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Conservation of Functional and Pharmacological Properties in the Distantly Related Temperature Sensors TRPV1 and TRPM8

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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. Because of 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 transient receptor potential melastatin 8 (TRPM8) and compared its behavior with that of its distant relative, the heat-sensitive capsaicin-gated transient receptor potential vanilloid 1 (TRPV1). It is remarkable to find that TRPM8, in addition to its behavior as an outwardly rectifying, nonselective cation channel, shares many functional and pharmacological properties with TRPV1. TRPM8 exhibits prominent time- and voltage-dependent behavior, a property that 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, N-(4-tertiarybutyl-phenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide (BCTC), (2R)-4-(3-chloro-2-pyridinyl)-2-methyl-N-[4-(trifluoromethyl)phenyl]-1-piperazinecarboxamide (CTPC), and N-(2-bromophenyl)-N'-{2-[ethyl(3-methylphenyl)amino]ethyl}-urea (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 N-(3-methoxyphenyl)-4-chlorocinnamide (SB-366791), at least, represents an apparently selective antagonist for TRPV1 suggests that this goal is attainable.

The transient receptor potential (TRP) superfamily of ion channels, of which there are now more than 25 members, seems to have evolved to subserve a diverse array of cellular processes (Clapham, 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 thermo-TRPs; 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 (McKemy et al., 2002; Benham et al., 2003; 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 nonselective cationic behavior and also potentially defines the outwardly rectifying current-voltage profile noted for many of these channels (Caterina et al., 1997, 1999; Gunthorpe et al., 2002; McKemy et al., 2002; Peier et al., 2002; Clapham, 2003). Because of their roles as cellular sensors, many TRPs represent good targets for therapeutic intervention. Hence, much recent work has focused on the functional and pharmacological characterization of these channels with a view to identifying selective tools with which to better validate their roles in health and disease and provide starting points for drug discovery.

ABBREVIATIONS: TRP, transient receptor potential; TRPM8, transient receptor potential melastatin 8; TRPV1, transient receptor potential vanilloid 1; FLIPR, fluorometric imaging plate reader; PCR, polymerase chain reaction; h, human; r, rat; SAR, structure-activity relationship; HEK, human embryonic kidney; 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.

TRPV1 (previously named vanilloid receptor-1) was the first heat-activated ion channel to be cloned. It has an activation threshold ($\sim$43°C) in the noxious heat range (Caterina et al., 1997) and was the defining member of the TRPV family (Gunthorpe et al., 2002). TRPV1 is predominantly expressed in peripheral sensory neurons of the dorsal root and trigeminal ganglia and is a truly polymodal receptor; it can be activated or modulated by chemical ligands like capsaicin (the "hot" ingredient of chili peppers), protons (acidic pH), the endocannabinoid anandamide, and ethanol (Caterina et al., 1997; Tominaga et al., 1998; Gunthorpe et al., 2002; Trevisani et al., 2002). TRPV1 is therefore a key sensory transducer in the pain pathway and is the subject of intense research to develop novel therapeutic agents that may have use for the treatment of pain, migraine, asthma, and a range of other indications (Appendino et al., 2003; Rami and Gunthorpe, 2004). Three other close relatives of TRPV1 have now also been shown to act as thermosensors with thresholds extending from the warm ($\sim$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 $\sim$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 (McKemy et al., 2002; Clapham, 2003; Patapoutian et al., 2003). TRPM8 can now also be considered to be a polymodal receptor because its activity is also controlled by a range of diverse chemical and physical stimuli. TRPM8 exhibits an activation threshold in the range of 22 to 27°C (McKemy et al., 2002; Peier et al., 2002), is gated by a range of synthetic "cooling" compounds such as icilin and eucalyptol (McKemy et al., 2002; Peier et al., 2002; Behrendt et al., 2004), and is reportedly inhibited by acid (Behrendt et al., 2004).

TRPM8, like TRPV1, is also expressed in sensory neurons in the peripheral nervous system, especially in dorsal root, trigeminal, and nodose ganglia (McKemy et al., 2002; Peier et al., 2002; Reid et al., 2002; Reid and Flonta, 2002; Nealen et al., 2003; Thut et al., 2003; Zhang et al., 2004). This, combined with clinical reports that document the beneficial effects of menthol treatment in patients suffering from postherpetic neuralgia and irritable bowel syndrome (Pittler and Ernst, 1998; Davies et al., 2002), suggests that TRPM8 may also be an exciting new target for the development of novel analgesics and 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 article 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 nonselective) tools with which to study TRPM8 function.

