, and temperature (37). Several recent studies (13, 40) reported that although the mRNA of most TRPV and TRPM subtypes can be detected with PCR in arterial smooth muscles, TRPV2, -V4, -M4, -M7, and -M8 appear to be predominantly expressed channels. For example, in rat aorta and pulmonary artery the major isoforms are TRPV4 and TRPM8. Activation of these channels with specific ligands induces significant Ca^{2+} elevations in isolated vascular myocytes, indicating their functional expression in vascular smooth muscles (VSMs; Ref. 40).

Several TRPV and TRPM members are now well recognized as primary temperature sensors that mediate responses of small-diameter sensory neurones to thermal stimuli. TRPV1 and TRPV2 are sensors for warm and noxious hot temperatures, respectively, whereas TRPV3 and -V4 can sense moderate temperature changes covering the region from ~25 to 39°C (37). TRPM8 has been extensively characterized as a cold receptor (3, 21, 23, 28), although originally it was cloned as a gene upregulated in prostate cancer (35). The presence of these thermosensitive TRPs in various VSMs raises numerous intriguing questions as to their functional roles, not only in the thermal behavior of blood vessels, but also in chemical signaling. Indeed, thermo TRPs are known to respond to various chemical stimuli as well (e.g., capsaicin and protons activate TRPV1, while menthol and icilin activate TRPM8; Ref. 37). Several studies (21, 31) have also shown that phosphatidylinositol 4,5-bisphosphate plays a central role in TRPM8 activation by both cold and menthol, suggesting that the role for phosphatidylinositol 4,5-bisphosphate is sensory cold transduction in vivo. Addressing the apparently paradoxical expression of TRPM8 in tissues not exposed to any essential temperature variations, we have recently characterized a novel biochemical pathway for TRPM8 activation. This involves multiple cascades of Ca^{2+} store depletion, Ca^{2+}-independent phospho-

Address for reprint requests and other correspondence: C. D. Johnson, Cardiovascular Biomedical Research Centre, Medical Biology Centre, Queen's Univ. of Belfast, 97 Lisburn Rd, Belfast, UK, BT9 7BL (e-mail: c.johnson@qub.ac.uk).

lipase A_2 ($iPLA_2$) activation, and synthesis of lysophospholipids that can activate TRPM8 at physiological temperature (1).

Thus TRPM8 can be potentially involved in the regulation of vascular tone even at constant physiological temperature. Even more intriguing is the role of TRPM8 in thermoregulation and control of skin blood flow. Indeed, in cutaneous circulation complex interplay between physical (cooling) and chemical (e.g., via neurotransmitter release, receptor activation, phospholipid signaling, and Ca^{2+} store release) modes of TRPM8 regulation can be naturally realized, but possible TRPM8 contribution to the vascular effects of local cooling has not yet been studied.

Local temperature-dependent control of cutaneous circulation has been the subject of considerable research showing that local cooling can induce both vasoconstriction and vasodilatation, the latter especially evident at very cold temperatures. The regulatory mechanisms are also very complex and involve a combination of sensory, autonomic, and direct effects, including transmitter release and VSM contractile function. While the initial vasoconstriction is believed to be mainly caused by the release of sympathetic neurotransmitters, there are also nonneurogenic vasodilator and vasoconstrictor components of cold response of unknown origin (15, 32).

Recent identification of TRPM8 as a cold-sensitive Ca^{2+} permeable channel expressed in the vasculature has now opened a new prospect for better understanding of the mechanisms of vascular effects of local cooling. Thus in the present study we intended to identify the role of TRPM8 in the regulation of vascular tone, with the hypothesis that TRPM8 channels are present in VSM and can affect tonic vasoconstriction/dilation. Since cooling has effects at multiple levels (e.g., neurotransmitter synthesis and release, Ca^{2+} homeostasis, adrenergic receptor function, or ability of VSM to contract), we performed experiments at constant physiological temperature and used menthol as a selective TRPM8 agonist. We investigated both molecular expression and in vitro functionality of TRPM8 channels in several rat vessels. In this context, we also examined the action of menthol on blood flow to human skin. Some of this data has been presented in an abstract form (24, 29).

METHODS

Rat studies

All experimental procedures involving animals were in accordance with UK Animal Scientific Procedures Act (1986) and were approved by the Queen's University Animal Welfare and Ethics Committee. Experiments were performed on vessels freshly dissected from 8- to 10-wk-old 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 isometric contraction studies, and the rest was used for semiquantitative PCR. Approximately 2 cm of thoracic aorta were used for contractile studies, the rest being used for semiquantitative PCR. Mesenteric artery was dissected from the aorta to the first branches.

Determination of TRPM8 expression by semiquantitative PCR, Western blotting, and immunocytochemistry

Semiquantitative PCR. Aorta, mesenteric, tail, and femoral arteries were examined with prostate, dorsal root ganglia, and liver used as positive controls. RNA was extracted from tissue using TRI Reagent following the recommended protocol. RNA concentration was then determined using a biophotometer (Eppendorf, Hamburg, Germany). Isolated RNA was reverse transcribed into cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions and was incubated in a thermal cycler (Techne, Burlington, NJ) at 37°C for 2 h, followed by 85°C for 5 min. Assay was performed using Reaction-Ready HotStart "Sweet" PCR master mix (SA Biosciences, Frederick, MD) with TRPM8 primer assay PPR52298A-200 (SA Biosciences). Reactions of 25 μl were subjected to 15 min at 95°C to activate HotStart Taq DNA polymerase and 35 cycles of PCR at 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Ten microliters of each reaction were electrophoresed on a 2% agarose gel. TRPM8 amplicon size was 81 base pairs.

Western blotting. Vascular tissue (aorta, mesenteric, tail, and femoral arteries) and prostate gland (for positive control) were removed and homogenized in ice-cold RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The homogenate was centrifuged at 4°C with 14,000 *g* for 10 min, the supernatant was collected, and the protein concentration was estimated using bicinchoninic acid protein assay (Pierce, Rockford, IL). The protein sample (30 μg) was resolved in an 8% SDS-PAGE gel and electrotransferred onto a nitrocellulose membrane (PerkinElmer, Waltham, MA). The membrane was blocked with 5% (wt/vol) BSA in PBS containing 0.1% Tween 20 for 1 h at room temperature, followed by incubation at 4°C overnight with the specific antibody polyclonal rabbit anti-TRPM8 (1:1,000; Alomone Labs, Jerusalem, Israel). The nitrocellulose membrane was then washed with PBS containing 0.1% Tween 20. After being washed, the membrane was incubated with peroxidase-conjugated goat-anti-rabbit secondary antibody (1:3,000 dilution; Bio-Rad, Hercules, CA) at room temperature for 1 h. Excess secondary antibody was again washed, and the bound secondary antibody was detected with ECL plus (GE Healthcare, Chalfont, St. Giles, UK). Protein loading was checked by examining β-tubulin levels.

Immunocytochemistry. Ventral tail arteries were isolated and transferred to Ca^{2+}-free physiological salt solution containing the following (in mM): 120 NaCl, 6 KCl, 1.2 $MgCl_2$, 10 HEPES, and 12 glucose, pH 7.4 (adjusted with NaOH). The proximal artery was cleaned free of connective tissue and longitudinally cut, and the endothelium was removed by gentle rubbing of the luminal surface. The tissue was then sectioned into ~5-mm lengths before being transferred to dissociation medium containing the following (in mM): 110 NaCl, 5 KCl, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 10 Na_2HCO_3, 10 HEPES, 10 taurine, 0.5 EDTA, 10 glucose, 2 $MgCl_2$, and 0.16 $CaCl_2$, pH 7.4 (adjusted with NaOH). The tissue was digested at 37°C for 20 min in dissociation medium 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 physiological salt solution to stop digestion. Single myocytes were dispersed by trituration with a small-bore pipette, and the cell suspension was placed on glass coverslips.

