TRPM8-independent Menthol-induced Ca\(^{2+}\) Release from Endoplasmic Reticulum and Golgi*

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Menthol, a secondary alcohol produced by the peppermint herb, *Mentha piperita*, is widely used in the food and pharmaceutical industries as a cooling/soothing compound and odorant. It induces Ca\(^{2+}\) influx in a subset of sensory neurons from dorsal root and trigeminal ganglia, due to activation of TRPM8, a Ca\(^{2+}\)-permeable, cold-activated member of the TRP superfamily of cation channels. Menthol also induces Ca\(^{2+}\) release from intracellular stores in several TRPM8-expressing cell types, which has led to the suggestion that TRPM8 can function as an intracellular Ca\(^{2+}\)-release channel. Here we show that menthol induces Ca\(^{2+}\) release from intracellular stores in four widely used cell lines (HEK293, lymph node carcinoma of the prostate (LNCaP), Chinese hamster ovary (CHO), and COS), and provide several lines of evidence indicating that this release pathway is TRPM8-independent: 1) menthol-induced Ca\(^{2+}\) release was potentiated at higher temperatures, which contrasts to the cold activation of TRPM8; 2) overexpression of TRPM8 did not enhance the menthol-induced Ca\(^{2+}\) release; 3) menthol-induced Ca\(^{2+}\) release was mimicked by geraniol and linalool, which are structurally related to menthol, but not by the more potent TRPM8 agonists icilin or eucalyptol; and 4) TRPM8 expression in HEK293 cells was undetectable at the protein and mRNA levels. Moreover, using a novel TRPM8-specific antibody we demonstrate that both heterologously expressed TRPM8 (in HEK293 cells) and endogenous TRPM8 (in LNCaP cells) are mainly localized in the plasma membrane, which contrast to previous localization studies using commercial anti-TRPM8 antibodies. Finally, aequorin-based measurements demonstrate that the TRPM8-independent menthol-induced Ca\(^{2+}\) release originates from both endoplasmic reticulum and Golgi compartments.

Menthol is a naturally occurring compound responsible for the minty flavor and smell of the mint plant (*Mentha piperita*). Whereas the first descriptions of the use of menthol as a cooling/soothing compound and odorant date back to ~2000 years ago, menthol is still extensively used as an additive in a wide variety of products ranging from ointments and candies to cigarettes. The soothing, refreshing and invigorating feature of the oil made from the peppermint herb is useful in massage for muscle fatigue. It is also used in the treatment of asthma, colic, exhaustion, fever, flatulence, headache, nausea, scabies, sinusitis, and vertigo (1). Currently the best described molecular target of menthol is TRPM8, a member of the melastatin branch of the TRP superfamily of cation channels. TRPM8 was initially identified in a screening procedure aimed at identifying mRNAs that are up-regulated in prostate cancer (2). Subsequently, TRPM8 expression was also demonstrated to be strongly up-regulated in several other primary tumor types including breast, colon, lung, and skin (2). The following studies identified TRPM8 in a subset of dorsal root and trigeminal neurons (3), and found it to function as a plasma membrane Ca\(^{2+}\)-permeable cation channel activated by cold temperatures (<28 °C) and by compounds such as menthol, eucalyptol, geraniol, linalool, and icilin (4–6). These findings strongly suggest that TRPM8 is involved in cold sensation by the somatosensory system, and provide a straightforward explanation for the cool sensation evoked by menthol. Yet, the function of TRPM8 in non-sensory cells and particularly its relation to the pathophysiology of cancer cells are currently unknown. Recent studies have suggested that TRPM8, besides its established function as a plasmalemmal channel, can also be found in the ER membrane of the androgen-responsive LNCaP prostate cell line, where it may function as a menthol- and cold-sensitive intracellular Ca\(^{2+}\)-release channel (7, 8). In addition, menthol-induced release of Ca\(^{2+}\) from intracellular stores has been reported in other different cell types, including skeletal muscle (9), tracheal epithelial cells (10), and dorsal horn neurons (11). These data raise the possibility that TRPM8 plays a more general role as an intra-
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Cellular Ca\(^{2+}\) release channel. However, in the absence of TRPM8-deficient mice or TRPM8-specific inhibitors, equaling all menthol-induced Ca\(^{2+}\) responses to TRPM8 activation may be premature.

