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Characterization of the mouse cold-menthol receptor TRPM8 and vanilloid receptor type-1 VR1 using a fluorometric imaging plate reader (FLIPR) assay

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1 TRPM8 (CMR1) is a Ca^{2+}-permeable channel, which can be activated by low temperatures, menthol, eucalyptol and icilin. It belongs to the transient receptor potential (TRP) family, and therefore is related to vanilloid receptor type-1 (VR1, TRPV1). We tested whether substances which are structurally related to menthol, or which produce a cooling sensation, could activate TRPM8, and compared the responses of TRPM8 and VR1 to these ligands.

2 The effects of 70 odorants and menthol-related substances on recombinant mouse TRPM8 (mTRPM8), expressed in HEK293 cells, were examined using a FLIPR® assay. In all, 10 substances (linalool, geraniol, hydroxycitronellal, WS-3, WS-23, FrescolatMGA, FrescolatML, PMD38, CoolactP and Cooling Agent 10) were found to be agonists.

3 The EC_{50} values of the agonists defined their relative potencies: icilin ($0.2\pm0.1\,\mu$M) > FrescolatML ($3.3\pm1.5\,\mu$M) > WS-3 ($3.7\pm1.7\,\mu$M) (−)menthol ($4.1\pm1.3\,\mu$M) frescolatMAG ($4.8\pm1.1\,\mu$M) > cooling agent 10 ($6\pm2.2\,\mu$M) (+)menthol ($14.4\pm1.3\,\mu$M) > PMD38 ($31\pm1.1\,\mu$M) > WS-23 ($44\pm7.3\,\mu$M) > Coolact P ($66\pm20\,\mu$M) > geraniol (5.9 ± 1.6 mM) > linalool (6.7 ± 2.0 mM) > eucalyptol (7.7 ± 2.0 mM) > hydroxycitronellal (19.6 ± 2.2 mM).

4 Known VR1 antagonists (BCTC, thio-BCTC and capsazepine) were also able to block the response of TRPM8 to menthol (IC_{50}: 0.8 ± 1.0, 3.5 ± 1.1 and $18\pm1.1\,\mu$M, respectively).

5 The Ca^{2+} response of hVR1-transfected HEK293 cells to the endogenous VR1 agonist *N*-arachidonoyl-dopamine was potentiated by low pH. In contrast, menthol- and icilin-activated TRPM8 currents were suppressed by low pH.

6 In conclusion, in the present study, we identified 10 new agonists and three antagonists of TRPM8. We found that, in contrast to VR1, TRPM8 is inhibited rather than potentiated by protons.
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Keywords: TRPM8; CMR1; cold; menthol; VR1; odorants; proton activation; FLIPR; pain

Abbreviations: ANA, anandamide; BCTC, *N*-(4-tert.butyl-phenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2*H*)-carboxamide; $[Ca^{2+}]_i$, intracellular calcium concentration; cDNA, complementary DNA; CMR1, cold-menthol receptor 1; FLIPR®, fluorometric imaging plate reader; HEK293, human embryonic kidney cells; NADA, N-arachidonoyl-dopamine; thio-BCTC, *N*-(4-tert.butyl-phenyl)-4-(3-chloropyridin-2-yl) tetrahydro-pyrazine-1(2*H*)-(thio) carboxamide; TRPM8, transient receptor potential melastatin subfamily channel 8; VR1, vanilloid receptor type-1

Introduction

The recent cloning and characterization of the cold-menthol receptor (TRPM8; CMR1) (McKemy *et al*., 2002; Peier *et al*., 2002) was a major breakthrough in the study of thermosensation. TRPM8 is activated by menthol, eucalyptol and icilin, and by temperatures below ~25°C. It belongs to the 'long', or melastatin, subfamily of the transient receptor potential (TRP) family of ion channels (Montell *et al*., 2002), and shows pronounced outward rectification with a relatively high permeability for Ca^{2+} ions, and little selectivity between monovalent cations. The TRPM8 channel is expressed specifically in a subset of temperature-sensing trigeminal and dorsal root ganglion neurones (Peier *et al*., 2002; Reid *et al*., 2002a, b; Nealen *et al*., 2003). Recently, a second cold receptor, ANKTM1, has been identified (Story *et al*., 2003), which, in contrast to TRPM8, is coexpressed with VR1 in a different subset of pain- and temperature-sensing trigeminal and dorsal root ganglion neurones. ANKTM1 is activated by icilin, but not menthol. These TRP channels play a major role in thermosensation (McKemy *et al*., 2002; Patapoutian *et al*., 2003).

