Conservation of functional and pharmacological properties in the
distantly related temperature sensors TRPV1 and TRPM8

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ABBREVIATIONS:
TRP, Transient Receptor Potential; TRPM8, Transient Receptor Potential Melastatin 8; TRPV1, Transient Receptor Potential Vanilloid 1; FLIPR® FLuorometric Imaging Plate Reader; HEK293, human embryonic kidney 293 (cells).

CHEMICAL NAMES:
SB-452533, N-(2-bromophenyl)-N'-{2-[ethyl(3-methylphenyl)amino]ethyl} urea
SB-366791, (N-(3-methoxyphenyl)-4-chlorocinnamide)
CTPC, (2R)-4-(3-chloro-2-pyridinyl)-2-methyl-N-[4-(trifluoromethyl)phenyl]-1-piperazinecarboxamide
BCTC, N-(4-tertiarybutyl-phenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide
Abstract

Members of the Transient Receptor Potential (TRP) superfamily of ion channels have now been defined as molecular transducers capable of reproducing the spectrum of temperature sensation exhibited by mammals. Due to their pivotal role in sensory transduction, many of these channels represent good targets for drug discovery. With a view to gaining further insight into the functional and pharmacological properties of these channels, we have used the whole-cell patch clamp technique to study the human cold-sensitive menthol receptor TRPM8 and compared its behaviour with that of its distant relative, the heat-sensitive capsaicin-gated TRPV1. Remarkably, we find that TRPM8, in addition to its behaviour as an outwardly rectifying, non-selective cation channel, shares many functional and pharmacological properties with TRPV1. TRPM8 exhibits prominent time- and voltage-dependent behaviour, a property which may underlie the conserved rectification or gating mechanisms exhibited by these channels. We also show that TRPM8 is modulated by ethanol, but unlike TRPV1 is insensitive to extracellular acidification. There is also significant overlap in the antagonist pharmacology of these channels with many TRPV1 antagonists such as capsazepine, BCTC, CTPC and SB-452533 exhibiting similar activity at TRPM8. Overall, the degree of pharmacological overlap between TRPV1 and TRPM8 has implications for the interpretation of studies conducted with these ligands to date, and highlights a clear challenge for the design of selective TRP channel antagonists. Our finding that SB-366791, at least, represents an apparently selective antagonist for TRPV1 suggests that this goal is attainable.
Introduction

The Transient Receptor Potential (TRP) superfamily of ion channels, of which there are now over 25 members, appears to have evolved to subserve a diverse array of cellular processes (for review, see Clapham et al., 2003). One rather exquisite role, namely that of temperature sensation, is now known to involve members of the TRPV (Vanilloid), TRPM (Melastatin) and TRPA (Ankyrin, ANKTM1) subfamilies (usefully grouped as thermoTRPs; Patapoutian et al., 2003) which together are able to reproduce the spectrum of temperature sensation, from noxious cold to noxious heat, with which we are all familiar (Benham et al., 2003; McKemy et al., 2002; Patapoutian et al., 2003).

All TRPs share a similar molecular architecture, most likely consisting of a symmetrical arrangement of four subunits, each of six transmembrane spanning domains, arranged around a central ion conducting pore (Clapham, 2003). The structure of the integral channel no doubt endows the receptor with its characteristic non-selective cationic behaviour and also potentially defines the outwardly rectifying current-voltage profile noted for many of these channels (Caterina et al., 1997; Caterina et al., 1999; Clapham, 2003; Gunthorpe et al., 2002; McKemy et al., 2002; Peier et al., 2002). Due to their roles as cellular sensors, many TRPs represent good targets for therapeutic intervention. Hence, much recent work has focussed on the functional and pharmacological characterisation of these channels with a view to identifying selective tools with which to better validate their roles in health and disease, and also provide starting points for drug discovery.

TRPV1 (previously named Vanilloid Receptor-1, VR1) was the first heat-activated ion channel to be cloned. It has an activation threshold (~43°C) in the noxious heat range (Caterina et al., 1997), and was the defining member of the TRPV family (Gunthorpe et al., 2002). TRPV1 is predominantly expressed in peripheral sensory neurones of the dorsal root and trigeminal ganglia and is a truly polymodal receptor: It can be activated or modulated by chemical ligands like capsaicin (the ‘hot’ ingredient of chilli peppers), protons (acidic pH), the endocannabinoid anandamide, and ethanol (Caterina et al., 1997; Gunthorpe et al., 2002; Tominaga et al., 1998; Trevisani et al., 2002). TRPV1 is therefore a key sensory transducer in the pain pathway and is the subject of intense research to develop novel therapeutic agents which may have utility for the treatment of pain, migraine, asthma and a range of other indications (for review, see
Three other close relatives of TRPV1 have now also been shown to act as thermosensors with thresholds extending from the warm (~30°C) for TRPV3 and TRPV4 to the high threshold noxious heat range for TRPV2 (>50°C; Benham et al., 2003).

TRPM8 was recently identified as a distant relative of TRPV1 (the receptors show only ~20% identity in protein sequence) by a bioinformatics approach (Peier et al., 2002) and an expression cloning strategy using the natural product menthol (McKemy et al., 2002). The definition of TRPM8 and, subsequently TRPA1 (Story et al., 2003), as cold-activated ion channels extended the exclusivity of the temperature sensor group to include members of the TRP channel superfamily beyond the TRPV class, and further highlights that many, if not all, of these channels are amazingly well equipped for their divergent roles as cellular sensors (Clapham, 2003; McKemy et al., 2002; Patapoutian et al., 2003). TRPM8 can now also be considered to be a polymodal receptor since its activity is also controlled by a range of diverse chemical and physical stimuli. TRPM8 exhibits an activation threshold in the range 22-27°C (McKemy et al., 2002; Peier et al., 2002) and is also gated by a range of synthetic ‘cooling’ compounds such as icilin and eucalyptol (Behrendt et al., 2004; McKemy et al., 2002; Peier et al., 2002) and is reportedly inhibited by acid (Behrendt et al., 2004).