With a view to gaining further insight into the functional and pharmacological properties of human TRPM8, we used the whole-cell patch-clamp technique to study this receptor in detail and compared its behavior 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 compared with TRPM8 provides encouraging evidence that selective compounds for these thermo-TRP channels can be developed.

Materials and Methods

Cloning and Expression of Human TRPM8. hTRPM8 was cloned by PCR-based methods using TRPM8-specific primers derived from the published sequence described by Tsavaler et al. (2001). Because full-length hTRPM8 amplification proved to be problematic, hTRPM8 was amplified in two fragments that overlapped a unique EcoRI restriction site (bases 1579 to 1584, AY090109). Fragment 1 was amplified by nested PCR with human prostate Marathon-Ready cDNA (BD Biosciences Clontech, Palo Alto, CA) using the following primers: first-round reaction, 5′-GTCCGCCCACGCGTCCG-3′ and 5′-GGCCAGAGTCTTCAGAAGCTTGC-3′; and nested reaction, 5′-CACCATGTCCTTTCGGGCAGCCAGGC-3′ and 5′-CGAGTAATAG-GAGACACGTCGTGG-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, San Jose, CA] using the following primers: 5′-CAGTGAGCAAGACAAG-GATAACTGG-3′ and 5′-CAGTTTTATTTGATTTTATTAGC-3′. PCR products were visualized on 1% agarose gels and purified using the Qiaquick PCR purification kit (QIAGEN, Valencia, CA) before being subcloned into pcDNA3.1D/V5/His-TOPO or pcDNA3.1/V5/His-TOPO (Invitrogen, Carlsbad, CA). The resulting plasmids were transformed into chemically competent TOP10 cells (Invitrogen). Plasmid DNA was prepared using the Qiaprep Spin MiniPrep Kit (QIAGEN). After confirmation of the hTRPM8 sequence by full-length double-stranded sequencing, fragments 1 and 2 were isolated by digestion with EcoRI and EcoRV and were used to generate the full-length human TRPM8 coding sequence by ligation using the Ligafast T4 DNA ligase system (Promega, Madison, WI). The integrity of the full-length TRPM8 sequence was verified by comparison with the National Center for Biotechnology Information's published sequences (GenBank 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 article differs from the original trp-p8 (AY090109) sequence (Tsavaler et al., 2001) by one amino acid change (Ile58 to threonine). This amino acid change is also encoded by the sequences deposited as GenBank accession numbers AY328400, AX267803, and AB061779 and human genomic DNA.

Human embryonic kidney (HEK) 293 cells were transfected using Lipofectamine Plus (Invitrogen) 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 Eagle's medium with Earle's salts and supplemented with 10% fetal bovine serum, nonessential amino acids, and 0.2 mM L-glutamine while being maintained under 5% CO_2 at 37°C. For electrophysiological experiments, cells were plated at a density of 30,000 cells/cm^2 onto 19-mm glass coverslips coated with poly-L-lysine with experiments being performed 24 to 72 h thereafter.

Whole-Cell Patch-Clamp Electrophysiology. Electrophysiological studies were conducted according to methods described previously (Hayes et al., 2000; Trevisani et al., 2002; Gunthorpe et al., 2004b). All recordings were performed at room temperature (20–24°C) using an Axopatch 200B amplifier controlled via the pClamp8/pClamp9 software suite (Axon Instruments Inc., Union City, CA) using standard whole-cell patch-clamp methods. The recording chamber was continuously perfused with extracellular solution consisting of 137 mM NaCl, 5.4 mM KCl, 10 mM D-glucose, 10 mM HEPES, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, adjusted to pH 7.3 with NaOH. Patch pipettes (resistance, 2–5 $M\Omega$) were fabricated on a Sutter instruments P-87 electrode puller (Sutter Instrument Company, Novato, CA) and filled with intracellular solution of the following composition: 140 mM CsCl, 10 mM EGTA, 2 mM MgATP, 10 mM HEPES, and 1 mM $CaCl_2$, adjusted to pH 7.4 with 1 M CsOH. Antagonist solutions were prepared as 10 mM stock solutions in dimethyl sulfoxide before dilution in extracellular solution. To minimize problems arising from the poor solubility of menthol, it was prepared as a 100 mM stock solution in ethanol before dilution in extracellular solution (final ethanol concentration = 0.1%) (Reid and Flonta, 2002). For studies examining the effect of acid on TRPM8, the pH of the extracellular solution was adjusted to 6.3 or 5.3 by the addition of HCl. Data were acquired at 8.3 to 10 kHz and filtered at 2 to 5 kHz. Series resistance compensation of up to 80% was used where appropriate. Agonists and antagonists were applied to cells in a controlled fashion using an automated fast-switching solution exchange system (SF-77B; Warner Instrument, Hamden, CT).