Although treatment with menthol or eucalyptol, or with cold temperatures, is a traditional method of pain relief (Wright, 1870; Green & Mcanliffe, 2000; Davies *et al*., 2002; Galeotti *et al*., 2002; Shanghai Medicinal Herbs, Essential Balm), little is known about the underlying analgesic mechanisms. It has been demonstrated that menthol blocks Na^+ and Ca^{2+} channels in dorsal root ganglion cells (Swandulla *et al*.,

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1987; Haeseler *et al*., 2002). Others have postulated that the analgesic activity of (–)menthol is mediated by selective activation of κ-opioid receptors (Galeotti *et al*., 2002).

The cold receptor TRPM8 is distantly related to the well-characterized heat-sensitive vanilloid receptor VR1 (or TRPV1). VR1 also belongs to the TRP channel family, but is activated by temperatures >42°C, or by ligands such as capsaicin and resiniferatoxin (RTX). Two endogenous VR1 agonists have been identified, anandamide (ANA) and *N*-arachidonoyl-dopamine (NADA) (Zygmunt *et al*., 1999; Di Marzo *et al*., 2001; Huang *et al*., 2002). Various VR1 antagonists have also been reported, for example, capsazepine, iodo-resiniferatoxin (I-RTX) and *N*-(4-tert.butyl-phenyl)-4-(3-chloropyridin-2-yl) tetrahydro-pyrazine-1(2H)-carboxamide (BCTC). These have analgesic effects *in vivo* (Bevan *et al*., 1992; Walpole *et al*., 1994; Catarina *et al*., 1997; 2000; Tominaga *et al*., 1998; Wahl *et al*., 2001; Pomonis *et al*., 2003; Rigoni *et al*., 2003).

Protons act as endogenous activators and modulators of VR1 responses. Low pH enhances the apparent VR1-binding affinity of capsaicin, and potentiates the channel gating of VR1 receptors (Caterina *et al*., 1997; 2000; Tominaga *et al*., 1998; Olah *et al*., 2001; Ryu *et al*., 2003). Since inflammation leads to acidification of the inflamed tissue, VR1 is thought to play a major role in the transduction of inflammatory pain.

As VR1 and TRPM8 are distantly related, and no antagonists have been described for TRPM8, we tested the effects of VR1 antagonists on TRPM8. Further, we investigated whether the responses of VR1 and TRPM8 towards agonists are influenced by pH.

Methods

Materials

Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS) and all cell culture reagents were obtained from Invitrogen (Karlsruhe, Germany). (−)Menthol, capsaicin, capsazepine, ruthenium red, eugenol, 4α-phorbol 12,13-didecanoate (4α-PDD) and probenecid were obtained from Sigma-Aldrich (Taufkirchen, Germany). (+)Menthol was purchased from Fluka-Sigma-Aldrich (Taufkirchen, Germany). Linalool, hydrocitronellal and citronellal were obtained from Henkel (Düsseldorf, Germany). WS-3 was obtained from Givaudan (Dubendorf, Switzerland). Icilin was purchased from Tocris (Ellisville, MO, U.S.A.). Frescolat ML and MGA were obtained from Haarmann & Reimer GmbH (Holzminden, Germany). WS-23 was obtained from Millennium Chemicals (Jacksonville, FL, U.S.A.). Cooling Agent 10, Coolact P and PMD38 were obtained from Takasago (Paris, France). BCTC and thio-BCTC (*N*-(4-tert.-butyl-phenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2*H*)-(thio) carboxamide) were synthesized according to published methods, and tested as free bases (Pomonis *et al*., 2003).

Cloning and expression of mTRPM8 and hVR1 receptors in HEK293 cells

mTRPM8 cDNA (GenBank accession NM_134252) was a generous gift of Ardem Patapoutian, Scripps Institute, La Jolla, U.S.A. This was subcloned into the *Nhe*I and *Kpn*I sites of the pcDNA5-Vector (Invitrogen, Karlsruhe, Germany), as described previously (Peier *et al*., 2002). The hVR1 cDNA (GenBank accession AJ272063) was cloned in pcDNA3.1 in a manner similar to that described previously (Hayes *et al*., 2000; Smart *et al*., 2000). HEK 293 cells were transiently transfected with mTRPM8 and hVR1 using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

Cell culture

HEK293 cells were routinely grown as monolayers in minimum essential medium (MEM) supplemented with non-essential amino acids, 10% fetal calf serum and 0.2 mM L-glutamine, and maintained under 95% O_2 / 5% CO_2 at 37°C.