TRPM8, like TRPV1, is also expressed in sensory neurones in the peripheral nervous system, especially in dorsal root, trigeminal, and nodose ganglia (McKemy et al., 2002; Nealen et al., 2003; Peier et al., 2002; Reid et al., 2002; Reid and Flonta, 2002; Thut et al., 2003; Zhang et al., 2004). This, combined with clinical reports which document the beneficial effects of menthol treatment in patients suffering from post herpetic neuralgia and irritable bowel syndrome (Davies et al., 2002; Pittler and Ernst, 1998) suggests that TRPM8 may also be an exciting new target for the development of novel analgesics as well as agents to treat a range of other conditions (Caterina et al., 1997; McKemy et al., 2002; Peier et al., 2002). To date, no selective TRPM8 antagonists have been reported, however, the recent paper by Behrendt et al. (2004), demonstrating that the TRPV1 antagonists BCTC (Valenzano et al., 2003) and capsazepine (Bevan et al., 1992; Valenzano et al., 2003) are also antagonists of mouse TRPM8, provides an exciting breakthrough in this field, providing the first (albeit non-selective) tools with which to study TRPM8 function.
With a view to gaining further insight into the functional and pharmacological properties of human TRPM8, we have used the whole-cell patch-clamp technique to study this receptor in detail and compared its behaviour with that of its distant relative, TRPV1. We find that TRPM8 exhibits time- and voltage-dependent gating and is subject to modulation by ethanol suggesting that these receptors are much more similar than initially envisaged. We also extend the findings of Behrendt et al (2004) defining significant overlap in the pharmacological Structure-Activity-Relationship (SAR) of the two receptors and identify a number of potent antagonists of the human TRPM8 receptor. Finally, our demonstration that the potent TRPV1 antagonist SB-366791 shows little or no effect versus TRPM8 provides encouraging evidence that selective compounds for these thermoTRP channels can be developed.
Cloning and expression of human TRPM8. hTRPM8 was cloned by PCR-based methods using TRPM8-specific primers based on the published sequence described by Tsavaler et al. (2001). As full-length hTRPM8 amplification proved to be problematic, hTRPM8 was amplified in two fragments that overlapped a unique EcoRI restriction site (base 1579 to 1584, AY090109). Fragment 1 was amplified by nested PCR with human prostate Marathon-Ready™ cDNA (Clontech) using the following primers: first round reaction 5' - GTCCGCCACGCGTCG-3’ & 5’-G GCCAGAGTCTTCAGAAGCTTG-3’; nested reaction 5’-CACCATGTCTTTCGGGCAGCCAGGC-3’ & 5’- CGAGTAATAGGAGACACGTCGG-3’. Fragment 2 was amplified from SMART prostate CDNA (made from human prostate poly A+ mRNA using the SMART RACE cDNA amplification kit, BD Biosciences) using the following primers: 5’- CAGTGAGCAAGACAAGGATACTGG-3’ & 5’-CAGTTTTATTTGATTTTATTAGC-3’. PCR products were visualised on 1% agarose gels and purified using the Qiaquick PCR purification kit (Qiagen) before being sub-cloned into pcDNA3.1D/V5/His-TOPO or pcDNA3.1/V5/His-TOPO (Invitrogen). The resulting plasmids were transformed into chemically competent TOP10 cells (Invitrogen). Plasmid DNA was prepared using the Qiaprep Spin MiniPrep Kit (Qiagen). Following confirmation of the hTRPM8 sequence by full length double stranded sequencing, fragments 1 and 2 were isolated by digestion with EcoRI and EcoRV and used to generate the full-length human TRPM8 coding sequence by ligation using the Ligafast T4 DNA ligase system (Promega). The integrity of the full length TRPM8 sequence was verified by comparison with NCBI published sequences (Accession numbers AY090109, AY328400, NM_024080, AX267803 and AB061779) and by the sequencing of clones derived from independent PCR reactions. The verified TRPM8 sequence that has been used in the studies in this paper differs from the original trp-p8 (AY090109) sequence (Tsavaler et al., 2001) by one amino acid change (Ile58 to Thr). This amino acid change is also encoded by the sequences deposited as accession numbers AY328400, AX267803 and AB061779, and also human genomic DNA.

Human embryonic kidney (HEK293) cells were transfected using Lipofectamine Plus (Life Technologies) according to the manufacturer’s protocol and stably expressing clones.
(hTRPM8.HEK293 cells) were generated by selection using G418 as described previously (Hayes et al., 2000). Cells were cultured on plastic tissue culture dishes in modified Eagles's medium with Earle's salts and supplemented with 10% fetal bovine serum, non-essential amino acids and 0.2mM L-glutamine while being maintained under 5% CO₂ at 37°C. For electrophysiological experiments cells were plated at a 30000 cells/cm² density onto 19 mm glass coverslips coated with poly-L-lysine with experiments being performed 24-72 hrs thereafter.

**Whole-cell patch clamp electrophysiology.** Electrophysiological studies were conducted according to previously described methods (Gunthorpe et al., 2004b; Hayes et al., 2000; Trevisani et al., 2002). All recordings were performed at room temperature (20-24°C) using an Axopatch 200B amplifier controlled via the pClamp8/pClamp9 software suite (Axon Instruments) using standard whole-cell patch-clamp methods. The recording chamber was continuously perfused with extracellular solution consisting of (mM): NaCl 137, KCl 5.4, D-Glucose 10, HEPES 10, CaCl₂ 2, MgCl₂ 1, adjusted to pH7.3 with NaOH. Patch pipettes (resistance 2-5 MΩ) were fabricated on a Sutter instruments P-87 electrode puller and filled with intracellular solution of the following composition (in mM): CsCl 140, EGTA 10, MgATP 2, HEPES 10, CaCl₂ 1, adjusted to pH7.4 with 1 M CsOH. Antagonist solutions were prepared as 10 mM stock solutions in dimethylsulphoxide, prior to dilution in extracellular solution. To minimise problems arising from the poor solubility of menthol, it was prepared as a 100 mM stock solution in ethanol prior to dilution in extracellular solution (final ethanol concentration = 0.1%; see Reid and Flonta 2002). For studies examining the effect of acid on TRPM8, the pH of the extracellular solution was adjusted to pH6.3 or pH5.3 by addition of HCl. Data were acquired at 8.3-10 kHz, filtered at 2-5 kHz. Series resistance compensation of up to 80% was used, where appropriate. Agonists and antagonists were applied to cells in a controlled fashion via an automated fast-switching solution exchange system (SF-77B, Warner Instruments)