Data Analysis. Data were analyzed using the Clampfit (Axon Instruments), Excel (Microsoft, Redmond, WA), and Origin (Origin LabCorp, Northampton, MA) 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, Essex, UK) (chemical structures are depicted in Fig. 5). All other compounds and cell-culture media were obtained from Sigma (Gillingham, UK) and Tocris Cookson Inc. (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. Because the rat and mouse orthologs of TRPM8 have been reported to be activated by menthol (McKemy et al., 2002; Peier et al., 2002) in a fashion similar to that first described for the native receptor expressed in dorsal root ganglion neurons (Reid and Flonta, 2001, 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 deactivated upon cessation of the menthol application (Fig. 1A). These effects of menthol were specific to TRPM8 because 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 nonselective TRPV poreblocker 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. 1, B and C), confirming the pharmacological profile expected for this receptor (Behrendt et al., 2004).
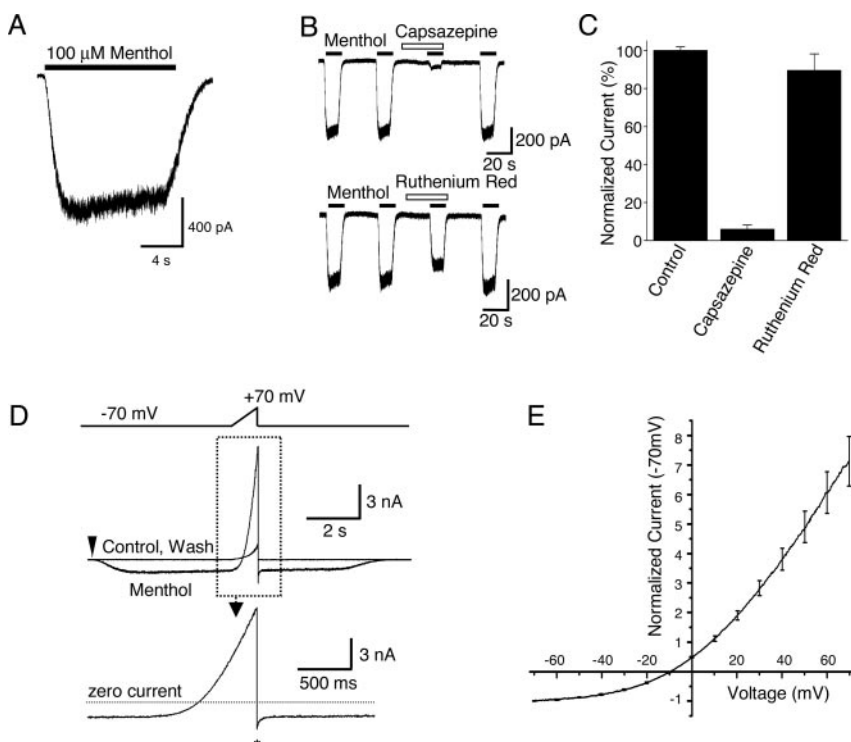

Fig. 1. Electrophysiological characterization of menthol-induced currents in HEK293 cells expressing human TRPM8. A, a representative recording of a whole-cell current recorded in response to menthol (100 μM; ■, 10-s duration) at a holding potential of −70 mV. B, repeated menthol applications resulted in little or no TRPM8 receptor desensitization 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, 1 s), which is defined at the top. 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 arrowhead), and currents were recorded before (control), during (menthol), and after agonist application (wash). Net menthol-gated currents (bottom) 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 shown in D. Currents at each holding potential were normalized to the current recorded at −70 mV before averaging across cells. Error bars (± 1 S.E.M.) are shown at 10-mV intervals.

Rectification Properties of TRPM8. To evaluate the rectification properties of human TRPM8, we carried out a series of voltage-ramp experiments (Fig. 1, D and E). Voltage-ramps from −70 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 orthologs of the receptor (McKemy et al., 2002; Peier et al., 2002) and many other TRP channels (Gunthorpe et al., 2002; Clapham, 2003).