Measurement of $[Ca^{2+}]_i$ using the FLIPR® assay

mTRPM8- and hVR1-transfected HEK293 cells were seeded into black-walled clear-base poly-D-lysine-coated 96-well plates (Becton Dickinson, Meylan Cedex, France) at a density of 25,000 cells per well in MEM, supplemented as described above, and cultured overnight. The cells were then incubated with MEM containing the cytoplasmic calcium indicator Fluo-4AM (4 μM; Molecular Probes, Eugene, Oregon, U.S.A.) at 37°C for 30 min. The cells were washed twice with HBSS supplemented with 2.5 mM probenecid and 20 mM HEPES, resuspended in the same buffer, and incubated for 15 min at 37°C. Subsequently, the plates were inserted into a fluorometric imaging plate reader (FLIPR®; Molecular Devices, Sunnyvale, CA, U.S.A.), and the fluorescence ($\lambda_{ex} = 488$ nM, $\lambda_{em} = 510$–570 nM) from $[Ca^{2+}]_i$ was determined before and after the addition of various concentrations of test compounds (Sullivan *et al*., 1999; Jerman *et al*., 2000).

In experiments designed to define the influence of low pH on mTRPM8 and hVR1 currents in HEK293 cells, contributions from the endogenous hASICa (acid-activated channel) are conceivable. This channel is desensitized by short exposures to low pH (Gunthorpe *et al*., 2001). Consequently, transfected cells were incubated at pH 6.3 for at least 1 min prior to measurements.

Data analysis

EC_{50} values were determined as the concentration of test substance required to produce half-maximal increases in $[Ca^{2+}]_i$. Maximal $[Ca^{2+}]_i$ responses were measured as peak fluorescence intensity (FI) minus basal FI, and expressed as percentages of the maximum response to icilin. Data are given as means ± s.e.m., unless otherwise stated. Curve fitting and parameter estimations were performed with Microsoft Excel 97 and Graph Pad Prism 3.01 (GraphPad Software Inc., CA, U.S.A.).

Results

Identification of TRPM8 agonists

mTRPM8 cDNA was cloned into a mammalian expression vector as described in 'Methods', and this was used to transfect HEK293 cells transiently with mTRPM8 for functional

studies. Ca^{2+} fluorescence was measured using the FLIPR® assay. The known agonists (−)menthol, icilin and eucalyptol caused increases in $[Ca^{2+}]_i$ in mTRPM8-transfected HEK293 cells (Figure 1).

Compounds from a library of odorants, or which were chemically related to menthol, were screened. In addition, the compound libraries of the fragrance industries were searched for compounds that produce cooling sensations. In all, 70 compounds were investigated at two concentrations (50 μM and 10 mM). Of these, 10 (linalool, geraniol, hydroxycitronellal, WS-3, WS-23, Frescolat MGA, Frescolat ML, PMD 38, Coolact P and Cooling Agent 10) produced increases in $[Ca^{2+}]_i$ in mTRPM8-transfected HEK293 cells, and were studied in more detail.

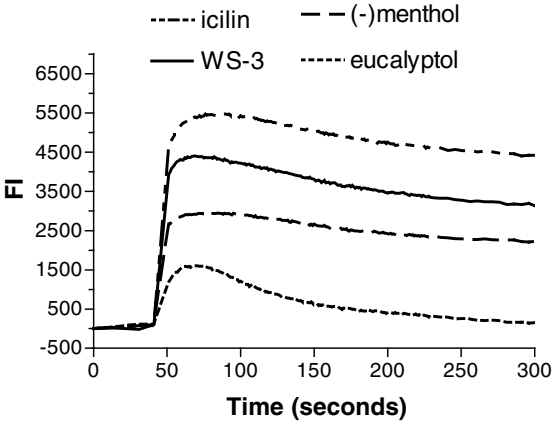


Figure 1 $[Ca^{2+}]_i$ responses to TRPM8 agonists. $[Ca^{2+}]_i$ fluxes induced by icilin (5 μM), WS-3 (30 μM), (−)menthol (30 μM) and eucalyptol (5 mM) were monitored using the FLIPR® assay in mTRPM8-transfected HEK293 cells. $[Ca^{2+}]_i$ responses were measured as changes in fluorescence intensity (FI) before and after the addition of agonists. The data shown are representative plots of the fluorescence signals against time during assays.

Various concentrations of these agonists were tested on mTRPM8-transfected, nontransfected and hVR1-transfected HEK293 cells. All of the identified TRPM8 agonists led to concentration-dependent increases in $[Ca^{2+}]_i$ in mTRPM8-transfected HEK293 cells, but not in nontransfected or hVR1-transfected HEK293 cells (data not shown), proving that the compounds are specific agonists of mouse TRPM8. The efficacies and potencies of linalool, geraniol, hydroxycitronellal, WS-3, WS-23, Frescolat MGA, Frescolat ML, PMD 38, Coolact P and Cooling Agent 10 are shown in Figure 2 and in Table 1.