**Data analysis.** Data were analysed using the Clampfit (Axon Instruments), Excel (Microsoft) and Origin (Microcal) software packages. Unless otherwise stated, data are presented as mean ± S.E.M. and Student’s t test was used to assess statistical significance, with a value of $p < 0.05$ being regarded as significant.
**Materials.** BCTC, CTPC, SB-452533 and SB-366791 were obtained from the Department of Medicinal Chemistry, GlaxoSmithKline, Harlow (chemical structures are depicted in Fig 5). All other compounds and cell culture media were obtained from Sigma (Gillingham, UK) and Tocris Cookson (Bristol, UK). The single stereoisomer (-)-menthol was used for all studies.
Results

**Activation of human TRPM8 by menthol.** We used the whole-cell patch clamp technique to study the functional and pharmacological properties of the human TRPM8 receptor in detail. Since the rat and mouse orthologues of TRPM8 have been reported to be activated by menthol (McKemy et al., 2002; Peier et al., 2002), in a similar fashion to that first described for the native receptor expressed in dorsal root ganglion neurones (Reid and Flonta, 2001; Reid and Flonta, 2002), we used this agent to reliably activate the hTRPM8 channel. Menthol, applied at a concentration of 100 µM, to hTRPM8.HEK293 cells at a holding potential of –70 mV, evoked large (mean current = 1115 ± 106 pA, n = 95), rapidly activating, inward currents which de-activated upon cessation of the menthol application (Fig. 1A). These effects of menthol were specific to TRPM8 since no currents were detected in the control wild-type HEK293 cells under similar conditions (n=5, data not shown).

We found that the menthol-evoked TRPM8 currents showed limited desensitization and tachyphylaxis, a property which fortuitously simplifies their biophysical and pharmacological characterization (Fig. 1B). As part of our initial characterization of the properties of human TRPM8 we tested the antagonistic activity of the competitive TRPV1 antagonist capsazepine, which has recently been reported to be a weak inhibitor of mouse TRPM8 (IC$_{50}$ = 18 µM; Behrendt et al., 2004), and the rather non-selective TRPV pore-blocker ruthenium red (Gunthorpe et al., 2002). Capsazepine, at a concentration of 10 µM, almost completely inhibited menthol-gated human TRPM8 currents (94 ± 2 %, n=5), whereas ruthenium red at the same concentration caused little or no effect (11 ± 9 % inhibition, n = 4; Fig. 1B & 1C), confirming the pharmacological profile expected for this receptor (Behrendt et al., 2004).

**Rectification properties of TRPM8.** In order to evaluate the rectification properties of human TRPM8, we carried out a series of voltage-ramp experiments (Fig. 1D&E). Voltage-ramps from –70 mV to +70 mV were applied before, during and after the application of 100 µM menthol and were timed to coincide with the steady-state phase of the agonist-induced current (Fig. 1D). Figure 1E shows pooled normalized data defining the net menthol-induced current. The data reveal that TRPM8 exhibits pronounced outward rectification (I$_{+70mV}$/I$_{-70mV}$ = 7.1 ± 0.8) and a reversal potential of –9.4 ± 0.5 mV (n = 10), similar to the properties of the rodent
orthologues of the receptor (McKemy et al., 2002; Peier et al., 2002) and many other TRP channels (Clapham, 2003; Gunthorpe et al., 2002).

**TRPM8 exhibits time- and voltage-dependent behaviour.** The fact that ‘tail currents’ were evident in the experiments conducted to examine the TRPM8 current-voltage relationship (indicated by an asterisk in Fig. 1D) suggests that this channel exhibits a non-instantaneous component of rectification, a property which may underlie the gating behaviour of TRPV1 (Ahern and Premkumar, 2002; Gunthorpe et al., 2000; Vlachova et al., 2003). To determine whether or not TRPM8 shows similar behaviour we therefore carried out a series of voltage-step experiments to characterise the rectification properties of TRPM8 in more detail. The response to a single voltage-step from -70 mV to +70 mV during menthol application is shown in Figure 2A and highlights a clear non-instantaneous, exponentially rising, component of TRPM8 receptor rectification (Fig. 2(i), arrow). The rising phase was best fitted with a bi-exponential curve (Fig. 2(ii)) with time constants (tau) of 4.0 ± 1.0 ms and 31.4 ± 5.4 with the fast component contributing 60.4 ± 10.2% of the current amplitude. Mirroring this effect, repolarization from a steady-state potential of +70 mV (maintained for 300 ms) back to the holding potential of −70 mV led to the appearance of a pronounced inward 'tail current' (Fig. 2A(i) open arrow). Although similar in appearance, the magnitude of the tail current observed is clearly greater in the voltage-step experiments (Fig 2A) than that observed in the voltage-ramps (Fig 1D), reflecting the influence of the underlying kinetics of the voltage-dependent transition. The subsequent relaxation from this potentiated or ‘dis-inhibited state’ of TRPM8 was also well described by a bi-exponential function with a fast time-constant of 5.8 ± 0.3 ms and a slow-time constant of 30.1 ± 0.7 ms, with the latter contributing 51.9 ± 9.1% of the current amplitude (Fig. 2A(ii)).

To examine the effect of the degree of depolarization on the level of ‘dis-inhibition’ of TRPM8 attainable, we measured the amplitude of the tail current that arises upon repolarization following steps to a series of different test potentials. A representative trace of the net menthol-induced current recorded in response to a series of eight voltage-steps between 0 and +70 mV is shown in Fig. 2B. It is clear from these data that depolarizations to 0 mV are sufficient to uncover the time-dependent behaviour (resulting in 1.8 ± 0.2 fold potentiation of TRPM8 function) and that further depolarization can result in yet greater effects on receptor function with the biggest increase at +70 mV representing a potentiation to 2.7 ± 0.3 times the steady-
state current level (n=6; Fig. 2C). Hence, TRPM8, like TRPV1 is intrinsically voltage-sensitive and its responsiveness will therefore be influenced by recent fluctuations in membrane voltage.