When cells adhered to coverslips, they were fixed in 4% paraformaldehyde in PBS for 3 min at room temperature. Cells were then washed four times for 15 min for 1 h before being incubated with 1% BSA in PBS for 1 h at room temperature. Cells were then incubated with rabbit anti-TRPM8 channel antibody (ACC-049; Alomone Labs; dilution of 1:200) in PBS containing 1.0% BSA and 0.05% Triton-X overnight at 4°C, washed, and incubated for 1 h with Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen Molecular Probes, Paisley, UK; dilution of 1:200). After a further wash, coverslips were mounted on slides with mounting medium containing DAPI and examined under a confocal microscope.

Contractile studies

Unless specified, tissue had the endothelium removed by passing a fine wire down the vessel lumen. Rings of proximal tail artery, thoracic aorta, or mesenteric artery (3- to 4-mm long) were mounted

on stainless-steel hooks within 4-ml tissue baths perfused (at 2 ml min^{-1}) with Krebs-Hansleit solution of the following composition (in mM): 118.4 NaCl, 4.75 KCl, 25 Na_2HCO_3, 1.19 KH_2PO_4, 1.18 $MgSO_4$, and 0.95 $CaCl_2$. Successful removal of the endothelium was confirmed by complete failure of ACh (10 μM) to elicit vasorelaxation after 10 min of contraction with KCl (60 mM; data not shown). Bath contents were kept at 37°C and continually bubbled with 5% CO_2-95% O_2 (pH of 7.4). Segments of vessels were suspended with cotton thread attached to hooks in baths and attached to force transducers (Piodem, UF1, 25 g; Digitimer, Welwyn Garden City, UK). Vessel mechanical responses were amplified (Neurolog NL108; Digitimer) and digitized using a laboratory interface (Micro 1401; C.E.D., Cambridge, UK) and data acquisition software (Spike 2, C.E.D.). Resting tensions on vessel segments were set as follows: 0.75 g of tail artery, 0.75 g of mesenteric artery, and 1 g of aorta, and they were left to stabilize for 1 h.

Protocols

The condition of the tissue was initially tested by adding KCl (60 mM) and rejecting tissue that did not responded with a robust contraction: tail and aorta of >0.5 g tension and mesenteric artery of >0.3 g. Then, one of three basic protocols was conducted. *1*) Vessels were examined simply for a contractile response to menthol (300 μM). *2*) Vessels were contracted with either KCl (60 mM) or with the α_1-adrenoceptor agonist phenylephrine (2 μM). Menthol (300 μM) or icilin (50 μM) was added either during contraction (having allowed 10 min for contraction to stabilize) or 3 min before contraction. *3*) Electrical field stimulation was delivered by two parallel platinum wires placed either side of the vessel (5-mm separation) with a supramaximal stimulus (5 impulses at 20 Hz, 6V, 1-ms duration, every 90 s). Evoked responses are abolished by tetrodotoxin (1 μM) or guanethidine (10 μM).

To investigate a possible contribution of the vascular endothelium to TRPM8 channel agonist responses, an additional set of experiments examined effects of menthol after 20 min incubation with the nonspecific muscarinic receptor antagonist atropine (1 μM) or an inhibitor of nitric oxide production, N^{ω}-nitro-L-arginine methyl ester (L-NAME; 20 μM). Thoracic aorta was used for this protocol, as it was easier to keep endothelium intact when required. Endothelium was deemed intact when addition of ACh (10 μM) resulted in >50% reduction of a KCl-induced precontraction.

Having found vascular responses evoked by TRPM8 agonists, a final set of experiments was performed where tail artery segments were preincubated (25–40 min) with inhibitors of the $iPLA_2$ isoforms, $iPLA_2\beta$ (localized in cytosol) and $iPLA_2\gamma$ (localized in cell membrane), using two isoforms of bromoenol lactone (BEL), (*S*)-BEL (100 μM) and (*R*)-BEL (100 μM), respectively.

Human study

Eleven subjects (6 males and 5 females; 23 ± 6 yr old) participated. All were nonsmokers, were not taking prescribed medication (except oral contraceptives), and had no history of cardiovascular disease. All protocols were approved by Queen's University School of Medicine and Dentistry Ethics Committee and conformed to guidelines contained within the Declaration of Helsinki. Written and verbal consent was obtained from each subject before participation. Subjects were asked to refrain from drinking alcohol for 24 h before the experiment and were asked not to participate in vigorous exercise, eat a large meal, or drink caffeine within 2 h.

Experimental methods

Experiments were performed in a small temperature-controlled room kept at 21 ± 1°C. Subjects sat upright in a comfortable and supportive chair with their left arm supported at heart level. Cutaneous red cell flux (RCF) was recorded from the ventral surface of the left forearm using a laser Doppler perfusion monitor (DRT4; Moor Instruments, Axminster, England) and expressed as arbitrary perfusion units (PU). Drugs were applied to the area of recording either by passive diffusion (menthol, L-NAME) or iontophoresis. For both methods, a Perspex ring-shaped electrode chamber (Moor Instruments) was fitted to the ventral surface with adhesive rings. An indifferent electrode was mounted in a saline-soaked strap that was placed around the left wrist. The chamber held the Doppler probe allowed injection of drug into the chamber (0.71 cm^2 surface area exposed) before recording. Iontophoresis was driven by a control unit (MIC1-e; Moor Instruments) attached to the electrode chamber and indifferent electrode. Data acquisition and the iontophoretic unit were controlled by software (moorSOFT/DRT4 v2.0) on a laptop computer. The forearm was chosen as the recording site primarily because baseline RCF was less labile than that recorded from the fingers or the hand.

Blood pressure was measured using automatic brachial auscultation (Critikon Dinamap 845, Tampa, FL) via an inflation cuff attached to the opposite (right) arm recording at the end of a 20-min acclimatization period before a protocol began, then in the middle, and in the last minute of the protocol. As blood pressure values were not affected by drug application (see Table 3), an increase in RCF represents a vasodilatation.

Protocols

The overall strategy was to determine, first, whether the TRPM8-specific agonist menthol (3% solution in 25% ethanol and sterile water) had an effect on RCF and then to examine possible mechanisms by which any effects might occur by pretreatment of recording site with atropine (10 mM) and L-NAME (100 nM) before subsequent application of menthol. Two recording sites were used in each protocol within 5 cm of each other. If RCF values were different (±10% of each other), a different site was chosen until flux measurements were comparable. In the first protocol, menthol was applied to the first chamber and its vehicle to the second chamber. In the second protocol, the effects of ACh were examined as a positive control for subsequent actions of atropine and L-NAME: pilot experiments indicated, first, that iontophoresis of ACh for 30 s at 70 μA (see Ref. 17 for similar protocol) produced a robust and reproducible increase in RCF, and, second, that iontophoresis of atropine for 30 s at 70 μA for 12 min before ACh application and then removed from the chamber by saline flush, was sufficient to block the affects of ACh (17); similarly, passive pretreatment with L-NAME for 12 min caused maximal inhibition of ACh response (see Ref. 7 for similar protocol). In the final protocol, the affects of these two compounds on the response to menthol were examined.

Drugs

All drugs were supplied by Sigma-Aldrich, except for the following: *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide (BCTC; Biomol International); (*R*)- and (*S*)-BEL (Cayman Chemical, Detroit, MI); tetrodotoxin (Tocris, Bristol, UK); and tetracaine gel (4%; Ametop, Smith & Nephew, UK). In the rat study, 100 mM menthol stock solution in ethanol were used to achieve the final bath concentration of 300 μM. Icilin was made up to 50 μM in the tissue bath, initially dissolved in DMSO. For humans, a 3% solution of menthol was made by dissolving in 25% ethanol and made up with sterile distilled water (192 mM). ACh was made up as a 1% solution with 3% mannitol in sterile water. Atropine and L-NAME were made up as 10 mM and 100 nM solutions, respectively, in sterile water.