Analysis of the $[Ca^{2+}]_i$ response curves showed that application of the agonists led to one of two different types of Ca^{2+} influx kinetics in mTRPM8-transfected HEK293 cells (Figure 1). For the first group of agonists (icilin, menthol, WS-3, WS-23, Frescolat MGA, Frescolat ML, PMD 38, Coolact P and Cooling Agent 10), the $[Ca^{2+}]_i$ response was typified by an initial very rapid onset (within ca. 1 s) and fast rate of increase. This reached a peak value after ca. 20–30 s, followed by a gradual slight decline over the course of the assay (Figure 1). The high $[Ca^{2+}]_i$ level was maintained for at least 4 min in the continued presence of the agonists.

The second type of agonist effect was produced by the odorants linalool, geraniol, hydroxycitronellal and eucalyptol (Figure 1). These induced a slower initial increase in $[Ca^{2+}]_i$, which peaked at roughly the same time as the response to the first type of agonist, but declined much more rapidly. Indeed,

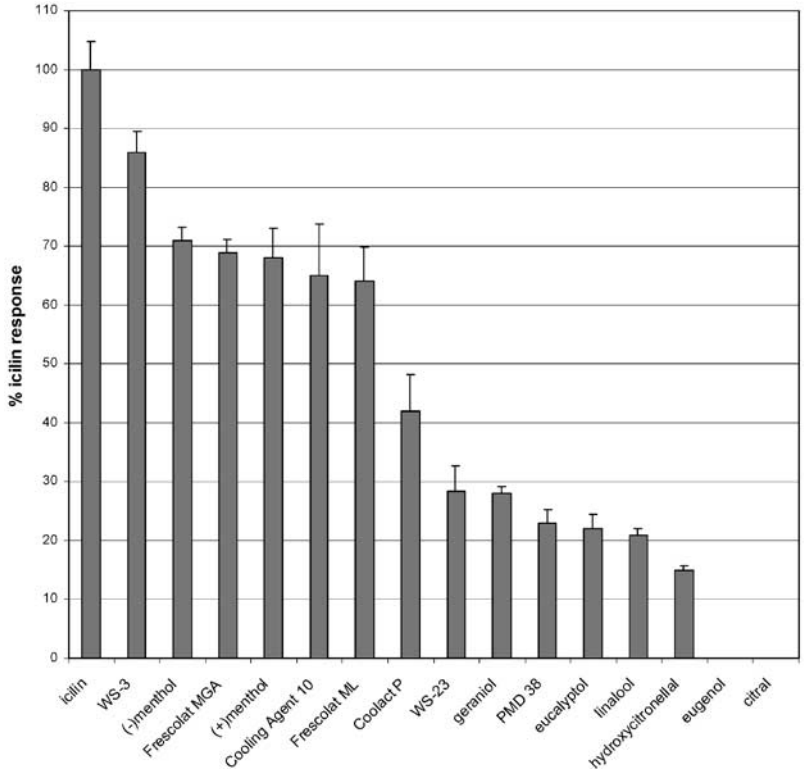


Figure 2 Efficacy of TRPM8 agonists. $[Ca^{2+}]_i$ responses were measured as maximal increases in fluorescence, expressed as percentages of the maximum icilin response. They are given as means ± s.e.m. ($n = 4$–8).

the increase in $[Ca^{2+}]_i$ above pre-test levels was negligible at the end of the assay period.

TRPM8 agonist potency and efficacy

The EC_{50} values of the agonists are listed in Table 1, ranked by potency. The order of potency, from the most to least potent was: icilin ($0.2\pm0.1\,\mu$M) > frescolatML ($3.3\pm1.5\,\mu$M) > WS-3 ($3.7\pm1.7\,\mu$M) > (−)menthol ($4.1\pm1.3\,\mu$M) > frescolatMAG ($4.8\pm1.1\,\mu$M) > Cooling Agent 10 ($6\pm2.2\,\mu$M) > (+)menthol ($14.4\pm1.3\,\mu$M) > PMD38 ($31\pm1.1\,\mu$M) > WS-23 ($44\pm7.3\,\mu$M) > Coolact P ($66\pm20\,\mu$M) > geraniol (5.9 ± 1.6 mM) > linalool (6.7 ± 2.0 mM) > eucalyptol (7.7 ± 2.0 mM) > hydroxycitronellal (19.6 ± 2.2 mM).

The efficacies of the TRPM8 agonists are shown in Figure 2 as percentages of the maximal response to the most potent agonist, icilin (Wei & Seid, 1983).

The rank order of efficacy for the agonists was: icilin > WS-3 > (−)menthol > FrescolatMAG > (+)menthol > Cooling Agent 10 > FrescolatML > CoolactP > WS-23 > geraniol > PMD38 > eucalyptol > linalool > hydroxycitronellal.

The efficacies of WS-3, both menthol isomers, Frescolat ML, Frescolat MGA and Cooling Agent 10 were slightly lower than that of icilin, but they were markedly less potent.

