ANKTM1, a TRP-like Channel Expressed in Nociceptive Neurons, Is Activated by Cold Temperatures

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Summary

Mammals detect temperature with specialized neurons in the peripheral nervous system. Four TRPV-class channels have been implicated in sensing heat, and one TRPM-class channel in sensing cold. The combined range of temperatures that activate these channels covers a majority of the relevant physiological spectrum sensed by most mammals, with a significant gap in the noxious cold range. Here, we describe the characterization of ANKTM1, a cold-activated channel with a lower activation temperature compared to the cold and menthol receptor, TRPM8. ANKTM1 is a distant family member of TRP channels with very little amino acid similarity to TRPM8. It is found in a subset of nociceptive sensory neurons where it is coexpressed with TRPV1/VR1 (the capsaicin/heat receptor) but not TRPM8. Consistent with the expression of ANKTM1, we identify noxious cold-sensitive sensory neurons that also respond to capsaicin but not to menthol.

Introduction

John Updike wrote, "Cold is an absence, an absence of heat, and yet it feels like a presence" (Updike, 1980). The ability to sense cold as a distinct presence resides in specialized neurons within the peripheral nervous system that detect environmental temperature. The cell bodies of these sensory neurons are clustered in ganglia located in the vertebral column and cranium, and their projections extend for long distances to peripheral tissues such as the skin of the trunk and head. It is hypothesized that channels present at the end of these projections are activated by physical stimuli such as temperature and pressure (Hensel, 1981).

The cloning of TRPV1 (VR1: a capsaicin- and heat-activated channel) has proven this hypothesis and ignited research into thermosensation at the molecular level (Caterina et al., 1997). TRPV1 is activated near 43°C, a temperature most mammals perceive as noxious. Three other TRPV channels with greater than 40% amino acid level identity to TRPV1 have since been cloned and characterized as thermosensors. These channels are activated at various heat thresholds, ranging from 33°C (warm) for TRPV3 to 55°C (high-threshold noxious heat) for TRPV2/VRL1 (Peier et al., 2002b; Smith et al., 2002; Xu et al., 2002; Caterina et al., 1999). TRPV4, originally described as an osmo-sensor, has also been shown to be activated by warm temperatures (Guler et al., 2002; Liedtke et al., 2000; Strotmann et al., 2000). Much less is known about channels that sense cold. Recently, the cloning of a menthol- and cold-activated channel, TRPM8 (CMR1) was described (McKemy et al., 2002; Peier et al., 2002a). The threshold of TRPM8 activation is ~25°C, consistent with the pleasant/cool feeling conveyed by menthol products. Since TRPM8 is initially activated at cool rather than cold temperatures, it has been postulated that other cold-activated channels exist. Indeed, recent reports (and this study) identify a population of neurons that respond to cold but not menthol (M.L Nealen et al., 2002, and D.W. Beacham et al., 2002, Soc. Neurosci., abstract).

All known temperature-activated receptors belong to the transient receptor potential (TRP) family of non-selective cation channels. TRP channels are divided into three subclasses designated TRPC, TRPV, and TRPM (Montell et al., 2002). All have six putative transmembrane domains with a proposed pore region between transmembrane domains five and six. TRP channels are thought to have cytoplasmic N- and C termini. The three classes of TRP channels are distinguished according to overall similarity as well as several unique characteristics. TRPV and TRPC members contain two to four N-terminal ankyrin domains thought to be involved in linking transmembrane proteins to the cytoskeleton. TRPC and TRPM members have a TRP box (with unknown function) following the sixth putative transmembrane domain. The involvement of TRP channels in sensory function has been evident since the original TRPC channel was cloned in *Drosophila* due to its essential role in the photoreceptor response to light (Montell and Rubin, 1989). OSM-9 and other TRPV-family members in *C. elegans* are required for various sensory responses (Tobin et al., 2002). In mammals, TRPC2 is required for the response of vomeronasal neurons to pheromones (Stowers et al., 2002). However, unlike the known mammalian thermo-activated TRPs, these channels do not seem to "sense" stimuli directly but act downstream of various G protein-coupled receptors (GPCRs), most probably via the phospholipase C pathway (Montell, 2001). Most of the 21 mammalian classical TRP channels described to date have diverse expression patterns and

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varied roles not related to sensory perception, including functions in male fertility and vasorelaxation.