Analysis of results

Rat study. Data are means ± SE. Raw data were analyzed by one-way ANOVA (followed by Tukey's post hoc test where appro-

priate) or paired Student's *t*-tests (where stated). Summary data for experiments where menthol/icilin was given during contraction are presented as percentage of contraction 10 min after application of menthol/icilin relative to that immediately before the drug application. Summary data for experiments where menthol/icilin was given 3 min before contraction are presented as contraction in the presence of menthol/icilin compared with the contraction in their absence.

Human study. Data are means ± SE. For RCF recordings in the presence of menthol, ethanol vehicle alone had no affect on RCF; therefore, the effects of menthol plus vehicle data were used for analysis. All statistical analyses were performed on RCF measurements. Comparisons within a drug treatment over time and between menthol and vehicle were made by ANOVA for repeated measures followed by Tukey's post hoc test where appropriate. Paired Students *t*-tests were used to compare peak in response to ACh, ACh plus atropine, and ACh and L-NAME, and for baseline values before addition of menthol, after pretreatment with atropine, L-NAME, or tetracaine. Differences at $P < 0.05$ were considered to be significant.

RESULTS

Identification of TRPM8 expression in rat vessels by semiquantitative PCR, Western blotting, and immunocytochemistry

Expression of TRPM8 receptor mRNA was confirmed by conventional semiquantitative PCR in rat tail artery, mesenteric artery, femoral artery, and aorta as well as prostate and liver for positive controls. Figure 1*A* shows the amplified products at ~81 base pairs generated after 35 cycles. The results were confirmed in four separate experiments. Expression of TRPM8 channel proteins in vascular tissue was further examined using Western blot analysis. The specific anti-TRPM8 antibody detected clear bands of expected ~150 kDa in all vascular tissue samples. Positive controls for TRPM8 were obtained from prostate tissue (Fig. 1*B*).

TRPM8 protein expression was also confirmed by immunocytochemistry in isolated tail artery myocytes. TRPM8 labeling yielded a thin intense signal on the perimeter of the cell, which can be attributed to its plasmalemmal and subplasmalemmal localization. In addition a more diffuse, but clearly clustered signal, was visible throughout the cytosol (Fig. 1*C*). These results are consistent with the view that TRPM8 can be expressed in both the plasma membrane and sarcoplasmic reticulum membrane where it mediates, correspondingly, Ca^{2+} influx and Ca^{2+} release (6). This confirms and greatly extends findings from the studies by Yang et al. (40) and Inoue et al. (13).

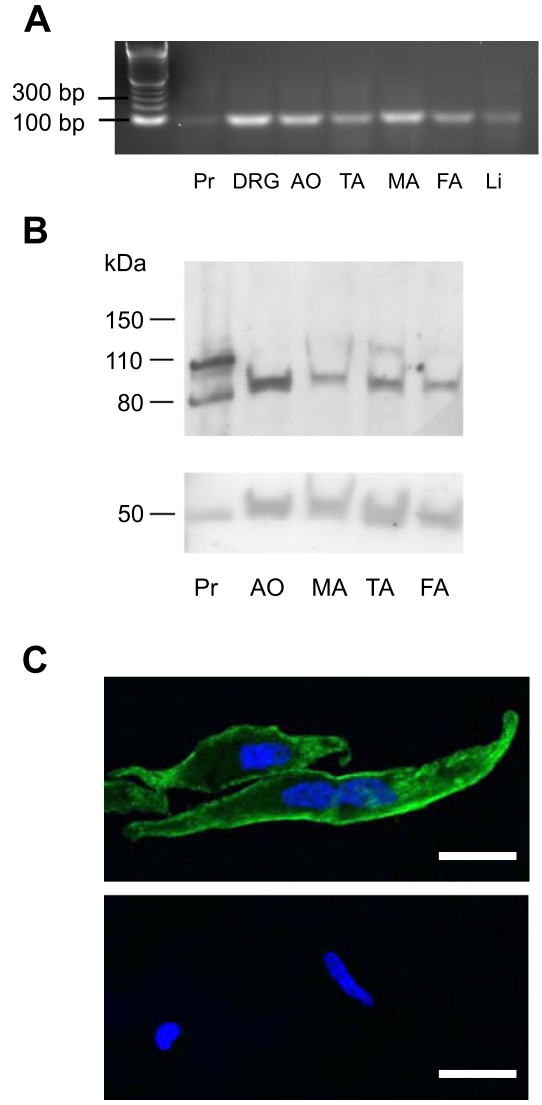

Fig. 1. *A*: transient receptor potential melastatin 8 (TRPM8) mRNA expression by semiquantitative PCR in rat blood vessels with endothelium removed. Prostate tissue (Pr), dorsal root ganglia (DRG), and liver (Li) were used as positive controls. Predicted size of PCR product was 81 base pairs. AO, aorta; TA, ail artery; MA, mesenteric artery; FA, femoral artery. *B, top*: Western blot analysis of TRPM8 protein expression in rat vascular tissue. Prostate protein was used as positive control. Expected size of TRPM8 was <150 kDa. *B, bottom*: β-tubulin used to demonstrate protein loading (at 50 kDa). *C, top*: immunocytochemical staining of the TRPM8 protein within isolated rat tail artery myocytes. Greater green fluorescence in the cell periphery suggests the channel may be located on subplasmalemmal sarcoplasmic reticulum and/or surface membrane. *C, bottom*: negative control performed by omitting the primary TRPM8 antibody. Nuclei appear blue in both images. Scale bars = 25 μm in *top* and *bottom*.

Rat isometric contraction study

In the pretensed, relaxed tail artery with endothelium removed, addition of menthol (300 μM) caused a small but consistent contraction that rose to a peak in the first minute of application (to 0.061 ± 0.007 g or 10 ± 1% of 60 mM KCl contraction; $n = 21$; $P < 0.001$, paired Student's *t*-test; Fig. 2*A*).

However, contractions in tail artery evoked by electrical stimulation of sympathetic nerves were significantly reduced by menthol ($P < 0.05$; $n = 11$, paired Student's *t*-test; Figs. 2*B* and 3*B*; Table 1). Addressing the possible mechanism(s) of the inhibitory action of menthol, we tested the effects of TRPM8 activation on KCl- or phenylephrine-induced contractions. When menthol was added after 10 min of precontraction induced by phenylephrine (2 μM, to 0.83 ± 0.12 g; $n = 10$) or KCl (60 mM, to 0.79 ± 0.07 g; $n = 16$), there was a marked relaxation to 34 ± 7 and 31 ± 5%, respectively, of the control contraction ($P < 0.001$ in each case, paired Student's *t*-test; Figs. 2*C* and 3*A* and Table 1). Preincubation of arteries with menthol caused a similar reduction of contractions caused by KCl and phenylephrine ($P < 0.001$, paired Student's *t*-test; Fig. 3*A*; Table 1). Almost identical results were gained using icilin (50 μM; Figs. 2*D* and 3*A*; Table 1). As icilin is a compound that is structurally unrelated to menthol, it is un-