Table 1 Chemical structures, efficacies and potencies of mTRPM8 antagonists and agonists

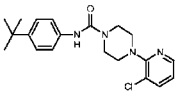
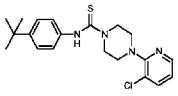
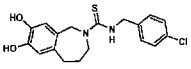
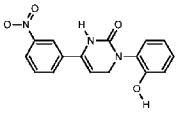
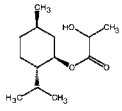
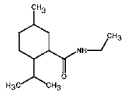
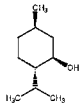
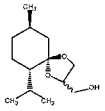
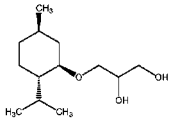
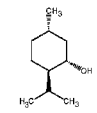
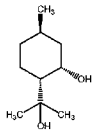
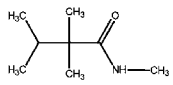
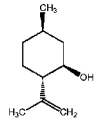
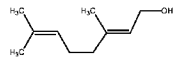
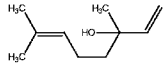
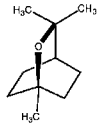
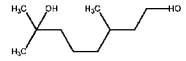
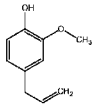
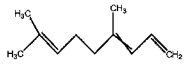
Compound antagonists	*Structure*	*Efficacy*	*Potency* IC_{50} (μM)
BCTC			0.8 ± 1.0
thio-BCTC			3.5 ± 1.1
Capsazepine			18 ± 1.1
Agonists		*% max icilin response*	*EC_{50}*
Icilin		$100\pm4.7\%$	$0.2\pm0.1\,\mu$M
Frescolat ML		$64\pm5.9\%$	$3.3\pm1.5\,\mu$M
WS-3		$86\pm3.4\%$	$3.7\pm1.7\,\mu$M
(−)menthol		$71\pm2.3\%$	$4.1\pm1.3\,\mu$M
Frescolat MGA		$69\pm2.2\%$	$4.8\pm1.1\,\mu$M
Cooling-agent 10		$65\pm8.7\%$	$6\pm2.2\,\mu$M
(+)menthol		$68\pm4.1\%$	$14.4\pm1.3\,\mu$M

Table 1 (*continued*)

Compound antagonists	*Structure*	*Efficacy*	*Potency* IC_{50} (μM)
PMD-38		23±2.2%	31±1.1 μM
WS-23		28±4.4%	44±7.3 μM
Coolact P		42±6.2%	66±1.2 μM
Geraniol		28±1.1%	5.9±1.6 mM
Linalool		21±1.1%	6.7±2.0 mM
Eucalyptol		23±2.4%	7.7±2.0 mM
Hydroxy-citronellal		15±0.7%	19.6±2.2 mM
Eugenol		Inactive	Inactive
Citral		Inactive	Inactive

Linalool, geraniol, hydroxycitronellal, WS-3, WS-23, FrescolatMGA, FrescolatML, PMD38, CoolactP and Cooling Agent 10 were identified as novel partial TRPM8 agonists. The efficacies and potencies of mTRPM8 antagonists and agonists are given. Changes in $[Ca^{2+}]_i$ were measured using the FLIPR® described in Methods ($n=4$–8).

Coolact P, PMD38 and WS-23 were rather weak agonists, with substantially reduced efficacies. The odorants linalool, geraniol and hydroxycitronellal were extremely weak agonists with rather low efficacies (Table 1, Figure 2).

Partial overlap of ligands for TRPM8 and VR1

The heat receptor VR1 is distantly related to TRPM8, and is well characterized as a pain target (Caterina *et al*., 1997; 2000). For this reason, we investigated whether TRPM8 and VR1 have common pharmacological aspects. It was shown that the VR1 antagonists capsazepine, BCTC and thio-BCTC inhibited the $[Ca^{2+}]_i$ response of mTRPM8 to 20 μM menthol in a concentration-dependent manner. IC_{50} values for this inhibition were 18±1.1 μM for capsazepine, 0.8±1.0 μM for BCTC and 3.5±1.1 μM for thio-BCTC, as shown in Figure 3.

Although these antagonists displayed the same potency ranking for both hVR1 and mTRPM8 (BCTC > thio-BCTC > capsazepine), their antagonistic potencies for mTRPM8 were much lower than for the hVR1 receptor (IC_{50} mTRPM8 vs hVR1: capsazepine, 18±1.1 vs 2.6±1.2 μM (Smart *et al*., 2001); thio-BCTC, 3.5±1.1 μM vs 54.3±21.8 nM (data not shown); BCTC 0.8±1.0 μM vs 34.9±19.4 nM (Valenzano *et al*., 2003)).