Whereas performing simultaneous whole cell patch clamp recordings and intracellular Ca\(^{2+}\) measurements on HEK293 cells overexpressing human TRPM8, we observed that not only activates plasma membrane TRPM8 currents, but also induces Ca\(^{2+}\) release from intracellular stores. Our results demonstrate that this intracellular Ca\(^{2+}\) release originates from both ER and Golgi compartments, is not related to TRPM8 expression, and is also observed in several other widely used cell types, pointing to a more universal TRPM8-independent menthol-activated release pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney cells (HEK293) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% (v/v) fetal calf serum (FCS) (Sigma), 4 mM L-alanyl-L-glutamine (Glutamax\textsuperscript{TM}, Invitrogen), 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, and MEM non-essential amino acids (1 times) (Invitrogen) at 37 °C in a humidity controlled incubator with 10% (v/v) CO\(_2\). When indicated, HEK293 cells were transiently transfected with human TRPM8 cloned in the bicistronic pCAGGS-IRES-GFP vector using TransIT\textsuperscript{TM}-293 transfection reagent (Mirus Corporation) following the manufacturer’s protocol. Lymph node carcinoma of the prostate (LNCaP) cells were grown in RPMI 1640 medium (Invitrogen) containing 10% (v/v) FCS (Sigma), 4 mM L-alanyl-L-glutamine (Glutamax, Invitrogen), 85 units/ml penicillin, and MEM non-essential amino acids (1 times) (Invitrogen) at 37 °C in a humidity controlled incubator with 5% (v/v) CO\(_2\). African monkey kidney (COS) cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) FCS (Sigma), 3.8 mM L-alanyl-L-glutamine (Glutamix, Invitrogen), 85 units/ml penicillin, 85 \(\mu\)g/ml streptomycin, and MEM non-essential amino acids (1 times) (Invitrogen) at 37 °C in a humidity controlled incubator with 5% (v/v) CO\(_2\).

RNA Extraction, cDNA Synthesis, and Reverse Transcriptase-PCR—Total RNA from cultured HEK293 cells was prepared using the RNeasy MiniKit (Qiagen) according to the protocol provided by the manufacturer. Reverse transcription of 1 \(\mu\)g of total RNA was performed with Ready-to-Go\textsuperscript{TM} You-Prime-First-Strand-Beads (Amersham Biosciences) using random primers. Amplification of specific TRPM8 fragments was performed on a 50-\(\mu\)l PCR, containing Taq DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl\(_2\), and 0.01% gelatin), 0.2 mM dNTPs, 2.5 units of Taq DNA polymerase (New England Biolabs), 0.5 pmol of each of the specific TRPM8 or actin primers (see Table 1) and the cDNA. The cycling protocol comprised denatur-

Protein Extractions and Differential Centrifugation—Whole cell extracts from TRPM8-transfected HEK293 cells were prepared using cold phosphate-buffered saline (10 mM phosphate buffer, pH 7.4, 137 mM NaCl, 2.7 mM KCl) supplemented with 1.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors mixture (10 \(\mu\)g/ml leupeptin and antipain, 2 \(\mu\)g/ml chymostatin and pepstatin) as described in Ref. 12. To investigate intracellular distribution of TRPM8, TRPM8-transfected HEK293 cells were lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and protease inhibitors mixture) and subjected to differential centrifugation at 20,000 \(\times\) g and 100,000 \(\times\) g, using a protocol described in Ref. 13. Protein concentrations were determined by the biecinchoninic acid assay method (14) using bovine serum albumin as a standard. Protein samples (20 \(\mu\)g) were solubilized in a 3-fold concentrated sample buffer (240 mM Tris, pH 6.8, 30% glycerol, 6% SDS, 3% dithiothreitol, 0.015% bromophenol blue) by heating to 56 °C for 15 min and were subsequently separated by 8% SDS-PAGE.

Anti-TRPM8 Antibody Generation and Immunodetection—For immunodetection, two different anti-TRPM8 antibodies were used: we compared commercially available anti-TRPM8 (Novus) to a newly generated anti-TRPM8 antibody designed in our laboratory. To generate the latter, rabbits were immunized with a synthetic peptide corresponding to a highly conserved region in the TRPM8 C terminus (C + \(^{1079}\)MRHRFRLDTKNL\(_{1095}\)) coupled to the keyhole limpet hemocyanin (Eurogentec). TRPM8 antibodies were purified from serum by peptide chromatography (Eurogentec). For characterizing the distribution of TRPM8 in different membrane fractions, nitrocellulose (Schleicher & Schuell) or polyvinylidine fluoride (Bio-Rad) membranes with separated proteins were probed with purified polyclonal rabbit anti-TRPM8 (1:2000 dilution), anti-plasma membrane Ca\(^{2+}\)-ATPase (1:2000 dilution), or monoclonal mouse anti-inositol trisphosphate receptor (IP\(_3\)R) isoform 3 (1:1000 dilution) antibodies (BD Biosciences) for 1 h at room temperature. Immunoreactive complexes were visualized by chemiluminescence and exposure to a Hyperfilm ECL (Amersham Biosciences), using either anti-rabbit or anti-mouse IgG antibodies conjugated to horseradish peroxidase (1:4000 dilution; Amersham Biosciences). When required, the bound antibodies were removed using Re-Blot Plus mild antibodies stripping solution according to the manufacturer’s instructions (Chemicon International, Inc.). Immunocytochemistry on TRPM8-transfected HEK293 cells and LNCaP cells was performed as described previously (12). Briefly, cells were fixed in 3.7% fresh formaldehyde for 10 min and permeabilized in 0.2% Triton X-100 for 10 min (all incubation and washing steps throughout the experiments were in phosphate-buffered saline with the indicated supplementation). After 2 h blocking with 3% bovine serum albumin, coverslips were incubated with anti-TRPM8 antibodies.
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(1:1000 dilution) overnight at 4°C. Secondary Alexa Fluor® 594-labeled goat anti-rabbit antibodies (Molecular Probes, Inc.) were used in a 1:1000 dilution and incubated for 1 h. Stained samples were treated with VectaShield® mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc.) to retard photobleaching and visualize nuclei.