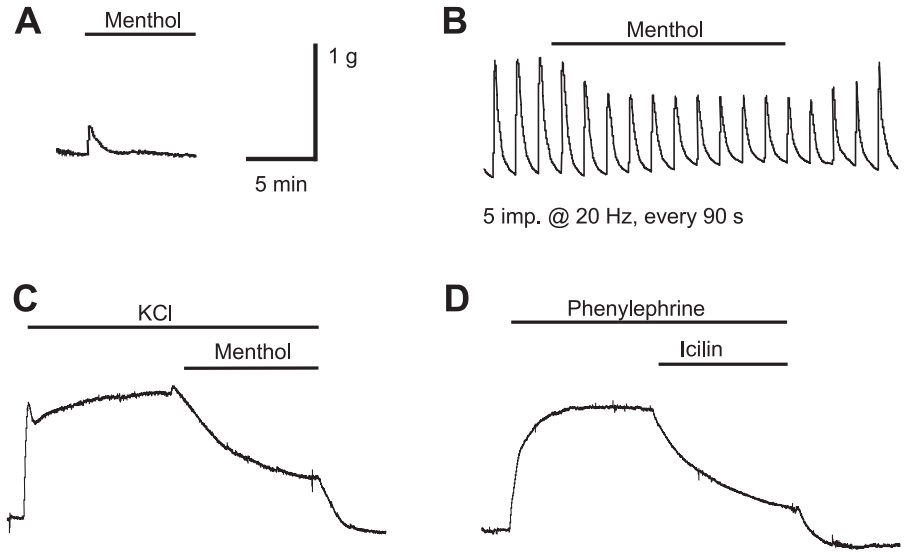

Fig. 2. *A*: menthol (300 μM) caused a moderate contraction in pretensed, relaxed tail artery. *B*: when applied during electrical field stimulation of sympathetic nerves, menthol reduced the size of contractile responses. *C* and *D*: vessels precontracted with either KCl (60 mM) or penylephrine (2 μM) relaxed in the presence of menthol (300 μM) and icilin (50 μM), respectively. Scale bar in *A* applies to *A*–*D*.

likely that these are nonspecific actions of these compounds. Thus our results show two responses to menthol agonists: a moderate vasoconstriction when vessels have low vasoconstrictor tone, and a marked vasodilatation when vasoconstrictor is high.

We attempted to block the agonist responses using three commercially available putative TRPM8 antagonists: BCTC (10–30 μM), 1,10 phenanthroline (0.4–1 mM), and capsazepin (30–100 μM). Preincubation with any of these agents strongly inhibited the initial KCl- or phenylephrine-induced contraction (by 60–100%, results not shown), indicating the nonspecific effects of these blockers, possibly on voltage- and agonist-stimulated Ca^{2+} entry pathways. Under these circumstances, it was not possible to assess reliably whether the responses to TRPM8 agonists are inhibited and discouraged us from pursuing this approach further.

Between vessels, the tail artery was affected to a greater extent by TRPM8 agonists compared with the aorta and mesenteric artery, although there were no significant differences between the aorta and mesenteric artery (Fig. 3*C*; Table 1). Preliminary recordings from femoral and renal arteries have also shown qualitatively similar effects of menthol and icilin on precontracted vessels (data not shown).

In thoracic aorta, the menthol-induced relaxation in precontracted vessels was significantly greater when the endothelium was left intact (by 15–20%), compared with denuded vessels (Fig. 3*D*; Table 2). Therefore, it appears that, apart from VSM, TRPM8 channels are present on the endothelium, which can contribute to vasodilatation when activated. However, this enhanced relaxation was unaffected by the additional presence of either L-NAME or atropine and appears to be independent of nitric oxide production or muscarinic receptor activation.

Table 1. *Responses of rat arterial vessels to TRPM8 agonists: absolute and normalized values*

	Tail Artery	Aorta	Mesenteric Artery
KCl	0.79±0.07 (16)	0.96±0.06 (19)	0.51±0.09 (10)
KCl + menthol	0.27±0.07 (16)	0.57±0.04 (19)	0.40±0.09 (10)
	31±5%[c]	63±4%[b,e]	74±7%[b,d]
KCl + icilin	0.40±0.07 (16)	0.57±0.06 (11)	0.40±0.10 (5)
	45±5%[c]	71±6%[b,d]	76±12%[b,d]
Menthol + KCl	0.39±0.10 (16)	0.57±0.07 (11)	0.50±0.12 (6)
	47±6%[c]	72±3%[b,d]	82±11%[e]
Icilin + KCl	0.32±0.06 (16)	0.64±0.06 (8)	0.45±0.13 (6)
	38±3%[c]	80±4%[c,d,e]	70±17%[d]
Phenylephrine	0.83±0.12 (10)	1.14±0.2 (5)	0.60±0.10 (4)
Phenylephrine + menthol	0.34±0.10 (10)	0.90±0.15 (4)	0.56±0.09 (4)
	34±7%[c]	75±3%[a]	83±6%[d]
Phenylephrine + icilin	0.26±0.10 (10)	0.52±0.11 (4)	0.35±.017 (3)
	28±9%[c]	59±8%[a]	77±11%[d]
Menthol + phenylephrine	0.62±0.12 (11)	0.88±0.21 (5)	0.57±0.13 (4)
	75±12%[a]	76±6%[a]	93±15%
Icilin + phenylephrine	0.34±0.12 (8)	0.49±0.14 (3)	0.32±0.15 (3)
	48±11%[b]	53±6%[a]	64±17%[a]
EFS	1.40±0.25 (11)	—	—
EFS + menthol	0.82±0.16 (11)		
	55±5%[a]	—	—

EFS, electrical field stimulation. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, respectively, control response vs. response in the presence of transient receptor potential melastatin 8 (TRPM8) agonist by paired Student's *t*-test. [d]$P < 0.05$, [e]$P < 0.01$, tail artery vs. aorta or mesenteric artery by one-way ANOVA. Number of measurements is shown in parentheses. Percentages refer to %control response remaining in the presence of TRPM8 agonist.

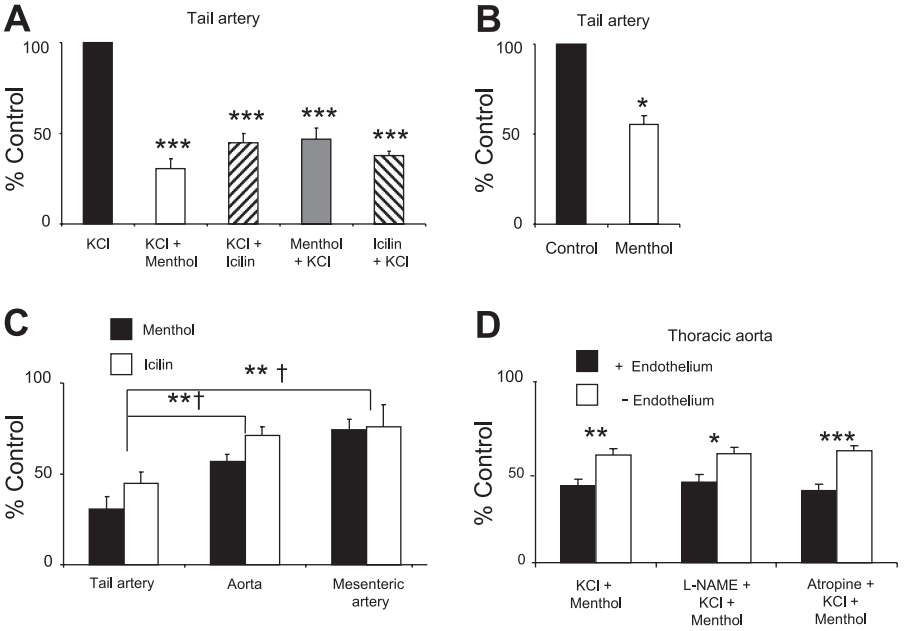

Fig. 3. *A*: summary of the effects of menthol or icilin on the contractile responses in tail artery to KCl (60 mM; $***P < 0.001$, one-way ANOVA). *B*: menthol reduced the response to electrical field stimulation (5 impulses at 20 Hz, every 90 s; $*P < 0.05$, paired Student's *t*-test). *C*: summary of the inhibitory effects of menthol (filled bars) and icilin (open bars) on the contractile responses to KCl (60 mM). These were reduced to a greater extent in tail artery than in aorta or mesenteric artery ($**P < 0.01$, menthol responses; $^{\dagger}P < 0.05$, icilin responses, one-way ANOVA). *D*: in thoracic aorta, menthol-induced relaxation (300 μM) of a KCl-induced contraction was enhanced when endothelium was kept intact ($**P < 0.01$, one-way ANOVA, filled bars) compared with responses after removal of endothelium (open bars). This enhancement was unaffected by preincubation of tissue with either N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 nM; $*P < 0.05$) or atropine (1 μM; $***P < 0.001$).