BCTC (10 μM) and thio-BCTC (10 μM) completely blocked the mTRPM8 response to 0.5 μM icilin, whereas capsazepine

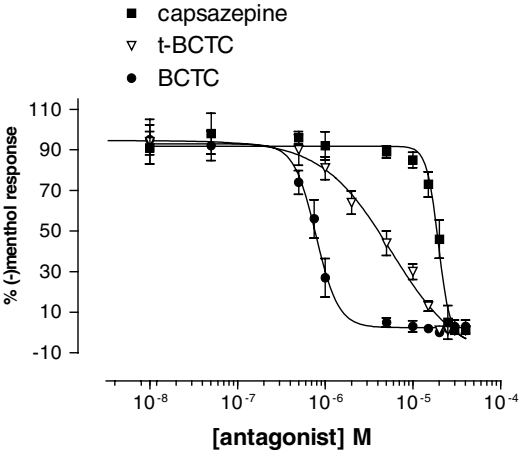


Figure 3 Antagonists of TRPM8. The VR1 antagonists (capsazepine, thio-BCTC and BCTC) inhibited the $[Ca^{2+}]_i$ increases induced by 20 μM (−)menthol *via* mTRPM8 channels in a concentration-dependent manner. $[Ca^{2+}]_i$ was monitored as described above. Responses were measured as peak increases in fluorescence, and expressed as percentages of the uninhibited response (mean ± s.e.m., $n=4$).

(30 μM) only blocked 40% of the response. Like Valenzano *et al.* (2003), we were not able to detect any quenching of Fluo-4 fluorescence by either BCTC or thio-BCTC. In contrast to their antagonism of icilin, neither BCTC nor thio-BCTC were able to block the $[Ca^{2+}]_i$ increase induced by 4α-phorbol 12,13-didecanoate (4α-PDD) in hTRPV4-transfected HEK293 cells; neither did they inhibit the ATP-induced $[Ca^{2+}]_i$ increase in CHO K1 cells (data not shown). This demonstrates that BCTC and thio-BCTC are selective antagonists for certain TRP channels.

Interestingly, the VR1 antagonist I-RTX had no influence on mTRPM8 currents (data not shown), and neither did the channel blocker ruthenium red (Peier *et al.*, 2002) nor the VR1 agonists capsaicin and RTX (data not shown).

pH sensitivity of TRPM8 and VR1

Protons act as endogenous activators or modulators of VR1 (Caterina *et al.*, 1997; Ryu *et al.*, 2003). When capsaicin (0.1 μM) was added to hVR1-transfected HEK293 cells at pH 6.3, a 1.6-fold increase in Ca^{2+} flux compared to that at pH 7.5 (Figure 4a) was observed. At pH 6.3, the VR1 response to the endogenous VR1 agonists ANA and NADA was even more markedly increased, by ca.3.5- to 4.0-fold (Figure 4a).

In contrast, the Ca^{2+} influxes induced by menthol and icilin through mTRPM8 channels were almost completely inhibited at pH 6.3 (Figure 4b); Ca^{2+} influx was also reduced when the agonists were applied at pH 8.0 rather than at pH 7.5. Thus, hVR1 and mTRPM8 channels react oppositely to acidic conditions: VR1 is potentiated, and TRPM8 is inhibited.