Electrophysiology—Patch clamp experiments were performed in the whole cell configuration using an EPC-9 amplifier and Pulse software (HEKA Elektronik). Electrode resistance was between 2 and 5 MΩ and 60% of the series resistance was compensated. All experiments were performed at 33°C and carried out between 16 and 24 h after transfection. The internal (pipette) solution contained (in mM) 140 CsCl, 10 HEPES, and 0.2 Fura-2, buffered at pH 7.2 with CsOH. The extracellular solution consisted of (in mM) 150 NaCl, 5 MgCl$_2$, 1 EGTA, and 10 HEPES, buffered at pH 7.4 with NaOH. Stimulation of TRPM8 currents was realized by application of 1 mM menthol (Sigma) to the bath solution. Current-voltage relationships were measured from linear 400-ms voltage ramps from −100 to +100 mV, which were applied every 5 s from a holding potential of +20 mV with a sampling interval of 0.8 ms.

Intracellular Ca$^{2+}$ Measurements—Intracellular Ca$^{2+}$ was measured using a monochromator based system consisting of a Polychrome IV monochromator (TILL Photonics) with an additional TILL photonics photomultiplier, both controlled by Pulse software (HEKA Elektronik). Cells were loaded with Fura-2 by incubating them in the culture medium with 2 µM Fura-2/AM (TefLabs) for 20 min at 37°C. Fluorescence was measured at alternating wavelengths between 350 and 380 nm, corrected by subtraction of the background fluorescence, and expressed as the ratio $R(F_{380}/F_{350})$.

The relationship between the fluorescence ratio $R$ and the [Ca$^{2+}$] was computed according to the Grynkiewicz equation (15). Additional correction on the $K_D$ of Fura-2 was performed for the different temperatures as described before (16). A SC-20 dual in-line heater/cooler (Warner Instruments) was used to control the temperature of the perfusate. A TA-29 thermistor (Thermometrics) positioned in close vicinity of the cell was used to monitor the bath temperature, which was recorded together with the fluorescent signal. The Ca$^{2+}$-containing extracellular solution consisted of (in mM) 150 NaCl, 5 MgCl$_2$, 1.5 mM CaCl$_2$, and 10 HEPES, buffered at pH 7.4 with NaOH. For the nominally Ca$^{2+}$-free solution, CaCl$_2$ was omitted. Different concentrations of menthol, linalool, geraniol, eucalyptol, or icilin were applied in this solution. Acetylcholine chloride (ACh) and 2,5-di-(tert-butyl)-1,4-benzo-hydroquinone (BHQ) were applied at concentrations of 100 and 50 µM, respectively. Pertussis toxin, U73122, and caffeine were used at concentrations of 500 ng/ml, 10 µM, and 10 mM, respectively. All reagents were obtained from Sigma. A stock solution of menthol and icilin was first made in ethanol resulting in a final concentration of the solvent not exceeding 0.3%. Ethanol itself does not evoke any intracellular Ca$^{2+}$ release at the concentrations used.

Aequorin Measurements—HEK293 cells, seeded on 13-mm gelatin-coated coverslips, were transfected with the bioluminescent protein aequorin constructs, targeted for the ER, Golgi, or cytosol (erAEQ, goAEQ, and cytAEQ, respectively) using GeneJuice® transfection reagent following the manufacturer’s protocol (Novagen). Experiments were performed at 37°C 1 day after transfection on a confluent cell layer (17).