Since TRPM8 activity can be induced at physiological temperature by $iPLA_2$ activation (1), we assessed the role of β- and γ-isoforms of $iPLA_2$, using (*S*)-BEL and (*R*)-BEL, respectively. First, since $iPLA_2$ shows some background activity (1), we investigated the effects of BEL on the inhibitory action of menthol on KCl-induced contractions (Fig. 4*A*). Menthol-evoked vasodilatation was significantly reduced in the presence of (*S*)-BEL [33 ± 3% of contraction remaining after menthol in control vs. 48 ± 4% after preincubation with (*S*)-BEL; 12 preparations from 6 rats; $P < 0.01$ by paired *t*-test]. However, there was no significant effect of (*R*)-BEL (47 ± 4% in control vs. 54 ± 4% after BEL; 8 preparations from 3 rats), suggesting that $iPLA_2\beta$, but not $iPLA_2\gamma$, inhibition is involved in these effects. Second, since agonists activate $iPLA_2$ (27), we also tested the effects of BEL on the inhibitory action of menthol on phenylephrine-evoked contractions as illustrated in Fig. 4, *B* and *C*. Consistent with the study by Park et al. (27), treatment with 100 μM (*S*)-BEL for 40–50 min severely reduced phenylephrine-induced contractions in five out of nine preparations obtained from four rats (55–98% reduction). In the remaining four preparations in which the agonist contractile response was preserved by >50% (65–100%), (*S*)-BEL almost abolished menthol-induced vasodilatation (94 ± 4% of control; $P < 0.03$, paired Student's *t*-test). In contrast, (*R*)-BEL under identical conditions inhibited neither the phenylephrine-induced contractions (108 ± 5%; $n = 9$) nor menthol-induced vasodilatation. We finally tried to reduce the inhibitory action of (*S*)-BEL on phenylephrine-induced contraction by reducing its concentration to 25 μM and time of treatment to 20 min. In this case, although the agonist-induced contractions were preserved in all preparations (106 ± 5% of control; $n = 6$), menthol-induced vasodilatation was also not affected. Thus (*S*)-BEL appears to inhibit in parallel both agonist-induced contractile responses and the effects of menthol.

Table 2. *Responses of rat aorta- endothelium intact and denuded*

	Aorta Endothelium Intact, g	Aorta Endothelium Denuded, g
KCl	1.21±0.08 (7)	1.14±0.06 (7)
KCl + menthol	0.46±0.03 (7)	0.61±0.05 (7)
	38±3%	54±4%†
KCl + menthol + atropine	0.40±0.03 (7)	0.68±0.06 (7)
	36±2%	56±4%*
KCl + menthol + L-NAME	0.50±0.04 (7)	0.66±0.06 (7)
	40±3%	55±4%‡

$*P < 0.05$, $\dagger P < 0.01$, $\ddagger P < 0.001$, respectively, control response in the presence of menthol versus response in the presence of menthol + atropine/N^{ω}-nitro-L-arginine methyl ester (L-NAME) by one-way ANOVA. Numbers in parentheses refer to number of vascular rings tested. Percentages refer to %control response remaining in the presence of menthol or menthol + atropine/L-NAME.

Human studies

Baseline blood pressures for all 12 subjects before and after the application of menthol, ACh, atropine, or L-NAME are shown in Table 3. There were no significant changes in blood pressure during any of the applications of drugs, so that an increase in RCF represents an increase in vascular conductance (decreased resistance) rather than pressure-related changes in flow. RCF was generally low (22 ± 3 PU) after the resting period, allowing more sensitive detection of the increases in RCF.

Application of menthol resulted in a gradual but marked increase in RCF in 11 out of 12 subjects after a delay of between 8 and 18 min, usually reaching a peak after 18–30 min (98 ± 19 PU after 30 min; $n = 12$; see Fig. 5*A*), indicating a marked vasodilatation. RCF measured concurrently from the vehicle control chamber showed no increase (24 ± 4 PU; $n = 12$) over the same time, so that menthol-induced increases in flow were highly significant compared with vehicle control ($P < 0.001$ at 30 min, ANOVA for repeated measures). Although diffusion delays may contribute to the rather slow onset of menthol action, it is interesting to note that in similar experiments responses to local cooling from 34 to 20°C also

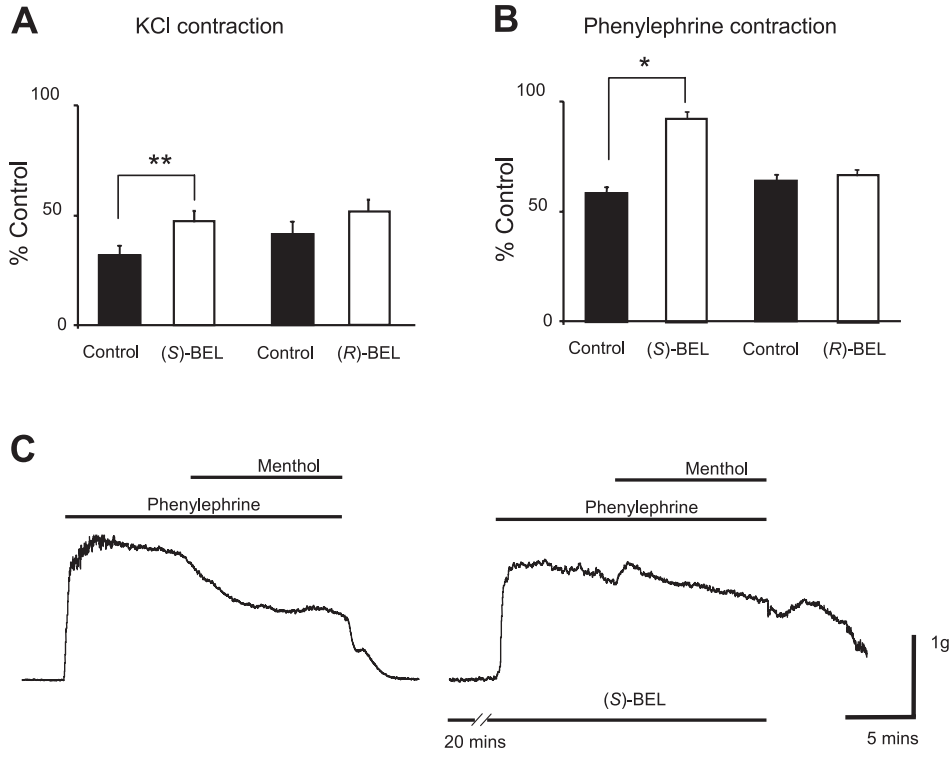

Fig. 4. *A* and *B*: relaxations of KCl-induced (60 mM; *A*) or phenylephrine-induced (2 μM; *B*) contractions in tail artery by menthol (filled bars) were significantly reduced (*$P < 0.05$, **$P < 0.01$, paired Student's *t*-test) after preincubation with (*S*)-BEL (100 μM) but not after preincubation with (*R*)-BEL (100 μM, open bars). Control KCl- and phenylephrine-induced contractions shown by hatched bars. *C*: raw data traces showing the control phenylephrine-induced contraction followed by the menthol-induced vasodilatation in at *left* and the loss of menthol-induced vasodilatation in the presence of (*S*)-BEL at *right*.

developed over a comparable time (15). However, in the latter case the principal response was vasoconstriction, while the vasodilator response was seen only at the sites treated with sympatholytics (see DISCUSSION).