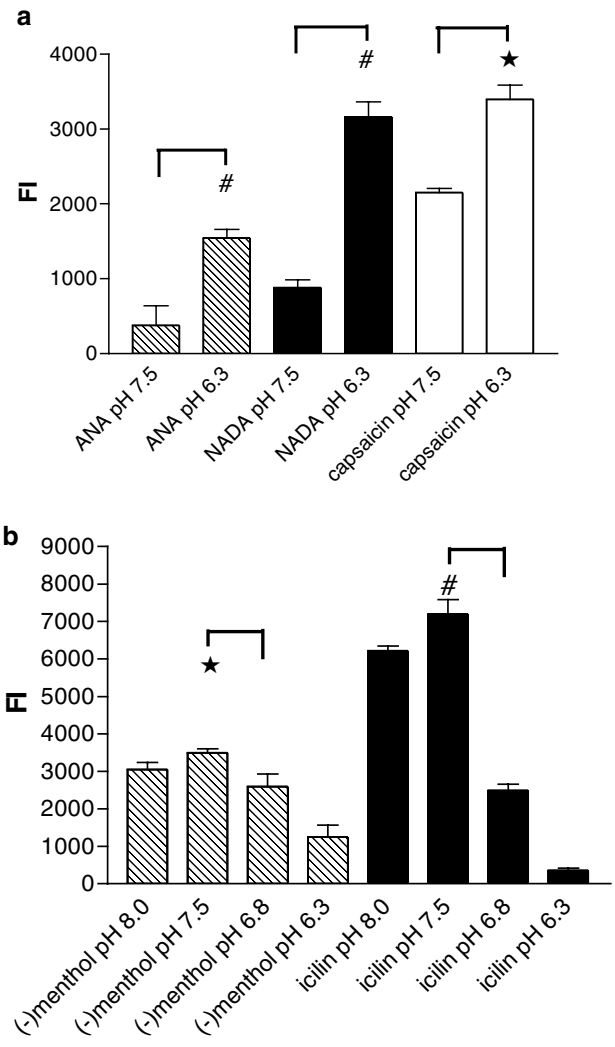


Figure 4 pH sensitivity of hVR1 and mTRPM8. (a) $[Ca^{2+}]_i$ was monitored in hVR1-transfected HEK293 cells before and after the addition of the agonists ANA (2 μM), NADA (2 μM), or capsaicin (0.1 μM). Responses were measured as increases in peak fluorescence intensity. Agonists were applied in buffers at either pH 7.5 or pH 6.3 ($n=3$). $^*P<0.01$ and $P<0.005$, unpaired *t*-test. (b) $[Ca^{2+}]_i$ was monitored in mTRPM8-transfected HEK293 cells before and after the addition of (−)menthol (20 μM) or icilin (0.5 μM). Agonist responses were measured as increases in peak fluorescence intensity. Agonists were applied at pH 8.0, pH 7.5, pH 6.8, or pH 6.3 ($n=3$). $^*P<0.05$ and $P<0.005$, unpaired *t*-test.

Discussion and conclusion

TRPM8 agonist potency and efficacy

The TRPM8 receptor is a transducer of cold stimuli in the somatosensory system (McKemy *et al.*, 2002; Peier *et al.*, 2002). However, due to the lack of specific and water-soluble ligands, only a limited pharmacological characterization has been possible to date. After screening a small library of 70 odorants, and other substances related to menthol (Eccles, 1994), we were able to identify 10 novel TRPM8 agonists. Consistent with the reported molecular pharmacology of TRPM8 (McKemy *et al.*, 2002; Peier *et al.*, 2002), menthol, eucalyptol and icilin increased $[Ca^{2+}]_i$ in mTRPM8-transfected HEK293 cells in the present study (Figure 1; Table 1). McKemy *et al.* did not specify the optical purity of the menthol they used; in our studies, the 1R,3R,4S form of (−)menthol was ca. 3.5-fold more effective than that of (+)menthol (Table 1), indicating that there is a slight preference for the (−) enantiomeric form.

Some of the identified TRPM8 agonists, namely WS-3, Coolact P, Cooling Agent 10, and PMD38, are used as cooling

agents in the food and cosmetics industry. This cooling sensation may in part be mediated by TRPM8. Manufacturers report the cooling strength of WS-3 to be ca. five-fold, Coolact P ca. four-fold, Cooling Agent 10 ca. 4.5-fold and PMD38 ca. 9.5-fold greater than that of (−)menthol. These estimates do not correlate with our results (Table 1). As an agonist for mTRPM8, WS-3 displayed a slightly higher efficacy and a similar potency to (−)menthol, but Coolact P and PMD38 displayed equal or lower efficacies and potencies. It is not unusual for *in vitro* and *in vivo* data to be discrepant. Probably, additional factors such as membrane permeability, metabolism, chemical stability, solubility, subjectivity and volatility of the tested compounds have also to be taken into account (Watson *et al*., 1978). There may also be differences between species in the ligand specificity of receptors (here, mouse vs human). Conceivably, these substances also activate other cold-transducing receptors such as ANKTM1, which is also activated by icilin (Story *et al*., 2003). Future studies will address the selectivity of the identified novel TRPM8 agonists.

Besides menthol and eucalyptol, we were able to identify three novel natural odorants (linalool, geraniol and hydroxycitronellal) that activate the mTRPM8 receptor. These natural odorants are found in formulations used in aroma therapies, for example, against headaches (Shanghai Medicinal Herbs, Essential Balm).

It is worth noting that an analgesic effect was reported recently for the novel TRPM8 ligand linalool, and also for menthol (Peana *et al*., 2003). Linalool is a fresh, pungent and flowery odorant found in plants such as *Convallaria majalis* (lily of the valley) and *Zingiber officinale* (ginger). We also found agonistic effects on mTRPM8 for geraniol, the main odorant component of roses, and hydroxycitronellal, a fresh citrus odorant.

The weak potency and efficacy of these odorants in our *in vitro* assays could be partially explained by their hydrophobicity and poor aqueous solubility. The observed concentration dependance of the responses to geraniol, linalool, eucalyptol and hydrocitronellal (Table 1) was in the same range as that reported for eucalyptol with TRPM8 (EC_{50}: 3.4 ± 0.4 mM; McKemy *et al*., 2002).

The evidence thus shows that menthol, eucalyptol, icilin and all of the newly identified agonists can produce cooling sensations, which may, at least in part, be explained by the activation of the TRPM8 cold receptor. Additionally, some of the compounds described here as agonists for TRPM8 have analgesic effects *in vivo*, suggesting a role for TRPM8 in pain relief.