Prior to measuring ER- or Golgi-aequorin signals, the culture medium was replaced with Krebs-Ringer buffer (KRB) (in mM) 135 NaCl, 5 KCl, 1 MgSO$_4$, 20 KH$_2$PO$_4$, 20 HEPES, and 5.5 glucose buffered at pH 7.4 with NaOH), containing 600 µM EGTA. Cells were incubated during 1 h with 5 µM ionomycin (Sigma) for Ca$^{2+}$ depletion and with 5 µM coelenterazine for reconstitution of the active aequorin. The cells were then washed extensively with KRB supplemented with 2% bovine serum albumin (Sigma) and 1 mM EGTA. After superfusion of the cells with KRB supplemented with 100 µM EGTA, the stores were loaded by superfusion with a KRB solution containing 1 mM CaCl$_2$. Finally, the cells were stimulated with 2 mM menthol diluted in the same solution.

HEK293 cells transfected with cytAEQ were incubated during 1 h in a 5% CO$_2$ incubator at 37°C with 5 µM coelenterazine (wild type) diluted in Dulbecco’s modified Eagle’s medium supplemented with 1% FCS. Measurements were performed in KRB supplemented with 1 mM CaCl$_2$ and cells were stimulated with 2 mM menthol in the same solution. The light signal was collected by a low-noise photomultiplier tube (Hamamatsu H7360-1, Hamamatsu Photonics K.K.) with a built-in amplifier-discriminator. The photomultiplier output was collected using a photon PCI-6601 counting board (National Instruments).

Statistical Analysis—Group data are expressed as mean ± S.E. Overall statistical significance was determined by analysis of variance. In the case of significance ($p < 0.05$), individual groups were compared by Student’s unpaired t test. Statistics was performed with Origin 7.0 software (OriginLab Corp.).

RESULTS

Menthol-induced Ca$^{2+}$ Release in HEK293 Cells—In HEK293 cells overexpressing human TRPM8, extracellular application of menthol induces typical outwardly rectifying whole cell currents, which have been described in detail in previous studies (4, 5). When combining whole cell patch clamp recordings at 33°C in Ca$^{2+}$-free extracellular solution with microfluorimetric Ca$^{2+}$-measurements using Fura-2, we observed that 1 mM menthol not only induced robust outward current activation but also a significant rise in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) (Fig. 1, A and B). Note the delay in the time course for the observed Ca$^{2+}$ rise: TRPM8 current is already desensitizing due to the high menthol concentration when [Ca$^{2+}$]$_i$ increases. As this set of experiments was performed in Ca$^{2+}$-free extracellular solution, these findings indicate that the menthol-induced rise in [Ca$^{2+}$]$_i$ originates from the release of Ca$^{2+}$ from intracellular stores. Both the whole cell current amplitude and [Ca$^{2+}$]$_i$, returned to the initial level upon wash-out of menthol.

However, whereas whole cell TRPM8 currents can be repetitively activated by menthol in Ca$^{2+}$-free solution (data not shown; see also Refs. 4 and 5), a rise in [Ca$^{2+}$]$_i$ was only observed during the first menthol application (Fig. 2A, for second application of 1 mM menthol at 33°C, Δ[Ca$^{2+}$]$_i$ = 5 ±
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TRPM8 is not only activated by menthol but also by cooling, and the effects of cold and menthol on TRPM8 are known to reinforce each other. This is illustrated in the experiment shown in Fig. 2C, where a TRPM8-transfected cell bathed in \( \text{Ca}^{2+} \)-containing solution was repeatedly stimulated with 10 \( \mu \text{M} \) menthol. At this low dose, menthol evokes a robust increase in \([\text{Ca}^{2+}]_i\) at 23 °C, whereas very little response is observed at 33 °C. To compare the temperature sensitivity of TRPM8 with that of the menthol-induced \( \text{Ca}^{2+} \)-release pathway, we measured menthol responses in both TRPM8-transfected and non-transfected HEK293 cells in \( \text{Ca}^{2+} \)-containing solution at 23 and 33 °C (Fig. 2, C and D), and observed two striking differences. First, the menthol responses in non-transfected cells were of smaller amplitude and required higher menthol concentrations. No responses were detectable to concentrations <100 \( \mu \text{M} \) menthol (data not shown). Second, we found that the menthol response in non-transfected cells was strongly counteracted by lowering the bath temperature to 23 °C (Fig. 2D), opposite to what we observed for a low dose of menthol in the TRPM8-transfected cells (Fig. 2C). In TRPM8-transfected cells, the concentration for half-maximal response (EC\(_{50}\)) increased from 33 ± 7 \( \mu \text{M} \) at 23 °C to 97 ± 2 \( \mu \text{M} \) at 33 °C. In addition, the maximal amplitude of the menthol response dropped from 3.5 \( \mu \text{M} \) at 23 °C to 2 \( \mu \text{M} \) at 33 °C (\( n = 4–7 \) for each data point) (Fig. 2E). We were unable to obtain EC\(_{50}\) values for the menthol response in non-transfected cells, as the responses did not saturate at the maximal menthol concentration that can be kept in solution (≤5 \( \text{mM} \)). Nevertheless, raising the temperature to 33 °C resulted in a 4–5-fold increase in the response to supramillimolar menthol concentrations, and induced significant responses at submicromolar menthol concentrations, which were not observed at 23 °C (\( n = 4–7 \) for each data point) (Fig. 2F). It should also be noted here that cooling down to 15 °C did not evoke a \( \text{Ca}^{2+} \) transient in non-transfected HEK293 cells (data not shown).