To specifically characterize the time- and voltage-dependence of the TRPM8-mediated ‘tail currents’ in detail we measured the relaxation kinetics exhibited by the ‘tail current’ upon repolarization to a series of negative potentials between -100 and -30 mV. Figure 2D shows a plot of the mean fast and slow rate-constants measured from individual bi-exponential curve fits to the individual current responses (n= 4). At all repolarization potentials between -100 and -30 mV, two similar time constants of around 5 and 30 ms were observed. These data reveal that the events mediating the time-dependent properties of TRPM8 observed upon depolarization and repolarization share broadly similar kinetics and hence may represent forward and reverse rates of the same mechanistic event. These data also highlight the influence of these properties in defining the non-instantaneous rectification behaviour of TRPM8 (Fig. 2E): At the approximate peak of the tail current (measured at 0.6 ms after the initiation of repolarization), the relationship between the tail current amplitude and the repolarization potential (i.e. the current-voltage relationship) was roughly linear whereas at the 200 ms timepoint (following tail current relaxation) the inward rectification observed for TRPM8 at steady-state is already apparent (Compare Fig 1D&E with Fig. 2E). This non-instantaneous rectification behaviour is therefore similar to that which we and others have previously described for TRPV1 and may have important implications for the gating and function of TRPM8 (see discussion).

**TRPM8 is not gated or modulated by acid.** Protons can act as direct activators and modulators of TRPV1 causing agonism with a pEC50 of ~5.3 (Gunthorpe et al., 2004a; Tominaga et al., 1998; Caterina and Julius, 2001). Furthermore, acid elicits significant potentiation of heat- or capsaicin-mediated activation of TRPV1 at sub-agonist concentrations (Caterina et al., 1997; Gunthorpe et al., 2004a; Tominaga et al., 1998). In contrast, TRPM8 mediated Ca\(^{2+}\) influx in response to menthol has been reported to be significantly decreased when the extracellular pH was maintained at 6.3 compared to 7.3, indicating acid-mediated inhibition (Behrendt et al., 2004). Since a measurement of Ca\(^{2+}\) influx into cells is an indirect method of studying channel function, we decided to use the patch-clamp technique to examine the effects of acid on TRPM8 more directly. We conducted experiments to assess the effects of reducing the extracellular pH from 7.3 to pH6.3 or pH5.3 and employed a protocol designed to
remove any contribution of the endogenous ASIC1a channels present in HEK293 cells by selective desensitization (See (Gunthorpe et al., 2001)) (Fig. 3A). In contrast to Behrendt et al., we found that TRPM8 responses to menthol exhibited little or no pH-dependence (Fig. 3B): The mean peak menthol-induced current was 104 ± 5% (n=5) or 94 ± 4% (n=9) of control at pH6.3 or pH5.3, respectively, and these values were not significantly different to control. Similarly, when we changed the extracellular pH from 7.3 to 5.3 during a sustained menthol application, only a slight reduction in the TRPM8 current was noted at either negative (-70 mV) or positive (+60 mV) membrane potentials (Fig. 3C), confirming our finding that TRPM8 shows little or no sensitivity to changes in extracellular pH.

**Ethanol inhibits TRPM8 function.** Ethanol has been shown to potentiate the response to chemical-(capsaicin, anandamide and acid)-mediated activation of TRPV1 and, through its effects on the heat-gating of TRPV1 effectively acts as an agonist of the receptor (Trevisani et al., 2002; Trevisani et al., 2004). Given the similarity in the rectification properties of these channels described above, we were therefore intrigued by the possibility that ethanol may also activate or modulate TRPM8. We found that co-application of 2% ethanol directly during a menthol stimulus strongly reduced the size of the menthol-evoked current (Fig. 4A). This effect occurred at both positive and negative potentials although we noted a slightly reduced effect at +60 mV. To characterise the effects of ethanol on TRPM8 in more detail, we used the same protocol as that employed in our antagonist studies and investigated the effects of concentrations ranging from 0.5-3% (Fig. 4B; concentrations which had no significant effect on the membrane current of wild type or TRPM8 expressing HEK293 cells, data not shown & Trevisani et al., 2002). Using this protocol, we found that ethanol caused a significant, concentration-dependent, inhibition of TRPM8 to 54 ± 7% (n =5), 19 ± 4% (n = 8) and 8 ± 2% (n = 4) of control, at concentrations of 0.5, 2 and 3%, respectively (Fig. 4C), in a fully reversible manner (Fig.4A & B). To gain insight into whether or not the affects of ethanol on the receptor were truly voltage-sensitive we established the current-voltage relationship of TRPM8 in the presence and absence of 2% ethanol (Fig. 4D). The paired data generated from these experiments indicates that ethanol does not cause a change in the reversal potential of the receptor (E_{rev} = -7.8 ± 1.0 and -8.4 ± 0.9 mV in the presence and absence of ethanol, respectively; p=0.5) but highlights a clear reduction (~40 %) in the modulatory effects of ethanol at positive membrane potentials, mimicking our previous experience with TRPV1 (Trevisani et al., 2002).
Common Structure-Activity-Relationships defining the ligand recognition properties of human TRPM8 and TRPV1. Since the cloning of TRPV1, and the realisation that it represents a good therapeutic target, there has been a huge expansion in the known pharmacology of this receptor (Appendino et al., 2003; Rami and Gunthorpe, 2004). In contrast, the more recently identified TRPM8, although an interesting therapeutic target in its own right (Peier et al., 2002), is rather poorly defined pharmacologically, and as yet, no selective antagonists have been reported. Recently, however, Behrendt et al., (2004) have described inhibition of mouse TRPM8 by the TRPV1 antagonists capsazepine (IC$_{50} = 18 \mu$M, as above), BCTC (IC$_{50} = 0.8 \mu$M) and thio-BCTC (IC$_{50} = 3.5 \mu$M). This finding was initially surprising, since apart from ruthenium red which appears to be a rather non-specific channel blocker of a number of TRP channels (Clapham, 2003), most of the TRPV1 ligands are reported to be selective over more closely related homologues such as TRPV2-4 (Gunthorpe et al., 2004b; Smith et al., 2002; Clapham, 2003; Gunthorpe et al., 2002). Based on this initial result of Behrendt and colleagues we therefore extended this pharmacological analysis to gain insight into the degree of overlap in the SAR of compounds at both receptors and ultimately define a compound that can distinguish between TRPV1 and TRPM8. In order to avoid the potential ambiguity that may occur due to non-specific effects on intracellular Ca$^{2+}$ concentration changes we applied whole-cell patch-clamp electrophysiology, rather than the FLIPR technique. We studied a selection of recently defined potent competitive TRPV1 antagonists (Fig. 5A) including BCTC (IC$_{50} = 35 \text{nM}$ vs rTRPV1; Valenzano et al., 2003) and a related piperazinyl urea compound ‘CTPC’ (see Appendino et al., 2003; (Dax et al., 2002). In an effort to identify a compound capable of discriminating between the two receptors we also widened our search to alternative TRPV1 templates such as the distinct TRPV1 chemical templates exemplified by SB-452533 (pA$_2 = 8.01$ (10 nM) vs hTRPV1; Rami et al., 2004) and SB-366791 (IC$_{50} = 6 \text{nM}$ vs hTRPV1; Gunthorpe et al., 2004b).