TRPM8 Exhibits Time- and Voltage-Dependent Behavior. 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 noninstantaneous component of rectification, a property which may underlie the gating behavior of TRPV1 (Gunthorpe et al., 2000; Ahern and Premkumar, 2002; Vlachova et al., 2003). To determine whether TRPM8 shows similar behavior, we therefore carried out a series of voltage-step experiments to characterize the rectification properties of TRPM8 in more detail. The response to a single voltage step from −70 to +70 mV during menthol application is shown in Fig. 2A and highlights a clear noninstantaneous, exponentially rising, component of TRPM8 receptor rectification (Fig. 2Ai, closed arrow). The rising phase was best fitted with a biexponential curve (Fig. 2Aii) with time constants (tau) of 4.0 ± 1.0 and 31.4 ± 5.4 ms, 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. 2Ai, 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 "disinhibited state" of TRPM8 was also well described by a biexponential 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. 2Aii).

To examine the effect of the degree of depolarization on the level of disinhibition of TRPM8 attainable, we measured the amplitude of the tail current that arises upon repolarization after 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 behavior (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 biexponential curve fits to the individual current responses (n = 4). At all repolarization potentials between −100 and −30 mV, two similar time constants of approximately 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 noninstantaneous rectification behavior 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 time point (after tail current relaxation), the inward rectification observed for TRPM8 at steady state is already apparent (compare Fig. 1, D and E, with Fig. 2E). This noninstantaneous rectification behavior is therefore similar to that which we and others have described previously 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 pEC$_{50}$ of ~5.3 (Tominaga et al., 1998; Caterina and Julius, 2001; Gunthorpe et al., 2004a). Furthermore, acid elicits significant potentiation of heat- or capsaicin-mediated activation of TRPV1 at subagonist concentrations (Caterina et al., 1997; Tominaga et al., 1998; Gunthorpe et al., 2004a). 