TRPM8 Expression and Localization in HEK293 and LNCaP Cells—This opposite temperature dependence indicates that the endogenous menthol-sensitive \( \text{Ca}^{2+} \)-release pathway differs from expressed TRPM8 at the thermodynamic level. However, solely based on these data we cannot fully exclude that HEK293 cells endogenously express TRPM8 protein on intracellular membranes with different temperature sensitivity than exogenous, overexpressed TRPM8. To rule out this possibility, we investigated the expression of TRPM8 in HEK293 cells at both the protein and mRNA levels.

First, we performed Western blotting using a commercially available anti-TRPM8 antibody (Novus) to probe the presence of TRPM8 protein in whole cell extracts from non-transfected and TRPM8-transfected HEK293 cells. Despite long exposures and the use of two different batches of antibody, we were unable to detect a clear band of the expected size in extracts from both non-transfected cells and TRPM8-transfected cells (Fig. 3A). As these results cast doubts about the applicability of the commercial anti-TRPM8 antibody in Western blotting, we produced and purified new anti-TRPM8 antibodies raised against a C-terminal epitope of TRPM8, 1078MRHRFRQLDKLNLDL1092. In whole cell
extracts isolated from TRPM8-transfected HEK293 cells, these anti-TRPM8 antibodies recognized a band of ≈120–125 kDa, which corresponds to the expected molecular mass of TRPM8 (Fig. 3B). No such band was detected in the whole cell extract isolated from non-transfected HEK293 cells (Fig. 3B). Similarly, non-transfected cells also did not display the TRPM8-specific immunodetection in low- and high-speed pellet membrane fractions obtained by differential centrifugation (Fig. 3C). In contrast, we found that more than 95% of TRPM8 was present in the low-speed pellet membranes obtained from TRPM8-transfected HEK293 cells (Fig. 3C). This fraction also contained the majority of the marker plasma membrane Ca$^{2+}$-ATPase 1 and a part of the ER membranes, indicated by IP$_{3}$R detection. These data clearly demonstrate that our new antibody detects overexpressed TRPM8 in Western blots and that the overexpressed TRPM8 is present to a large extent in a plasma membrane-enriched fraction, indicating that a significant part of the exogenously expressed TRPM8 protein is localized in the plasma membrane, although it may also be present in intracellular membrane fractions.

Next, we compared both antibodies in immunocytochemical staining of non-transfected and TRPM8-transfected HEK293 cells as well as in LNCaP cells, which endogenously express TRPM8. As shown in Fig. 3D, our new antibodies specifically stained TRPM8-expressing HEK293 cells, with only little background staining of non-transfected cells. The TRPM8-specific detection was consistent with localization of TRPM8 in the plasma membrane, although some intracellular staining was sometimes evident. In striking contrast, staining of TRPM8-expressing HEK293 cells with the commercial anti-TRPM8 (Novus) antibody resulted in equal staining of both non-transfected and TRPM8-transfected HEK293 cells, and the staining pattern was consistent with an intracellular localization of the epitope(s) (Fig. 3F). As previous studies reporting that endogenous TRPM8 in LNCaP cells is to a large extent or even fully restricted to intracellular compartments used the commercial anti-TRPM8 antibodies (7, 8), we repeated immunocytochemical staining of LNCaP using our new anti-TRPM8 antibody. In contrast to the results from these previous studies, the staining pattern that we obtained was consistent with a principally plasma-
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| Gene                | Primer | Size of amplicon |
|---------------------|--------|------------------|
| TRPM8 primer set 1  |        |                  |
| TRPM8 primer set 2  |        |                  |
| TRPM8 primer set 3  |        |                  |
| Actin               |        |                  |

| Gene | Forward | Reverse | Size of amplicon |
|------|---------|---------|------------------|
| TRPM8 primer set 1 | TGGTTTGGCCACCAGGAGTCG | CCACTTCAGCCTGGTGTC | 2000 |
| TRPM8 primer set 2 | CTGCCCCCTTTACGGGAGTG | CTTAATTCTGAAGATGGCCC | 300  |
| TRPM8 primer set 3 | TGTTTTGGCCACCAGGAGTCG | CCACTTCAGCCTGGTGTC | 800  |
| Actin | CAAACTGCACTGCTAATCG | GCACTAAGTCATATCCG | 350  |