The protocol we employed for all pharmacological experiments is similar to that used to confirm the finding that the thio-urea capsazepine inhibited TRPM8 (Fig. 1B), and is shown in Figure 5B. Using this protocol we found that BCTC was a potent TRPM8 antagonist ($83 \pm 8\%$ inhibition at $1 \mu$M; n=5) with an IC$_{50}$ of $143 \pm 19 \text{nM}$, similar to its activity at TRPV1 (Fig. 5C, D). Similarly, the related piperazinyl urea CTPC was also a potent inhibitor of TRPM8 ($98 \pm 1 \%$ inhibition at $1 \mu$M; n=3) with an IC$_{50}$ of $131 \pm 14 \text{nM}$ (Fig. 5C, D) providing initial insight
into the SAR tolerated at this receptor site. The more structurally divergent urea compound SB-452533 also showed activity at TRPM8 with an IC$_{50}$ of 571 ± 77 nM (Figure 5C, D; 99 ± 1 %, inhibition at 10 µM, n=10) whereas the cinnamide SB-366791 showed little or no activity at TRPM8, even up to concentrations of 10 µM (14 ± 2 % inhibition, n=12; Fig 5C, D). These data with a range of competitive TRPV1 antagonists clearly demonstrate that there is a large degree of overlap in the TRPV1 and TRPM8 pharmacophores. Encouragingly, our data also show that the two receptor activities can be separated and exemplifies SB-366791 as a suitable antagonist for the further study of TRPV1.
Discussion

The properties of the human TRPM8 receptor that we have defined in our whole-cell patch clamp experiments are similar to those of the rat and mouse orthologues described by McKemy et al., and Peier et al., respectively (McKemy et al., 2002; Peier et al., 2002). Human TRPM8 is robustly activated by menthol and exhibits an outwardly rectifying current-voltage relationship with a reversal potential close to 0 mV, consistent with its behaviour as a non-selective cation channel (McKemy et al., 2002; Peier et al., 2002). It was during this initial characterisation that we also noted that TRPM8 exhibited the hallmarks of time- and voltage-dependent behaviour that we have previously described for TRPV1 (Gunthorpe et al., 2000). We therefore characterised the rectification properties of the TRPM8 receptor in detail to gain insight into whether these underlying features were conserved between these two distantly related temperature sensors.

**TRPM8 exhibits time- and voltage-dependent behaviour.** Our results demonstrate that TRPM8 exhibits clear non-instantaneous rectification behaviour (Fig. 1D&2): Steps to positive potentials effectively potentiated TRPM8 responses with kinetics in the order of 4-30 ms and, following a return to negative resting potentials, currents relaxed to their pre-stimulus levels resulting in the appearance of ‘tail currents’. The timecourse of the tail currents could be approximated with bi-exponential curves with a fast and a slow time constant which closely resembled the kinetic characteristics of the tail currents described for rTRPV1 (Ahern and Premkumar, 2002; Gunthorpe et al., 2000; Vlachova et al., 2003). These biexponential kinetics are also consistent with the occurrence of multiple TRPM8 open and/or closed states, a feature which as now been rigorously defined for TRPV1 through in depth single channel analysis (Hui et al., 2003; Liu et al., 2003). It therefore appears that although TRPV1 and TRPM8 are gated with opposite polarities in terms of temperature sensitivity, the underlying gating mechanism may be conserved. We hypothesize that this behaviour can be explained by a voltage-dependent inhibition of the channel at negative potentials that is effectively removed with depolarization, and simply reinstated with repolarization (Gunthorpe et al., 2000). This intrinsic property of the receptor, which may actually take the form of a separate structural domain/gate, is therefore likely to explain a significant part or all of the outward rectification behaviour of TRPM8. Interestingly, this should also endow the TRPM8 receptor with an ability to respond in a manner which reflects recent activity of the cell (i.e. responses will be influenced by recent
depolarisations mediated by TRPM8 or other mechanism) and show a degree of coincidence detection (Gunthorpe et al., 2000). Importantly, the presence of this unusual feature in the rectification profile in both channels indicates that the activation mechanisms of TRPM8 and TRPV1 might be more similar than expected based on a simple comparison of their primary amino acid sequence or their relative positions in the TRP superfamily tree (Clapham, 2003). Indeed, recent reports now shed further light on this possibility, demonstrating that the voltage-dependence of each channel’s activation is a key determinant of their intrinsic temperature sensitivity (Voets et al., 2004; Brauchi et al., 2004).