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 with 7.3, indicating acid-mediated inhibition (Behrendt et al., 2004). Because 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 6.3 or 5.3 and used a protocol designed to remove any contribution of the endogenous ASIC1a channels present in HEK293 cells by selective desensitization (Gunthorpe et al., 2001) (Fig. 3A). In contrast to the findings of Behrendt et al. (2004), 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 pH 6.3 or 5.3, respectively, and these values were not significantly different from those of control. Likewise, 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, it effectively acts as an agonist of the receptor (Trevisani et al., 2002, 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 coapplication 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 characterize the effects of ethanol on TRPM8 in more detail, we used the same protocol as that used in our antagonist studies and investigated the effects of concentrations ranging from 0.5 to 3% (Fig. 4B) (concentrations which had no significant effect on the membrane current of wild-type or TRPM8-expressing HEK293 cells, data not shown and Trevisani et al., 2002).
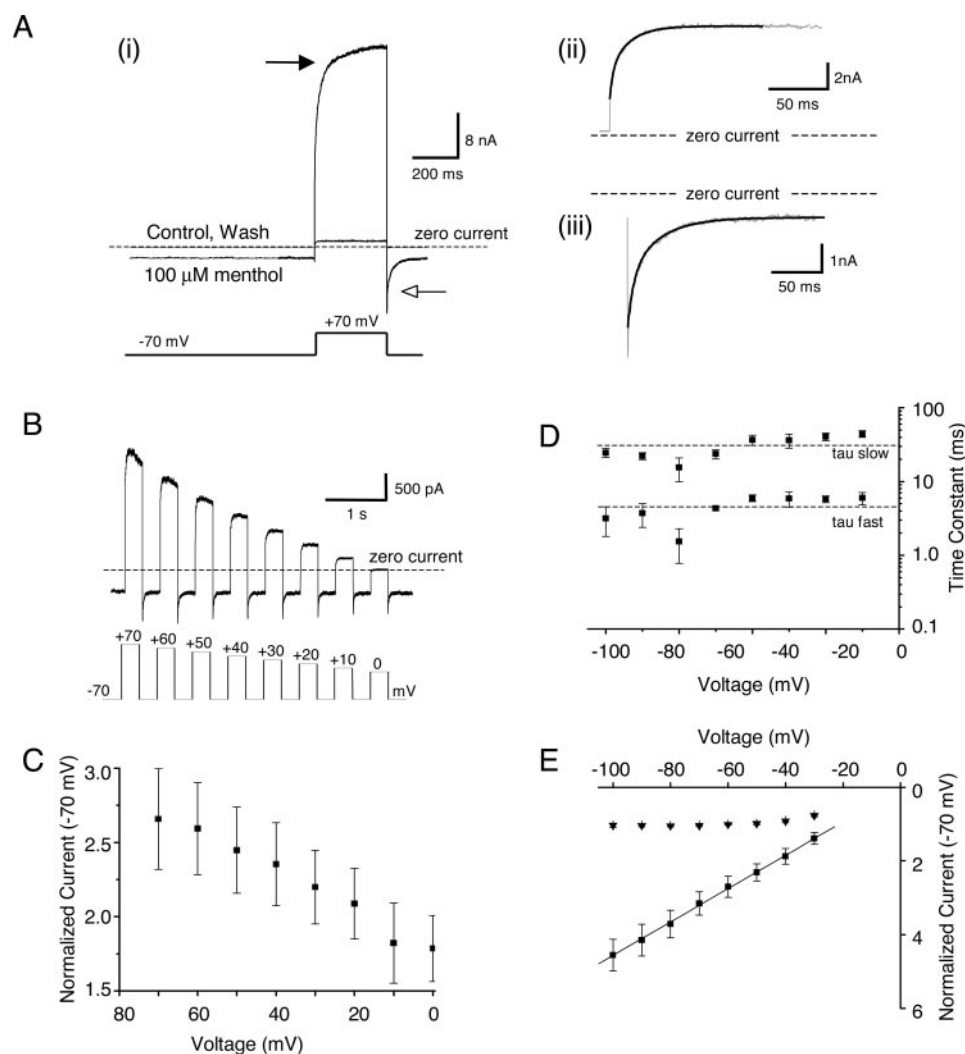
Fig. 2. Time-dependent components of TRPM8 rectification. Ai, an example of a whole-cell recording of the response to a single voltage step illustrating the time-dependent properties of TRPM8. The lower trace shows the voltage command with a 300-ms voltage step from −70 to +70 mV. The upper trace depicts three current traces that were collected in the following order: control, 100 μM menthol, and wash. The voltage step was applied during the steady-state nondesensitizing phase, approximately 5 s after the onset of the menthol response. The arrows indicate onset of the time-dependent behavior 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 after repolarization to −70 mV from positive potential. Aii, the activation kinetics of the current evoked by a depolarizing voltage step from −70 to +70 mV are shown in more detail. The exponentially rising component (top trace) can be fitted by a biexponential 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. Aiii, 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 (bottom trace) was also well-described by a biexponential 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 after a range of step depolarizations was studied using a voltage protocol (bottom 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 after 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 after 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 biexponential function with a slow-time constant of approximately 30 ms and a fast-time constant of approximately 5 ms, as indicated by the broken 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 (■) or 200 ms (▼) after the initiation of the repolarizing voltage step.