Pharmacological Properties of Menthol-induced Ca\(^{2+}\) Release—To investigate the nature of the menthol-sensitive Ca\(^{2+}\) stores in HEK293 cells in more detail, we tested whether stimulation with agents known to release Ca\(^{2+}\) from intracellular stores affected a subsequent response to menthol. HEK293 endogenously express muscarinic acetylcholine receptors (18), whose activation leads to phospholipase C-dependent IP\(_3\) production and subsequent Ca\(^{2+}\) release from IP\(_3\)-sensitive stores. In line herewith, application of 100 \(\mu\)M ACh in Ca\(^{2+}\)-free medium led to a fast and transient increase in [Ca\(^{2+}\)]\(_i\) (Fig. 5A). When 1 mM menthol was applied at 33 °C after stimulation with ACh, we recorded a significantly reduced menthol response (control, \(\Delta[Ca^{2+}]\_i = 106 \pm 8\) nM; ACh, \(\Delta[Ca^{2+}]\_i = 35 \pm 13\) nM, \(p = 0.04\)) (Fig. 5, A and F). A similarly reduced menthol response was observed after preincubation for 30 min with BHQ, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (BHQ, \(\Delta[Ca^{2+}]\_i = 22 \pm 5, p = 0.005\)) (Fig. 5, B and F).

From these data we conclude that at least part of the menthol-sensitive stores contain IP\(_3\) receptors and sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase pumps.

To investigate more in depth the nature of the menthol-induced Ca\(^{2+}\)-release pathway, we tested the effects of U73122 and pertussis toxin. U73122 is known to inhibit phospholipase C activation, and as such it is inhibiting the hydrolysis of PIP\(_2\) into IP\(_3\) and diacylglycerol. Simultaneous application of 10 \(\mu\)M U73122 with 1 mM menthol at 33 °C did not affect the amplitude or time course of the rise in intracellular Ca\(^{2+}\) (Fig. 5, C and F). Likewise, the menthol-induced Ca\(^{2+}\) release was unaffected after preincubation for 16 h with 500 ng/ml pertussis...
toxin, a potent inhibitor of the \( \alpha \) subunit of \( \mathrm{G}_\mathrm{i}, \mathrm{G}_\mathrm{o}, \) and \( \mathrm{G}_\mathrm{t} \) type trimeric G proteins (Fig. 5, D and F).

In line with previous studies suggesting that HEK293 cells express subsets of the ryanodine receptor (19), we found that application of 10 mM caffeine results in a small but significant increase in \( \left[ \mathrm{Ca}^{2+} \right]_i \). Subsequent application of menthol resulted in an unaffected \( \left[ \mathrm{Ca}^{2+} \right]_i \) release signal (Fig. 5, C and D), suggesting that ryanodine receptors are not involved in the menthol-induced \( \mathrm{Ca}^{2+} \) release.

Next, we tested whether other known TRPM8 agonists besides menthol can induce \( \mathrm{Ca}^{2+} \) release in HEK293 cell: 1) icilin, a synthetic compound and currently the most potent TRPM8 agonist; 2) geraniol, a monoterpenoid and an alcohol present in many essential oils; 3) eucalyptol, a cyclic ether and monoterpene derived from the eucalyptus tree; and 4) linalool, a naturally occurring terpene alcohol with a pleasant floral scent. Published EC\( _{50} \) values for activation of TRPM8 are, in order of potency, 0.2 \pm 0.1 \( \mu \mathrm{M} \) (icilin) > 5.9 \pm 1.6 mM (geraniol) > 6.7 \pm 2 mM (linalool) > 7.7 \pm 2 mM (eucalyptol) (6). No response was observed upon application of icilin (20 \( \mu \mathrm{M} \)) or eucalyptol (3 mM) at 33 \( ^\circ \mathrm{C} \) (Fig. 6, A and B). In contrast, application of linalool or geraniol caused a rise similar to that observed upon menthol application (Fig. 6, C and D). Importantly, the responses to both linalool and geraniol were significantly more pronounced at 33 \( ^\circ \mathrm{C} \) than at 23 \( ^\circ \mathrm{C} \), very similar to what we observed with menthol (Fig. 6, E and F). It is also interesting to note that linalool and geraniol, which both have structural similarity to menthol, induce \( \mathrm{Ca}^{2+} \) release, whereas the structurally unrelated TRPM8 agonists are without effect.