Iontophoretic application of ACh produced a rapid increase in RCF to a peak (102 ± 14 PU; $n = 12$) at ~4 min, with a gradual recovery to baseline (see Fig. 5*B*). Pretreatment of the recording area by passive application of L-NAME (100 nM) significantly reduced the ACh response (54 ± 12 PU; $n = 12$; $P < 0.05$, paired Student's *t*-test) to ~55 ± 24% of the ACh response. Iontophoretic coapplication of atropine (10 mM) caused a more profound reduction (27 ± 8 PU; $n = 12$; 26 ± 4%; $P < 0.01$, paired Student's *t*-test). Thus our experimental procedures were working as would be predicted from previous studies (7, 18), serving as positive controls for the following experiments. The response to menthol (Fig. 5*C*) was reduced to a similar degree in the presence of L-NAME (to 50 ± 11 PU after 30 min; $n = 12$; 52 ± 45%; $P < 0.05$, ANOVA for repeated measures), as was the menthol response in the presence of atropine (to 38 ± 7 PU after 30 min; $n = 12$; 47 ± 10%; $P < 0.001$, ANOVA for repeated measures).

In an attempt to test the involvement of sensory nerves in the blood flow changes in response to menthol, we used pretreatment of the recording area with the local anesthetic, tetracaine. This caused a marked increase in RCF (to 164 ± 32 PU; $n = 3$; data not shown). At both the control site in the presence of tetracaine alone and the test site in the presence of tetracaine and menthol, there was a gradual decrease in RCF over 30 min, with menthol appearing not to have any additional effect on the response (to 47 ± 11 PU with tetracaine alone, 44 ± 15 PU in the presence of menthol, after 30 min; $P < 0.05$ in each case, paired Student's *t*-test; 1 vs. 30 min; $n = 3$).

Discussion

Despite the use of menthol in culinary and medicinal preparations over the millennia (see Ref. 10), relatively little is known about its actions on the human cardiovascular system. In the first part of this study, we confirmed that TRPM8 channels are both expressed and functional in several rat arteries. The major effect was seen in precontracted vessels, where TRPM8-specific agonists caused a profound dilatation. These effects were predominantly attributable to direct stimulation of TRPM8 channels associated with VSM cells. The endothelium also contributed a significant relaxation (~15% of control contraction) on activation by menthol, although this enhancement of the menthol-induced relaxation appears to be independent of cholinergic/nitric oxide pathways. Neverthe-

Table 3. *Blood pressure and RCF values in human subjects*

	Baseline	After 30-min Menthol	After 12-min ACh	Baseline After Pretreatment with L-NAME/Atropine	After 30-min Menthol/ L-NAME/Atropine
RCF, PU	22±3	96±19	102±14	15±2/25±4*	49±11/38±7
Systolic pressure, mmHg	129±2	120±3	119±3	122±3	122±2
Diastolic pressure, mmHg	72±2	73±1	71±2	73±2	73±2
Mean ABP, mmHg	91±2	88±2	87±2	90±3	90±2

RCF, red cell flux; PU, perfusion units; ABP, arterial blood pressure. *$P < 0.05$, control baseline vs. L-NAME pretreatment baseline by paired Student's *t*-test.

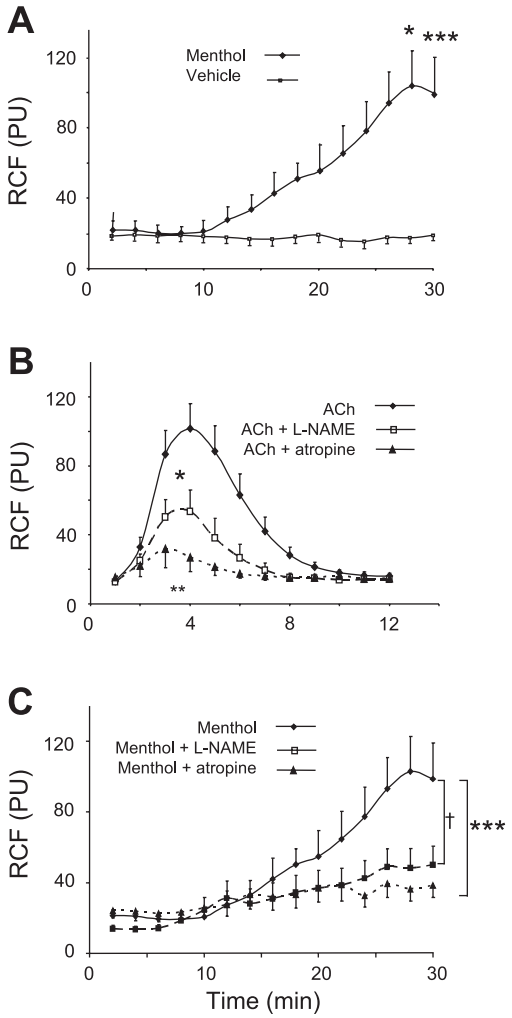

Fig. 5. Effects of menthol and ACh on human forearm cutaneous red cell flux. *A*: Vasodilatation after ~10 min passive menthol application (3% solution; solid line). Blood flow from the vehicle control site (25% ethanol; dashed line) recorded simultaneously showed no such increase ($*P < 0.05$ and $***P < 0.001$, respectively, menthol vs. vehicle control, ANOVA for repeated measures). *B*: iontophoretic application of ACh (1% solution, 1 × 30 s at 70 μA; solid line, ◆) caused a marked vasodilatation which was significantly attenuated by pretreatment with L-NAME (10 mM solution, 12 min, large dashes, □) or atropine (100 nM solution, 1 × 30 s at 70 μA; fine dashes, ▲). $*P < 0.05$ and $**P < 0.01$ levels for peak ACh response vs. peak response with L-NAME or atropine, paired Student's *t*-test. *C*: passive application of menthol (solid line, ◆) caused a marked vasodilatation, which was significantly attenuated by pretreatment with L-NAME (10 mM solution, 12 min, large dashes, ■) or atropine (100 nM solution, 1 × 30 s at 70 μA; fine dashes, ▲). $^{\dagger}P < 0.05$ and $***P < 0.001$ levels at 30 min for menthol vs. L-NAME or atropine, respectively, ANOVA for repeated measures.

less, we show that contractile action of sympathetic neurotransmitters can be antagonized by TRPM8 activation (Fig. 2, *B* and *D*). This observation is particularly relevant to our in vivo human studies.

In the second part of this study, we found that in human forearm cutaneous vessels, menthol caused a profound dilatation. The delay in action (8–18 min) may simply reflect the time taken for menthol to passively diffuse to blood vessels from which RCF measurements were made ~1 mm below the skin surface. This observation is of particular relevance in light of the findings from studies in rat arteries. However, in contrast to the rat studies, we have also demonstrated that at least part of the vasodilatation in humans involves activation of muscarinic receptors and/or production of nitric oxide. Thus it appears that there are several mechanisms by which TRPM8 activation can influence VSM cells and that the consequences of TRPM8 channel activation may vary according to prevailing vasomotor tone and autonomic innervation influences.

Many recent studies (6, 8, 34, 36) have implicated the TRPM8 channel as a regulator of intracellular calcium. Increased intracellular Ca^{2+} concentrations associated with channel activation (38) would be expected to cause contraction. The small contraction of nonstimulated tail artery is consistent with this (Fig. 2*A*). Slightly more puzzling is the observation of relaxation of precontracted vessels (by either phenylephrine or high potassium) in response to menthol and icilin. As icilin is a compound that is structurally unrelated to menthol, it is unlikely that this is a nonspecific action of these compounds. Similar observations of menthol effects in other smooth muscle tissues have been made previously [guinea-pig ileum (12), guinea-pig bronchus (39), and guinea-pig trachea (14)]. Notably, several studies (1, 6, 34, 41) have shown that the channel can be expressed both on plasma membrane and endoplasmic reticulum and can alter intracellular calcium (Ca^{2+}) fluxes through both routes. Its localization on the perimeter of the VSM cell and deeper in the cytosol is also consistent with such dual roles (Fig. 2*C*). TRPM8 localized in the sarcoplasmic reticulum membrane can support cold/menthol/icilin-induced Ca^{2+} release from the sarcoplasmic reticulum causing Ca^{2+} store depletion (1, 34). This seems to be one of the most likely mechanisms that can explain vasoconstrictor inhibition by TRPM8 agonists, but further studies are required to substantiate this view. Consistent with this is the observation that during patch clamp recording in rat tail artery myocytes the rate of spontaneous outward transient currents (STOCs, which reflect BK_{Ca} channel activation due to spontaneous localized calcium release events) was initially increased by application of menthol, while at the same time amplitude of STOCs was reduced until STOC activity ceased almost completely (24). It is also of interest that an early in vivo study demonstrated a fall in arterial blood pressure on systemic injection of menthol in rabbits and cats (30), suggesting that vasodilatation induced by systemically administered menthol can have significant effects on arterial blood pressure.