**TRPM8 is insensitive to changes in extracellular pH.** Recently, Behrendt et al., reported that menthol- and icilin-induced TRPM8 responses were strongly inhibited by acidification (Behrendt et al., 2004). We were intrigued by this finding, since it implies that the gating of TRPM8 and TRPV1 are subject to opposite control by protonation and has implications for the role of these channels in responses to local acidosis which can accompany inflammation (which may reach pH5.5; see (Reeh and Steen, 1996)). Since Behrendt et al used a Ca²⁺-based FLIPR assay, which is an indirect method of assessing channel function, we decided to use the patch-clamp technique to assess the effects of acid on TRPM8 in more detail. We found no significant effect of pH6.3 or even further acidification to pH5.3 on menthol-induced currents in human TRPM8 expressing HEK293 cells (Fig. 3) showing that TRPM8 is relatively insensitive to protons and suggesting that it does not play a key role in acid-evoked pain or inflammation. This finding is perhaps not that surprising since protons appear to be a somewhat specific regulator of TRPV1 rather than members of the TRP family in general (Clapham, 2003; Gunthorpe et al., 2002) and the key extracellular glutamate residue (E600) which has been shown to play a role in the acid-mediated potentiation of TRPV1 (Jordt et al., 2000) is not conserved in TRPM8 (McKemy et al., 2002; Peier et al., 2002; Tsavaler et al., 2001). Indeed, our data are consistent with the recent findings of Andersson et al., (2004) who find no evidence for the regulation of menthol-evoked mouse TRPM8 responses by extracellular acidification. However, Andersson et al., have shown that cold and icilin responses are affected by acidification but that this is most likely due to changes in intracellular pH, suggesting unforeseen complexity in the different transduction mechanisms leading to TRPM8 activation. Given this apparent complexity, whether or not (patho)physiological TRPM8 function *in vivo* is influenced by changes in pH remains to be fully determined.
Ethanol inhibits TRPM8 function. We have previously demonstrated that ethanol (0.1%-3%) causes marked potentiation of TRPV1 channels gated by capsaicin, protons and heat which results from its profound ability to lower the heat threshold for TRPV1 activation (Trevisani et al., 2002). Given the similarities in the rectification and gating behaviour of TRPM8 and TRPV1 we were intrigued by the possibility that TRPM8 may also be modulated by ethanol. We found that ethanol effectively inhibited TRPM8 function in a concentration-dependent fashion yielding ~50% reduction in menthol-evoked currents at 0.5% and almost complete inhibition at 3% ethanol (Fig. 4). Although these findings are in contrast to the potentiating effects that we noted for ethanol on TRPV1 (Trevisani et al., 2002), and were initially perplexing, they can perhaps be rationalised based on evidence which demonstrates that the gating mechanisms of TRPV1 and TRPM8 are highly conserved (see above; Brauchi et al., 2004; Voets et al., 2004). Our hypothesis is that a shared sensitivity to ethanol is therefore consistent with the conservation of the underlying gating machinery, and the direction of the effect (i.e. potentiation or inhibition) may simply relate to the differential tuning of the receptors to respond to heating or cooling, respectively.

This hypothesis fits well with the recent data of Voets et al., (2004) which links the temperature-sensitive properties of TRPM8 and TRPV1 to their voltage-dependence. Crucially, the opening and closing rates of TRPM8 and TRPV1 show opposite temperature-dependence such that, in the two state model presented, only the opening rate of TRPV1 or the closing rate of TRPM8 appears to be strongly temperature-dependent (Voets et al., 2004). These features effectively govern the gating properties of the receptors which can be considered to open in response to graded shifts in their voltage-dependent activation curves such that closing of TRPM8 and opening of TRPV1 are promoted by heat. If our hypothesis is correct, then ethanol may effectively replicate the action of heat on the receptors by affecting the same transitions that are now known to be temperature sensitive. This predicts that the effects of ethanol should also be intertwined with the voltage-dependent gating properties of the receptors. Indeed, in our studies completed to date we do find preliminary evidence of a voltage-dependence to ethanol action. The effects of ethanol on TRPV1 were more marked at negative potentials effectively being overcome and less striking at positive potentials (Trevisani et al., 2002). Similarly, inhibition of TRPM8 is also reduced at positive potentials (Fig. 4). Further work is now required to demonstrate that ethanol can alter the state of the receptor in the same way as heat and hence mirror the oppositely geared temperature-dependence that these receptors have.
evolved. A more detailed understanding the gating mechanisms of these channels in terms of their multiple closed and open states (Brauchi et al., 2004; Hui et al., 2003; Liu et al., 2003) would also aid further insight into the mechanism by which ethanol can modulate these and perhaps other TRP channels.

Given the clear effects of ethanol on TRPM8 receptor function, it is tempting to speculate on the relevance of this for understanding the effects of ethanol on the body from clinical and personal experience. This is difficult given the large number of receptors which are now known to be affected by ethanol combined with our relatively poor understanding of the biology of TRPM8. In the limited number of studies to date we note that, due to its rather lipophilic properties, most menthol treatments and medications used on patients are ethanol-based solutions with typical ethanol concentrations of more than 60% (Pittler and Ernst, 1998; Wasner et al., 2004; Yosipovitch et al., 1996). It is also noteworthy that ethanol is often used as a vehicle for capsaicin and menthol and hence may complicate the results of experiments with these ligands or in systems where thermoTRPs are either present and/or studied. Clearly past data could be re-interpreted and the design of future experiments improved based on a greater appreciation of the effects of ethanol on these receptors. Similarly, since TRPM8 is likely to be responsible for the effects of a number of known plant-derived or synthetic cooling agents (e.g. icilin, eucalyptol, WS3, cooling agent 10) which are used in the food and cosmetics industry, their combination with ethanol may be usefully reconsidered. However, since these agents are also likely to have effects via other receptor systems this may need to proceed on an empirical basis.