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. 4, A and B). To gain insight into whether 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 indicate that ethanol does not cause a change in the reversal potential of the receptor ($E_{rev} = -7.8 \pm 1.0$ 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 realization 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. Behrendt et al. (2004), however, have recently described the 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, because apart from ruthenium red, which seems to be a rather nonspecific channel blocker of a number of TRP channels (Clapham, 2003), most of the TRPV1 ligands are reported to be selective over more closely related homologs such as TRPV2–4 (Gunthorpe et al., 2002, 2004b; Smith et al., 2002; Clapham, 2003). 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. To avoid the potential ambiguity that may occur because of nonspecific 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 ($IC_{50} = 35$ nM versus rTRPV1) (Valenzano et al., 2003) and a related piperazinyl urea compound "CTPC" (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) versus hTRPV1; Rami and Gunthorpe, 2004) and SB-366791 ($IC_{50} = 6$ nM versus hTRPV1; Gunthorpe et al., 2004b).

The protocol we used for all pharmacological experiments is similar to that used to confirm the finding that the thiourea capsazepine inhibited TRPM8 (Fig. 1B) and is shown in Fig. 5B. Using this protocol, we found that BCTC was a potent TRPM8 antagonist (83 ± 8% inhibition at 1 μM; $n = 5$) with an IC_{50} value of 143 ± 19 nM, similar to its activity at TRPV1 (Fig. 5, C and D). Likewise, the related piperazinyl urea CTPC was also a potent inhibitor of TRPM8 (98 ± 1% inhibition at 1 μM; $n = 3$) with an IC_{50} value of 131 ± 14 nM (Fig. 5, C and 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 (Fig. 5, C and 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. 5, C and D). These data, with a range of competitive TRPV1
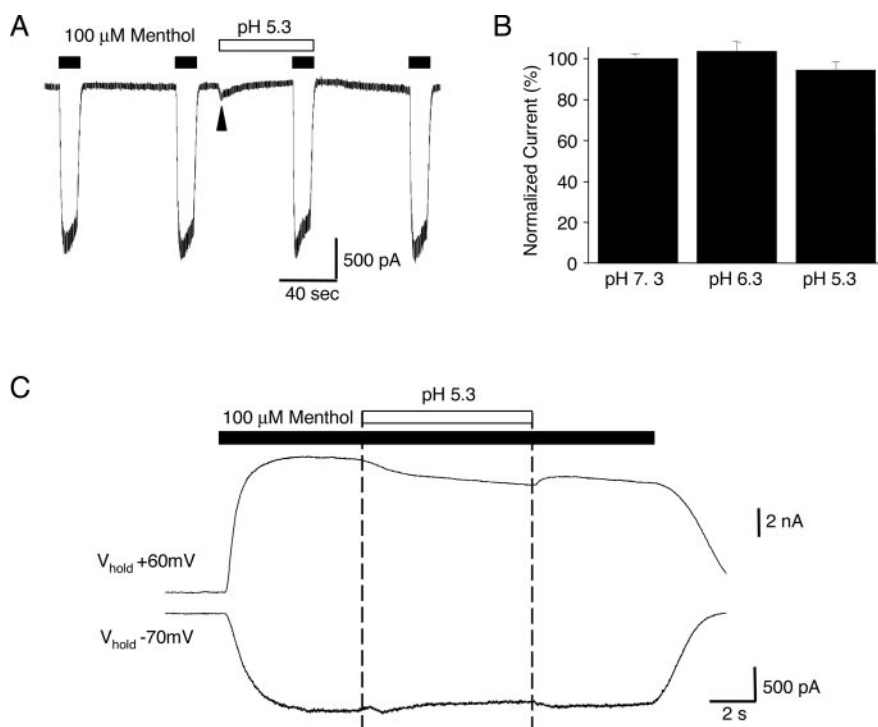
Fig. 3. Extracellular acidification does not modulate TRPM8 function. A, the effect of acidification to pH 5.3 on menthol-evoked TRPM8 currents was assessed after initial desensitization of any acid-sensing ion channel-mediated current present in the HEK293 cells (see text). In the exemplar trace shown, two control menthol pulses were recorded at pH 7.3 (standard extracellular solution) followed by a further menthol response recorded at pH 5.3. The final response was recorded at pH 7.3, 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 40-s pH 5.3 pretreatment time used is sufficient to desensitize the acid-sensing ion channel and remove its contribution from the peak of the menthol response recorded at pH 5.3. 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 pH 7.3 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.

antagonists, clearly demonstrate that there is a large degree of overlap in the TRPV1 and TRPM8 pharmacophores. It is encouraging that 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 orthologs described by McKemy et al. (2002) and Peier et al. (2002), respectively. 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 behavior as a nonselective cation channel (McKemy et al., 2002; Peier et al., 2002). It was during this initial characterization that we also noted that TRPM8 exhibited the hallmarks of time- and voltage-dependent behavior that we have described previously for TRPV1 (Gunthorpe et al., 2000). We therefore characterized 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 Behavior. Our results demonstrate that TRPM8 exhibits clear noninstantaneous rectification behavior (Figs. 1D and 2). Steps to positive potentials effectively potentiated TRPM8 responses with kinetics in the order of 4 to 30 ms, and after