The small but significant reduction in the vasorelaxing effect of menthol by $iPLA_2\beta$ inhibition (but not $iPLA_2\gamma$; Fig. 3*E*) supports the mechanism proposed by Abeele et al. (1) that involved $iPLA_2\beta$ activation and production of lysophospholipids; the latter can activate TRPM8 directly even at physiological temperature. However, significant relaxation of KCl-induced contractions remained in (*S*)-BEL-treated preparations suggesting that $iPLA_2$ activation (or rather its basal activity since no intervention was made in these experiments that could induce $iPLA_2$ activity) plays a relatively minor role in this process. In contrast, receptor agonists induce $iPLA_2$ activation (27). We found that both phenylephrine-induced contractions and menthol-induced inhibition of agonist-induced contractions were inhibited by (*S*)-BEL but not by (*R*)-BEL (Fig. 4). Taken together, these results imply that agonist-induced rather than basal activity of $iPLA_2\beta$ could play an important signaling role in TRPM8 activation in VSM (cf. to Refs. 1 and 27).

Apart from the small contribution of endothelium to menthol-induced relaxation already mentioned, there may have been a neural contribution to the responses observed in rat vessels. The major innervation to the rat tail artery specifically is a sympathetic vasoconstrictor supply (33). It is possible that TRPM8 channel activation in sympathetic terminals may have caused the release of neurotransmitter, contributing to the vasoconstriction under relaxed conditions. Regarding relaxation of preconstricted vessels caused by phenylephrine and KCl, there is no sympathetic dilator innervation documented to rat tail artery (11) and this possibility may be discounted. A further possibility is that TRPM8 channels are activated in sensory axon collateral nerves, which then release vasodilator substances, typically calcitonin gene-related peptide. However, this is also unlikely as capsaicin (a compound that selectively causes release of synaptic vesicles in sensory fibers) fails to produce a dilatation of tail artery preconstricted with KCl (Johnson, CD, unpublished observations) and calcitonin gene-related peptide levels detected are very low in rat tail artery (20).

Rat tail artery is an attractive model to work with, as it has no sympathetic vasodilator innervation, sensory innervation appears to be of little functional consequence (see above), and the endothelium can be removed. In contrast, control of the human forearm cutaneous circulation is more complex, having all of these elements active, in addition to sympathetic vasoconstrictor tone (15, 17, 19, 25, 33). It is possible that neuronal activation by TRPM8 channels may result in a vasodilatation. A recent study (25) used this vascular bed as a model to investigate sensory actions of menthol and other TRP channel agonists. They reported that although menthol caused vasodilatation at the site of application, it was localized to this area alone, implying that menthol did not evoke a sensory axon reflex to cause the release of vasodilator substances remote from the site of application, whereas TRPA1 channel agonism did. We attempted to investigate this by application of the local anesthetic tetracaine (results not shown). However, this approach was inconclusive as it resulted in a marked vasodilatation itself, presumably due to the concurrent blockade of sympathetic vasoconstrictor tone and possibly due to its effects on intracellular calcium stores (26). Although afferent nerve activity was also blocked, any vasodilatation that might occur independently from the sensory axon reflex on addition of menthol would be masked due to the preexisting vasodilatation.

A further possibility to consider is that menthol activated TRPM8 channels in sympathetic vasodilator fibers that are known to innervate the human forearm cutaneous circulation, mediated by release of ACh (17, 19). This would be consistent with our observation that both L-NAME and atropine markedly reduced the dilatation induced by menthol. This being the case, the response that remained (~30–50%) is difficult to interpret with the current methodology, as it may be due to direct TRPM8-mediated effects on smooth muscle as outlined already or simply that there was incomplete block with these agents.

Some recent studies (16, 18, 22) on isolated cells have identified effects elicited by menthol and icilin (although at higher, often millimolar, concentrations compared with the submillimolar concentrations used in our study) to produce activation of TRPA1 channels. It is thus possible that vascular responses to these agonists in the current study could be mediated, at least partially, via TRPA1 channels. However, as specific activation of TRPM8 and TRPA1 channels has different vascular effects in human forearm skin (25), it seems unlikely that our responses in either the rat or human study were mediated by TRPA1 channels. Additionally, to date no study has reported the presence of TRPA1 channels in vascular tissues.

In conclusion, our study shows that TRPM8 channels are present in rat blood vessels and can contribute to vasomotor tone when activated. The resultant vasoconstriction or vasodilatation depends on the existing degree of vasomotor tone and is mainly dependent on TRPM8 activation on smooth muscle cells directly, although the endothelium appears to enhance TRPM8-induced relaxation. The channels may contribute to the regulation of vasomotor tone in human cutaneous blood vessels, but the mechanism is less clear, with possible additional roles for a neuronal and an endothelial component.

Further studies are now required to identify which elements of this heterogeneous tissue are involved, what mediates natural activation of these channels in vivo, and to what extent they participate in control of vasomotor tone. TRPM8 channels may be involved in intracellular Ca^{2+} control in nontemperature-dependent tissues, and given the crossover between cold activation of the channel and its possible influence on cutaneous vessels, it will be of interest to find out the exact location of these channels and their contribution to vascular diseases such as Reynaud's disease.

ACKNOWLEDGMENTS

We thank Alamone Labs for the gift of antibodies.

GRANTS

We thank the School of Medicine, Dentistry and Biomedical Sciences, Queen's University of Belfast, and British Heart Foundation (Grant No. FS/07/040) for support.

REFERENCES

1. **Abeele FV, Zholos Bideaux G A, Shuba Y, Thebault S, Beck B, Flourakis M, Panchin Y, Skryma R, Prevaskaya N.** iPLA$_2$-dependent gating of TRPM8 by lysophospholipids. *J Biol Chem* 281: 40174–82, 2006.
2. **Albert AP, Large WA.** Signal transduction pathways and gating mechanisms of native TRP-like cation channels in vascular myocytes. *J Physiol* 570: 45–51, 2006.
3. **Bautista DM, Siemens J, Glazer JL, Tsuruda PR, Basbaum AI, Stucky CL, Jordt SV, Julius D.** The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature* 484: 204–209, 2007.
4. **Beech DJ, Muraki K, Flemming R.** Non-selective cationic channels of smooth muscle and the mammalian homologues of Drosophila TRP. *J Physiol* 559: 685–706, 2004.
5. **Beech DJ.** Emerging functions of 10 types of TRP cationic channel in vascular smooth muscle. *Clin Exp Pharmacol Physiol* 32: 597–603, 2005.
6. **Bidaux G, Flourakis M, Thebault S, Zholos A, Beck B, Gkika D, Roudbaraki M, Bonnal JL, Mauroy B, Shuba Y, Skryma R, Prevarskaya N.** Prostate cell differentiation status determines transient receptor potential melastatin member 8 channel subcellular localization and function. *J Clin Invest* 117: 1647–1657, 2007.
7. **Crompton R, Clifton VL, Bisits AT, Read MA, Smith R, Wright IMR.** Corticotrophin-releasing hormone causes vasodilatation in human skin via mast cell-dependent pathways. *J Clin Endocrinol Metab* 88: 5427–5432, 2003.
8. **De Petrocellis L, Starowicz K, Moriello AS, Vivese M, Orlando P, Di Marzo V.** Regulation of transient receptor potential channels of melastatin type 8 (TRPM8): effect of cAMP, cannabinoid CB1 receptors and endovanilloids. *Exp Cell Res* 313: 1911–1920, 2007.