**Menthol-induced \( \mathrm{Ca}^{2+} \) Release Originates from ER and Golgi**—To directly investigate whether calcium is released from intracellular stores in HEK293 cells, we used the calcium-sensitive bioluminescent protein aequorin targeted to either ER or Golgi or the cytosol as described earlier (17) (erAEQ, goAEQ, or cytAEQ respectively). After aequorin reconstitution in calcium-depleting conditions, the ER or Golgi compartments or cytosol were refilled by superfusing the cells with a solution containing 1 mM \( \mathrm{CaCl}_2 \). The refilling of the stores can be monitored as an increase in aequorin chemiluminescence, which reaches a maximum after \( \sim 100 \) s. After 100 s, the solution was switched to the same solution containing 2 mM menthol. Average curves \((n = 8–12, \text{measured on different days})\) representing chemiluminescence are plotted as a function of time (Fig. 7, A, ER, and C, Golgi). Time constants for exponential decay (\( \tau \)) were...
calculated with a monoexponential fit for each trace (Fig. 7, B and D). In control conditions an artifactual decrease of the signal corresponded to a time constant $\tau$ of $136 \pm 23$ s ($n = 8$) or $132 \pm 10.2$ s ($n = 10$) for ER and Golgi, respectively, due to aequorin consumption as described earlier (20). In menthol-treated cells, $\tau$ was significantly smaller ($\tau = 46 \pm 2.6$ s, $n = 9$, and $\tau = 44 \pm 1.7$ s, $n = 12$) for ER and Golgi, respectively (for both ER and Golgi measurements, $p < 0.001$). These data confirm that menthol is evoking a $\text{Ca}^{2+}$ release from the ER as well as from the Golgi compartment.

To rule out the possibility that menthol had a direct influence on the aequorin signal and to provide evidence that the rise in $\text{Ca}^{2+}$ was effectively the result of a $\text{Ca}^{2+}$ release from intracellular compartments, we performed measurements on HEK293 cells transfected with cytosol-targeted aequorin (cytAEQ) (Fig. 7E). In agreement with the Fura-2 measurements, we observed a rise in the amount of photons emitted upon addition of 2 mM menthol. Thus we conclude from the aequorin measurements that millimolar menthol concentrations lead to calcium release from both the ER and Golgi compartments.

**Menthol-induced $\text{Ca}^{2+}$ Release in Different Cell Lines**—Finally, to investigate whether the menthol-induced $\text{Ca}^{2+}$ release is restricted to HEK293 cells or rather a more ubiquitous phenomenon we tested menthol response in three other widely used cell lines, namely LNCaP, COS, and Chinese hamster ovary. In all three cell types we found that application of 3 mM menthol at 33 °C in the absence of extracellular $\text{Ca}^{2+}$ led to a distinct increase in $[\text{Ca}^{2+}]_i$. The amplitudes and time courses of the menthol responses in these three cell lines were comparable what we observed in HEK293 cells (Fig. 8, A–C). Moreover, both the dose dependence and the temperature sensitivity of the menthol response was similar to that in HEK293 cells (Fig. 8, D–F), indicating that a similar menthol-induced $\text{Ca}^{2+}$-release pathway is present in cell lines derived from different tissues and species.

**DISCUSSION**

In the present study we have described a novel pathway through which menthol causes intracellular $\text{Ca}^{2+}$ signals in different cell types. Our results demonstrate that menthol causes $\text{Ca}^{2+}$ release from ER and Golgi via a mechanism independent of TRPM8, the best described molecular target of menthol. Interestingly, this menthol-sensitive $\text{Ca}^{2+}$-release pathway is strongly potentiated at higher temperatures, opposite to the behavior of TRPM8. Linalool and geraniol, two other natural compounds with structural resemblance to menthol, also evoked intracellular $\text{Ca}^{2+}$ release with similar kinetics and temperature dependence.

The sensory effects of menthol have been known for ages. Its
minty taste, fresh smell, cooling effect, and analgesic properties have laid the foundations of its widespread use in the food industry and medicine. However, the molecular targets responsible for these menthol effects are only poorly understood. It is now generally believed that activation of TRPM8 in cold-sensitive neurons of trigeminal and dorsal root ganglia underlies the cooling effect of the menthol (3, 4), although characterization of a TRPM8-deficient mouse would be needed to fully prove this point. Moreover, menthol has been shown to inhibit voltage-dependent Na⁺ and Ca²⁺ channels, which may contribute to the antinociceptive and local anesthetic effects of menthol (21). Interestingly, the sensory impact of menthol on mucosa or skin is clearly biphasic: whereas low doses evoke a cooling sensation, higher concentrations of menthol lead to irritation and even induce a burning feeling (1). Menthol-containing lotions and shampoos typically contain up to 0.25% (w/w) menthol (1), which corresponds to concentrations of >15 mM. It is tempting to speculate that a menthol-induced Ca²⁺-release pathway like the one described here, may contribute to the irritating effects that can be observed at such high menthol doses.