**Significant overlap in the ligand recognition properties of human TRPM8 and TRPV1.** As a polymodal detector of noxious chemical and physical stimuli, TRPV1 represents an exciting target for the development of novel therapeutic agents (Appendino et al., 2003; Rami and Gunthorpe, 2004). To date, several classes of compounds have been identified that show biological activity at TRPV1 receptors, some of which are highly potent and selective (Rami and Gunthorpe, 2004). In contrast, the more recent cloning of TRPM8 means that its pharmacological characterisation lags behind that of TRPV1, and no selective antagonists of this receptor have been reported to date. The discovery that the TRPV1 antagonists capsazepine, BCTC and thio-BCTC are all (albeit fairly weak) inhibitors of the mouse TRPM8 receptor was therefore a key development (Behrendt et al., 2004) paving the way for the
identification of more potent and selective small molecule antagonists as well as compounds which, perhaps for therapeutic benefit, target multiple TRP channels. Our electrophysiological studies now confirm and extend these findings. We find that BCTC and capsazepine are in fact more potent human TRPM8 antagonists than expected from the work of Behrendt et al.. This may reflect an underestimate in the potency derived from the FLIPR-Ca$^{2+}$ based studies of Behrendt et al. or a species difference in potency of BCTC, capsazepine and other antagonists versus the rodent and human TRPM8 orthologues. What is clear is that these TRPV1 antagonists show little separation between their activity at TRPV1 and TRPM8 making the effects of these compounds in vitro and in vivo more difficult to interpret with accuracy.

In an attempt to identify compounds with a greater ability to discriminate between TRPM8 and TRPV1 we profiled further TRPV1 antagonists which have been described in the literature. We found that CTPC, a related piperazinyl urea of BCTC (Dax et al., 2002) was also a potent TRPM8 antagonist. Indeed, its IC$_{50}$ of 131±14 nM, means that it is effectively equipotent to BCTC (IC$_{50}$=143±19 nM; Fig. 5) and hence these molecules represent the most potent human TRPM8 antagonists reported to date. This result also confirms that TRPM8 activity is not readily removed by simple modification of this chemical template. This conclusion is also supported by the clear TRPM8 activity of the more divergent urea template exemplified by SB-452533 (IC$_{50}$=571±77 nM) demonstrating that a large number of (thio)urea based compounds are TRPM8 antagonists and that there is a large degree of overlap in the TRPV1 and TRPM8 pharmacophores. In this respect it is therefore pertinent that in moving away from the (thio)ureas, the cinnamide SB-366791, which is a well defined potent and selective TRPV1 antagonist (Gunthorpe et al., 2004b), was the first compound which we have identified which shows little or no activity versus TRPM8 (Fig. 5). This therefore defines SB-366791 as a selective tool antagonist for the study of TRPV1 and, by virtue of separating the two receptor activities, holds promise for the identification of antagonists selective for TRPM8 and indeed other TRPs.

In conclusion, we have provided functional and pharmacological evidence for a striking level of conservation in the functional properties of the distantly related TRPM8 and TRPV1 channels. In particular, the clear time- and voltage-dependent properties exhibited by TRPM8 and their modulation by ethanol suggests that the gating properties of these receptors are much more similar than initially envisaged. Whilst demonstrating the significant overlap in the
pharmacological Structure-Activity-Relationship (SAR) between the TRPM8 and TRPV1 receptors we have also identified a number of potent antagonists of the human TRPM8 receptor and exemplified SB-366791 as a selective tool antagonist for the study of TRPV1. All of these findings should provide new impetus to the further understanding of the biological functions of TRPM8.
Reference List


Fig. 1. Electrophysiological characterization of menthol-induced currents in HEK293 cells expressing human TRPM8. A, A representative recording of a whole-cell current recorded in response to menthol (100 µM; black bar, 10 sec duration) at a holding potential of -70 mV. B, Repeated menthol applications resulted in little or no TRPM8 receptor desensitisation or tachyphylaxis. C, Capsazepine (10 µM) robustly inhibited TRPM8 in a reversible manner (94 ± 2 % inhibition, n=5) whereas ruthenium red (10 µM) caused little or no inhibition of TRPM8 (11 ± 9 %, n = 4, p=0.2). D, The current-voltage relationship of TRPM8 was established using a voltage-ramp protocol (-70 to +70 mV, 1s) which is defined in the upper panel. The voltage-ramp was timed to coincide with the steady state of a menthol-evoked response (100 µM menthol application was triggered at the time indicated by the arrow head) and currents were recorded prior to (Control), during (Menthol) and following agonist application (Wash). Net menthol-gated currents (lower panel) for each cell were calculated by subtracting the control background current from the menthol-evoked current. E, A plot of the current-voltage relationship for TRPM8 derived from pooled normalized data from 10 experiments similar to and including that in D. Currents at each holding potential were normalized to the current recorded at –70 mV prior to averaging across cells. Error bars (± 1 S.E.M) are shown at 10 mV intervals.

Fig. 2. Time-dependent components of TRPM8 rectification. A(i), An example of a whole-cell recording of the response to a single voltage-step illustrating the time-dependent properties of TRPM8. The lower trace shows the voltage command with a 300 ms voltage step from –70 mV to + 70 mV. The upper trace depicts three current traces which were collected in the following order: control, 100 µM menthol and wash. The voltage step was applied during the steady-state non-desensitizing phase, approximately 5 seconds after onset of the menthol response. The arrows indicate onset of the time-dependent behaviour of the current in response to the instantaneous change in membrane voltage. This can be seen as a slowly increasing current after depolarization and as an overshoot inward 'tail current' following repolarization to –70 mV from positive potential. A(ii) illustrates in more detail the activation kinetics of the current evoked by a depolarizing voltage step from -70 to +70 mV. The exponentially rising component (upper trace) can be fitted by a bi-exponential curve with time constants of 31.4 ± 5.4 and 4.0 ± 1.0 ms with the fast time constant giving rise to 60.4 ± 10.2 % of the response. A(iii) exemplifies the kinetics of the 'tail current' in response to a repolarizing voltage step from
+70 to -70 mV. The traces show the net menthol current which was determined by subtraction of the control current from the menthol-gated current. The 'tail current' (lower trace) was also well described by a bi-exponential fit with time constants of 30.1 ± 0.7 and 5.8 ± 0.3 ms of approximately equal weight (fast constant weight = 51.9 ± 0.9 % of the response). B, The degree of potentiation of TRPM8 attainable following a range of step depolarisations was studied using a voltage protocol (lower trace) containing a series of 300 ms step depolarizations to test potentials between +70 and 0 mV. The trace shown is the net menthol-gated component of the current response recorded following subtraction of background leak currents and demonstrates the clear occurrence of 'tail currents' at all potentials tested. C, a graph comparing the magnitude of the 'tail current' observed at −70 mV following step depolarizations to test potentials between 0 and +70 mV. The data shown are pooled from six experiments similar to and including that shown in B, and were normalized to the steady-state menthol-evoked current at −70 mV. D, kinetic analysis of the tail currents elicited by repolarization from +70 mV to a range of negative test potentials between -100 and -10 mV. All tail current trajectories were best fitted with a bi-exponential function with a slow time constant of around 30 ms and a fast time constant of around 5 ms, as indicated by the dotted lines which represent the mean of these data. E, graph plotting instantaneous current-voltage relationships constructed for tail current amplitudes measured at either 0.6 ms (squares) or 200 ms (triangles) following the initiation of the repolarizing voltage step.