Partial overlap of ligands for TRPM8 and VR1

The VR1 antagonists capsazepine, BCTC and thio-BCTC, though not I-RTX, inhibited the Ca^{2+} influx induced through TRPM8 by 20 μM menthol in a concentration-dependent manner. The channel blocker ruthenium red, and the VR1 agonists capsaicin and RTX, had no effect on TRPM8. Capsaicin, capsazepine, BCTC, RTX and I-RTX are supposed to share the same binding pocket at transmembrane domains (TM) 2–3 of the VR1 receptor (Jordt & Julius, 2002; Valenzano *et al*., 2003). At the predicted capsaicin-binding region of VR1 (TM2 and TM3, Jordt & Julius, 2002), the amino-acid sequence identity of VR1 and TRPM8 was 36%, compared to an overall amino-acid sequence identity of 21%. Future studies will have to ascertain whether capsazepine, BCTC and thio-BCTC interact at a TRPM8 site corresponding to the capsaicin-binding site of VR1.

We have shown here for the first time that capsazepine is an antagonist of recombinant mTRPM8. Interestingly, Reid *et al*. (2002a) demonstrated that capsazepine is able to block native cold- and menthol-induced Ca^{2+} currents in rat dorsal root ganglion. This observation might be explained by the fact that capsazepine inhibits TRPM8. Our results extend the range of receptors, such as voltage-gated Ca^{2+}-channels (Docherty *et al*., 1997) and nicotinic acetylcholine receptors (Liu & Simon, 1997; Wardle *et al*., 1997), that are known to interact with capsazepine.

BCTC has until now been regarded as a highly specific VR1 antagonist, since no interactions with 60 other receptors were observed in a study by Valenzano *et al*. (2003). However, our results indicate that BCTC also antagonizes TRPM8 at submicromolar concentrations, which has implications for its use as a specific VR1 antagonist *in vivo*. BCTC seemed to be a more specific VR1 antagonist than capsazepine, as no interactions with TRP channels other than VR1 have been published to date. Our results show that BCTC acts as an antagonist for TRPM8, which may indicate that BCTC could be an inhibitor for other related TRP channels.

Looking at the chemical structures and potential pharmacophore, the similarities and differences between BCTC and icilin are obvious. Both molecules have two aromatic rings and a urea moiety in common. The distances between these pharmacophore elements are clearly different, which may partly explain why icilin acts as an agonist and BCTC as an antagonist. This has to be confirmed with studies using structural analogues of BCTC and icilin.

pH sensitivity of TRPM8 and VR1

The activation of VR1 by capsaicin was potentiated by low pH in this study. Caterina *et al*. (1997) reported that capsaicin-induced currents were ca. five-fold greater at pH 6.3 than at pH 7.6 in a *Xenopus* oocyte expression system. However, we only observed a 1.6-fold increase in Ca^{2+} flux compared to that at pH 7.5 (Figure 4a).

The effect of ANA, an endogenous CB1 and VR1 agonist (Di Marzo *et al*., 2001), was also strongly potentiated by low pH in this study (Figure 4a). Olah *et al*. (2001) also reported that acidification potentiates the activity of ANA, whereas others observed no potentiation (Smart *et al*., 2000; for review Ralevic *et al*., 2002). The difference may be due to methodological discrepancies. We also observed that another endogenous CB1 and VR1 agonist, NADA (Bisogno *et al*., 2000), was even more strongly potentiated by acid pH than ANA (Figure 4a).

In contrast to VR1, the TRPM8-mediated Ca^{2+} response to menthol and icilin was inhibited by low pH (Figure 4b). Thus, TRPM8 and VR1 are oppositely modulated by low pH. Under inflammatory conditions, when acidification of inflamed tissue occurs, both mechanisms may play a role in the development of hyperalgesia. The reduced pH could sensitize VR1 and thereby make the tissue more susceptible to pain stimuli, and increasing heat sensations; the same acidic conditions would inhibit TRPM8, and reduce 'pleasant cool' sensations. Thus, VR1 and TRPM8 may act in concert under inflammatory conditions, and cause an aggravation of thermal hyperalgesia.

In conclusion, we have identified 10 novel TRPM8 agonists. The identification of three natural odorants that activate TRPM8, together with the fact that TRPM8 is expressed in the trigeminus, which belongs to the sensory system of the olfactory epithelium, suggests that TRPM8 could be an important 'Chemosensory Trigeminal Nerve Receptor'. Agonistic TRPM8 responses to (−)menthol and icilin were inhibited dose-dependently by three well-known VR1 antagonists (capsazepine, thio-BCTC and BCTC). This suggests a partial overlap between the ligand specificities of TRPM8 and VR1, whereas the VR1 response to endogenous agonists was strongly potentiated by low pH, the TRPM8 response was inhibited.

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