a return to negative resting potentials, currents relaxed to their prestimulus levels, resulting in the appearance of tail currents. The time course of the tail currents could be approximated with biexponential curves with a fast- and a slow-time constant that closely resembled the kinetic characteristics of the tail currents described for rTRPV1 (Gunthorpe et al., 2000; Ahern and Premkumar, 2002; Vlachova et al., 2003). These biexponential kinetics are also consistent with the occurrence of multiple TRPM8 open and/or closed states, a feature which has now been rigorously defined for TRPV1 through in-depth single-channel analysis (Hui et al., 2003; Liu et al., 2003). It therefore seems 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 behavior 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 behavior of TRPM8. It is interesting that this should also endow the TRPM8 receptor with an ability to respond in a manner that reflects recent activity of the cell (i.e., responses will be influenced by recent depolarizations mediated by TRPM8 or other mechanism) and show a degree of coincidence detection (Gunthorpe et al., 2000). It is important to note that the
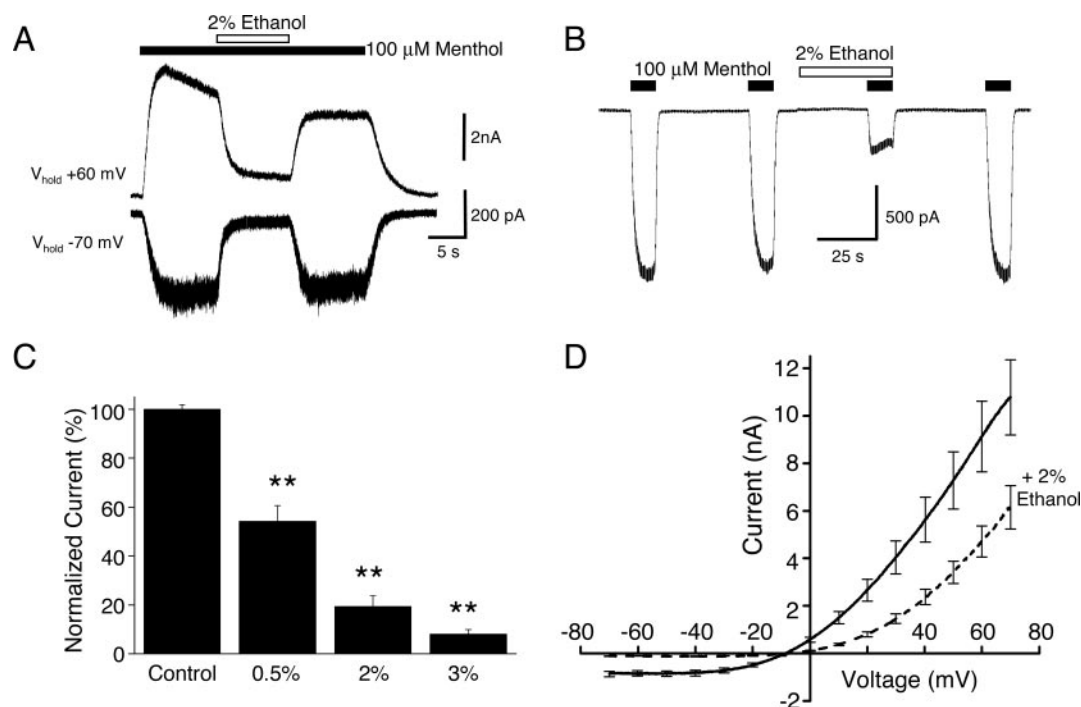
Fig. 4. Ethanol causes inhibition of TRPM8. A, representative whole-cell recording illustrating the design of the experiment used to examine the effects of ethanol on menthol-gated TRPM8 currents. Menthol (100 μM, 10 s) evoked responses in HEK293.hTRPM8 cells that were clearly inhibited by coapplication of 2% ethanol at both negative (-70 mV; 91 $\pm$ 4% inhibition, $n = 8$) and positive ($+60$ mV; 83 $\pm$ 5% inhibition, $n = 8$; significantly different from 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 wild-type or TRPM8-expressing cells (data not shown). B and 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 to 3% ethanol, yielding 54 $\pm$ 7% ($n = 5$), 19 $\pm$ 4% ($n = 8$), and 8 $\pm$ 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 with control ($p < 0.01$). D, Current-voltage relationships in response to menthol generated using a voltage ramp protocol (-70 to $+70$ mV, 1 s) in the presence and absence of 2% ethanol.

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 on the basis of 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 (Brauchi et al., 2004; Voets et al., 2004).

TRPM8 Is Insensitive to Changes in Extracellular pH. Behrendt et al. (2004) reported recently that menthol- and icilin-induced TRPM8 responses were strongly inhibited by acidification. We were intrigued by this finding, because 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 response to local acidosis, which can accompany inflammation (which may reach pH 5.5) (Reeh and Steen, 1996). Because Behrendt et al. (2004) used a Ca^{2+}-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 pH 6.3 or even further acidification to pH 5.3 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 because protons seem to be a somewhat specific regulator of TRPV1 rather than members of the TRP family in general (Gunthorpe et al., 2002; Clapham, 2003) 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 (Tsavaler et al.,

2001; McKemy et al., 2002; Peier et al., 2002). 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 caused by changes in intracellular pH, suggesting unforeseen complexity in the different transduction mechanisms leading to TRPM8 activation. Given this apparent complexity, whether (patho)physiological TRPM8 function in vivo is influenced by changes in pH remains to be fully determined.