9. **Dietrich A, Kalwa H, Fuchs B, Grimminger F, Weissmann N, Gudermann T.** In vivo TRPC functions in the cardiopulmonary vasculature. *Cell Calcium* 43: 233–244, 2007.
10. **Eccles R.** Menthol and related cooling compounds. *J Pharm Pharmacol* 46: 618–630, 1994.
11. **Häbler HJ, Jänig W, Krummel M, Peters OA.** Reflex patterns in postganglionic neurons supplying skin and skeletal muscle of the rat hindlimb. *J Neurophysiol* 72: 2222–2236, 1994.
12. **Hawthorn M, Ferrante J, Luchowski E, Rutledge A, Wei XY, Triggle DJ.** The actions of peppermint oil and menthol on calcium channel dependent processes in intestinal, neuronal and cardiac preparations. *Aliment Pharmacol Ther* 2: 101–118, 1988.
13. **Inoue R, Jensen LJ, Shi J, Morita H, Nishida M, Honda A, Ito Y.** Transient receptor potential channels in cardiovascular function and disease. *Circ Res* 99: 119–131, 2006.
14. **Ito S, Kume H, Shiraki A, Kondo M, Makino Y, Kamiya K, Hasegawa Y.** Inhibition by the cold receptor agonists menthol and icilin of airway smooth muscle contraction. *Pulm Pharmacol Ther* 21: 812–817, 2008.
15. **Johnson JM, Yen TC, Zhao K, Kosiba WA.** Sympathetic, sensory, and nonneuronal contributions to the cutaneous vasoconstrictor response to local cooling. *Am J Physiol Heart Circ Physiol* 288: H1573–H1579, 2005.
16. **Karashima Y, Damann N, Prenen J, Talavera K, Voets T, Nilius B.** Biomodal action of menthol on the transient receptor channel TRPA1. *J Neurosci* 27: 9874–9884, 2007.
17. **Kellogg DL Jr, Pérgola PE, Piest KL, Kosiba WA, Crandall CG, Grossmann M, Johnson JM.** Cutaneous active vasodilation in humans is mediated by cholinergic nerve cotransmission. *Circ Res* 77: 1222–1228, 1995.
18. **Kühn FJ, Kühn C, Lückhoff A.** Inhinition of TRPM8 by icilin distinct from desensitization induced by menthol and menthol derivitives. *J Biol Chem* 284: 4102–4111, 2009.
19. **Levick JR.** *An Introduction to Cardiovascular Physiology* (4th ed.). London: Arnold, 2003.
20. **Li Y, Duckles SP.** Effect of age on vascular content of calcitonin gene-related peptide and mesenteric vasodilator nerve activity in the rat. *Eur J Pharmacol* 236: 73–78, 1993.
21. **Liu B, Qin F.** Functional control of cold- and menthol-sensitive ion channels by phospatidylinositol 4,5-biphosphate. *J Neurosci* 25: 1674–1681, 2005.
22. **Macpherson LJ, Hwang SW, Miyamoto T, Dubin AE, Patapoutain A, Story GM.** More than cool: promiscuous relationships in menthol and other sensory compounds. *Mol Cell Neurosci* 32: 335–343, 2006.
23. **McKemy DD, Neuhausser WM, Julius D.** Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* 416: 52–58, 2002.
24. **Melanaphy D, Johnson CD, Dickson P, Anderson P, Zholos A.** Characterization of the TRPM8 calcium channel in rat aorta and tail artery. *Proc Physiol Soc* 7: PC2, 2007.
25. **Namer B, Seifert F, Handwerker HO, Maihöfner C.** TRPA1 and TRPM8 activation in humans: effects of cinnamaldehyde and menthol. *Neuroreport* 16: 955–959, 2005.
26. **Overend CL, Eisner DA, O'Neill SC.** The effect of tetracaine on spontaneous Ca^{2+} release and sarcoplasmic reticulum calcium content in rat ventricular myocytes. *J Physiol* 502: 471–479,1997.
27. **Park KM, Trucillo M, Serban N, Cohen RA, Bolotina VM.** Role of iPLA2 and store-operated channels in agonist-induced Ca2+ influx and constriction in cerebral, mesenteric, and carotid arteries. *Am J Physiol Heart Circ Physiol* 294: H1183–H1187, 2008.
28. **Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Early TJ, Dragoni I, McIntyre P, Bevan S, Patapoutain A.** A TRP channel that senses cold and menthol. *Cell* 108: 705–715, 2002.
29. **Purse A, Zholos AV, Johnson CD.** A possible role for transient receptor potential (melastatin) 8 (TRPM8) channels in human cutaneous blood flow. *Proc Physiol Soc* 11: C106, 2008.
30. **Rakieten N, Rakieten MR.** The effect of l-menthol on systemic blood pressure. *J Am Pharm Assoc (Wash)* 46: 82–84, 1957.
31. **Rohacs T, Lopes CM, Michailidis I, Logothetis DE.** $PI(4,5)P_2$ regulates the activation and desensitization of TRPM8 channels through the TRP domain. *Nat Neurosci* 8: 626–634, 2005.
32. **Roosterman D, Goerge T, Schneider SW, Bunnett NW, Steinhoff M.** Neuronal control of skin function: the skin as a neuroimmunoendocrine organ. *Physiol Rev* 86: 1309–1379, 2006.
33. **Sittiracha T, McLachlan EM, Bell C.** The innervation of the caudal artery of the rat. *Neuroscience* 21: 647–659, 1987.
34. **Thebault S, Lemonnier L, Bidaux G, Flourakis M, Bavencoffe A, Gordienko D, Roudbaraki M, Delcourt P, Panchin Y, Shuba Y, Skryma R, Prevarskaya N.** Novel role of cold/menthol-sensitive transient receptor potential melastatine family member 8 (TRPM8) in the activation of store-operated channels in LNCaP human prostate cancer epithelial cells. *J Biol Chem* 280: 39423–39435, 2005.
35. **Tsavaler L, Shapero MH, Morkowski S, Laus R.** Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. *Cancer Res* 61: 3760–3769, 2001.
36. **Tsuzuki K, Xing H, Ling J, Gu JG.** Menthol-induced Ca^{2+} release from presynaptic Ca^{2+} stores potentiates sensory synaptic transmission. *J Neurosci* 24: 762–771, 2004.
37. **Venkatachalam K, Montell C.** TRP channels. *Annu Rev Biochem* 76: 387–417, 2007.
38. **Wellman GC, Nelson MT.** Signaling between SR and plasmalemma in smooth muscle: sparks and the activation of Ca^{2+}-sensitive ion channels. *Cell Calcium* 34: 211–229, 2003.
39. **Wright CE, Laude EA, Grattan TJ, Morice AH.** Capcasin and neurokinin-A induced bronchoconstriction in the anaesthetized guinea-pig: evidence for a direct action of menthol on isolated bronchial smooth muscle. *Br J Pharmacol* 121: 1645–1650, 1997.
40. **Yang XR, Lin MJ, McIntosh LS, Shan JSK.** Functional expression of transient receptor potential melastatin- and vanilloid- related channels in pulmonary arterial and aortic smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 290: L1267–L1276, 2006.
41. **Zhang L, Barritt GJ.** Evidence that TRPM8 Is an androgen-dependent Ca^{2+} channel required for the survival of prostate cancer cells. *Cancer Res* 64: 8365–8373, 2004.