Even before cloning of TRPM8, it was already demonstrated that menthol induces Ca²⁺ release from intracellular stores in skeletal muscle cells and airway epithelia (9, 10). Later studies have reported similar menthol-induced Ca²⁺ release in TRPM8-expressing LNCaP cells and dorsal root ganglion neurons (4, 7, 8), leading to the hypothesis that TRPM8 can function as an intracellular Ca²⁺-release channel. Further support to this idea came from immunocytochemical stainings indicating that TRPM8 is significantly or even exclusively located on intracellular membranes in LNCaP cells (7, 8). However, our present data indicate that these results should be interpreted with caution. First, we found that menthol is also able to evoke a similar Ca²⁺ release in HEK293 cells, despite the fact that these cells do not express detectable levels of TRPM8. Second, in our hands, overexpression of TRPM8 in HEK293 cells had no effect on the time course or amplitude of the menthol-induced Ca²⁺ release. Finally, we found that the commercially available anti-TRPM8 antibody that was used in these previous studies was unable to reliably detect TRPM8 in protein extracts from TRPM8-overexpressing HEK293 cells, and caused diffuse cytosolic immunostaining in both non-transfected and TRPM8-transfected wild-type

![FIGURE 7. Aequorin measurements confirm menthol-induced Ca²⁺ release from intracellular stores. A, averaged time course for the counts of emitted photons in ER-targeted aequorin measurements. Upon menthol application, a faster Ca²⁺ release is observed from the ER. B, comparison of time constants as calculated from a monoexponential fit of the decay as shown in A. C and D, same as in A and B but now using aequorin targeted to the Golgi compartment. E, increase in cytosolic Ca²⁺ upon menthol application measured using cytosol-targeted aequorin, represented by the increase in emitted photons.](image-url)
HEK293 cells. Using a newly generated anti-TRPM8 antibody, which was able to specifically detect TRPM8 in transfected HEK293 cells, we obtained a clear plasma membrane staining in LNCaP cells.

At present, we have no information concerning the molecular nature of the menthol-sensitive Ca\(^{2+}\)-release pathway. Our finding in HEK293 cells that prior application of a high concentration of caffeine had no effect on the response to a subsequent menthol application indicates that a role for ryanodine receptors can be excluded. In contrast, prior stimulation with ACh led to a strong suppression of the menthol response, indicating that the menthol-induced Ca\(^{2+}\) release mainly originates from IP\(_3\)-sensitive stores. We also tested the effect of 2-aminoethoxydiphenyl borate and xestospongin C, two membrane permeant inhibitors of IP\(_3\)Rs, on menthol-induced Ca\(^{2+}\) release in HEK293 cells. However, these experiments did not yield clear answers on the involvement of IP\(_3\)Rs, as both 2-aminoethoxydiphenyl borate and xestospongin C by themselves evoked Ca\(^{2+}\) release in a significant proportion of cells (data not shown). Several unspecific effects of these compounds have been described earlier (22–25). Alternatively, menthol may activate a Ca\(^{2+}\) release pathway different from the well characterized IP\(_3\) and ryanodine receptors. Several recent studies have suggested that members of the TRP channel superfamily, including TRPP2 (polycystin 2) (26), TRPV1 (27), and TRPM8 (7, 8), can function as release channels on intracellular membranes. Although our experiments allow discarding TRPM8 as menthol target in our experiments with HEK293 cells, the involvement of other TRP channels in the menthol-induced release phenomenon mandates further investigation. Finally, it cannot be excluded that menthol leads to a passive loss of Ca\(^{2+}\) from ER and Golgi, either by inhibiting the mechanisms responsible for Ca\(^{2+}\) uptake into these compartments (i.e., the sarcomplasmic/endo-plasmic reticulum Ca\(^{2+}\)-ATPase and SPCA-type Ca\(^{2+}\)-ATPases) or by reducing the Ca\(^{2+}\)-buffering capacity of the stores.

Our present results also indicate that caution should be taken when using intracellular Ca\(^{2+}\) measurements to monitor the activity and menthol sensitivity of WT and mutant TRPM8. Indeed, Ca\(^{2+}\) responses at higher doses of menthol may be significantly contaminated by TRPM8-independent Ca\(^{2+}\) release from intracellular stores. In such cases, the opposite temperature sensitivity and differential sensitivity to icilin and eucalyptol can be employed to discriminate between TRPM8 and the TRPM8-independent menthol-sensitive release pathway.

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