Ethanol Inhibits TRPM8 Function. We have demonstrated previously 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 behavior 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 rationalized on the basis of the 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
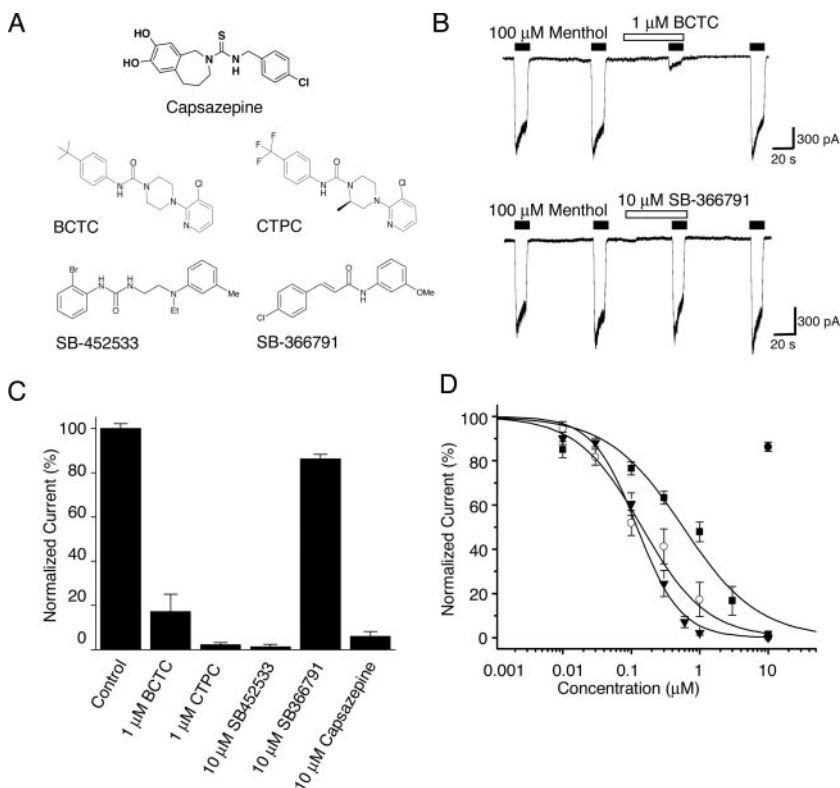
Fig. 5. Overlap in the ligand-recognition properties of TRPM8 and TRPV1. A, a comparison of the chemical structures of the TRPV1 antagonists capsazepine, BCTC, CTPC, SB-452533, and SB-366791. B, exemplar traces from experiments conducted to assess the antagonistic activity of the TRPV1 antagonists shown in A versus TRPM8. BCTC (1 μM) caused almost complete inhibition of TRPM8, whereas SB-366791 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 (○; IC_{50} = 143 ± 19 nM, n = 4–5 per concentration), CTPC (▼; IC_{50} = 131 ± 14 nM, n = 3–9 per concentration), and SB-452533 (■; IC_{50} = 571 ± 77 nM, 4–6 per concentration); the effects of SB-366791 are represented by a single data point (●; n = 12) at the test concentration of 10 μM.

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 link the temperature-sensitive properties of TRPM8 and TRPV1 to their voltage dependence. 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 seems to be strongly temperature-dependent (Voets et al., 2004). These features effectively govern the gating properties of the receptors that 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). Likewise, 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 of the gating mechanisms of these channels in terms of their multiple closed and open states (Hui et al., 2003; Liu et al., 2003; Brauchi et al., 2004) 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 that 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 because of its rather lipophilic properties, most menthol treatments and medications administered to patients are ethanol-based solutions with typical ethanol concentrations of more than 60% (Yosipovitch et al., 1996; Pittler and Ernst, 1998; Wasner et al., 2004). 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 in which thermo-TRPs are either present and/or studied. It is clear that past data could be reinterpreted and the design of future experiments improved from a greater appreciation of the effects of ethanol on these receptors. Likewise, because 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) that are used in the food and cosmetics industry, their combination with ethanol may be usefully reconsidered. However, because 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 characterization 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 orthologs. 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 that 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} value of 131 ± 14 nM means that it is effectively equipotent to BCTC ($IC_{50} = 143 \pm 19$ 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 ($IC_{50} = 571 \pm 77$ 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 that we have identified which shows little or no activity compared with 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 suggest that the gating properties of these receptors are much more similar than initially envisaged. We have demonstrated the significant overlap in the pharmacological SAR between the TRPM8 and TRPV1 receptors, and

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.

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Correction to "Conservation of Functional and Pharmacological Properties in the Distantly Related Temperature Sensors TRVP1 and TRPM8"

In the title of the above article [Weil A, Moore SE, Waite NJ, Randall A, and Gunthorpe MJ (2005) *Mol Pharmacol* **68:**518–527], because of an error during copyediting, two letters were transposed in one of the receptor names. The correct receptor name is TRPV1, not TRVP1.

The online version of the article was corrected in departure from the print version.

We regret this error and apologize for any confusion or inconvenience it may have caused.