Fig. 3. Extracellular acidification does not modulate TRPM8 function. A, The effect of acidification to pH5.3 on menthol-evoked TRPM8 currents was assessed following initial desensitisation of any ASIC-mediated current present in the HEK293 cells (see text). In the exemplar trace shown, two control menthol pulses were recorded at pH7.3 (standard extracellular solution) followed by a further menthol response recorded at pH5.3. The final response was recorded at pH7.3 allowing the stability of the recording to be assessed and any reversal of effects to be determined. The arrowhead indicates the activation of endogenous ASIC1a channels in this cell. The 40s pH5.3 pre-treatment time used is sufficient to desensitise the ASIC and remove its contribution from the peak of the menthol response recorded at pH5.3. B, Pooled, normalized data showing the effect of extracellular pH on menthol-gated currents from experiments similar to and including that shown in A. C, The effects of extracellular acidification from pH7.3 to 5.3 were also examined during a sustained menthol application. These experiments revealed only a slight reduction in the TRPM8 current at either negative (-
70 mV) or positive (+60 mV) membrane potentials confirming that TRPM8 shows little or no sensitivity to large changes in extracellular pH.

**Fig. 4.** Ethanol causes inhibition of TRPM8. A, Representative whole-cell recording illustrating the design of the experiment used to examine the effects of ethanol on menthol-gated TRPM8 currents. Menthol (100 μM, 10s) evoked responses in HEK293.hTRPM8 cells which were clearly inhibited by co-application of 2 % ethanol at both negative (-70 mV; 91 ± 4% inhibition, n=8) and positive (+60 mV; 83 ± 5% inhibition, n=8; significantly different to the level of inhibition obtained at -70 mV; Student’s paired t test, p < 0.001) membrane potentials in a reversible fashion. Application of ethanol alone (0.5 - 3 %) did not evoke any significant changes in response current in either WT or TRPM8 expressing cells (data not shown). B, C, The inhibitory effects of ethanol were studied in more detail using a conventional antagonist protocol and show a clear concentration-dependence over the range of 0.5-3% ethanol yielding 54 ± 7 % (n =5), 19 ± 4 % (n = 8) and 8 ± 2 % (n = 4) inhibition in the presence of 0.5, 2 and 3 % ethanol, respectively. Reduction of current at all three ethanol concentrations was significant compared to control (p < 0.01). D, Current-voltage relationships in response to menthol generated using a voltage ramp protocol (-70 to +70 mV, 1s) in the presence and absence of 2% ethanol.

**Fig. 5.** Overlap in the ligand recognition properties of TRPM8 and TRPV1. A, A comparison of the chemical structures of the TRPV1 antagonists capsazepine, BCTC, CTPC, SB-452533 and SB-366791. B, Exemplar traces from experiments conducted to assess the antagonistic activity of the TRPV1 antagonists shown in A versus TRPM8. BCTC (1 μM) caused almost complete inhibition of TRPM8, whereas SB366791 at a concentration of 10 μM had little or no effect. C, Pooled data quantifying the level of TRPM8 inhibition noted (at the highest concentration tested) for BCTC (1 μM; 83 ± 8%, n=5), CTPC (1 μM; 98 ± 1 %, n=3), SB-452533 (10 μM; 99 ± 1 %, n=10), SB-366791 (10 μM; 14 ± 2 %, n=12) and capsazepine (10 μM; 94 ± 2 %, n=5) from experiments similar to and including those in B. D, graph highlighting the concentration-response data generated for BCTC (open circles; IC\textsubscript{50}= 143 ± 19 nM, n=4-5 per concentration), CTPC (triangles; IC\textsubscript{50}=131 ± 14 nM (n=3-9 per concentration) and SB452533 (squares; IC\textsubscript{50}=571 ± 77 nM, 4-6 per concentration); the effects of SB366791 are represented by a single data point (filled circle; n=12) at the test concentration of 10 μM.


Figure 1
Figure 2
Figure 3

A. 100 µM Menthol pH 5.3

B. Normalized Current (%)

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<th>pH</th>
<th>Normalized Current (%)</th>
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C. 100 µM Menthol pH 5.3

- $V_{\text{hold}}$ +60mV
- $V_{\text{hold}}$ -70mV

2 nA

500 pA

2 s
Figure 4

Panel A: Graph showing the effect of 100 µM Menthol and 2% Ethanol on membrane currents with holding potentials of +60 mV and -70 mV.

Panel B: Graph showing the effect of 100 µM Menthol and 2% Ethanol on membrane currents over time.

Panel C: Bar graph showing normalized current (%) for Control, 0.5%, 2%, and 3% concentrations with significance levels indicated.

Panel D: Graph showing the relationship between voltage (mV) and current (nA) with 2% Ethanol and control conditions.

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Figure 5

A) Chemical structures of Capsazepine, BCTC, CTPC, SB-452533, and SB-366791.

B) Current traces showing the effect of 100 µM menthol and 1 µM BCTC on the ion channel.

C) Bar graph showing normalized current (%).

D) Dose-response curves for the effects of various concentrations of SB-366791, BCTC, and Capsazepine.

The figure illustrates the pharmacological properties of different compounds on an ion channel model.