It is now recognized that other classes of ion channels are homologous to the classical TRP channels, and a nomenclature system has been proposed to reflect this relationship (Montell, 2001). The new subtypes include TRPP for PKD2-like channels (PKD2 is mutated in polycystic kidney disease), TRPML for Mucolipidin-like channels (Mucolipidin mutations are responsible for some lysosomal storage disorders), and TRPN for NOMPC-like channels (NOMPC is required for mechanosensory function in flies). Here, we show that ANKTM1, a TRP-like channel, participates in thermosensation. ANKTM1 responds to cold temperatures but not to menthol when expressed in mammalian cell lines or *Xenopus* ooctyes. ANKTM1 is expressed in a small population of sensory neurons that also express TRPV1 but not TRPM8, arguing that at least two distinct groups of neurons sense cold temperature.

Results

To date, all five thermo-activated channels identified belong to the TRP family, all characterized by six transmembrane domains (6TM) and most containing N-terminal ankyrin domains (Montell, 2001). We took a combined bioinformatic and expression analysis approach to identify additional TRP channels involved in sensory detection. A search for predicted cDNA sequences that contain both ankyrin domains (PFAM00023) and 6TM domains (PFAM00520) led us to ANKTM1. Human ANKTM1 is an uncharacterized putative channel cloned from cultured fibroblasts (Jaquemar et al., 1999). A previous study found no significant expression of ANKTM1 in various tissues examined, although EST sequences suggest that ANKTM1 is upregulated in many human tumor cells. Full-length mouse ANKTM1 was amplified using RT-PCR from sensory neurons of the trigeminal and dorsal root ganglia (DRG) (data not shown). Theoretical translation of the nucleotide sequence predicts an 1125 amino acid protein very similar to human ANKTM1 (1119 amino acids) (GenBank accession number AY231177). Mouse ANKTM1 has 14 predicted N-terminal ankyrin domains followed by a 6TM domain. ANKTM1 seems to be the sole mammalian member of a distant subfamily of TRP channels (data not shown).

We analyzed overall tissue distribution of ANKTM1 by Northern blot analysis using a probe corresponding to nucleotides 590–1492 of mouse ANKTM1. ANKTM1 expression was not detected on a blot containing mouse tissues such as heart, lung, skeletal muscle, and kidney (Figure 1). We also probed a blot containing rat tissues relevant to sensory neurons and their target tissues such as DRG, spinal cord, and skin. A single mRNA species of approximately 5 kb was detected only in DRG (Figure 1). More detailed expression analysis of ANKTM1 in DRG was carried out via in situ hybridization and immunostaining. Digoxigenin-labeled ANKTM1 cRNA probes strongly hybridized to neurons in adult DRG and trigeminal ganglia (Figure 2 and data not shown). We found ANKTM1 expression restricted to a small sub-population of DRG neurons (59 of 1608; 3.6%). To further characterize its expression in sensory neurons, we examined
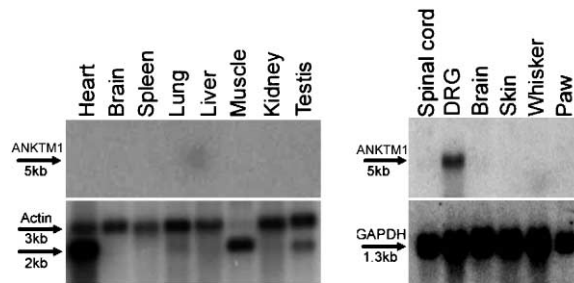

Figure 1. ANKTM1 Is Specifically Expressed in Sensory Neurons
Northern blot analysis of mouse (1st image) and rat (2nd image) tissues reveals expression of ANKTM1 in the dorsal root ganglia (DRG). Blots were hybridized with a ^{32}P-labeled fragment of ANKTM1 cDNA (top image) and with control cDNAs (bottom image).

whether other DRG markers colocalized with ANKTM1. ANKTM1 is not expressed in heavily myelinated neurons marked by NF-150 kd (Figures 2A–2C). This suggests that ANKTM1 is most likely expressed in the non-myelinated C- or lightly myelinated A_δ-fiber population of neurons that sense temperature and/or noxious stimuli. In accordance with this observation, we further discovered that ANKTM1 is expressed in Calcitonin gene-related peptide (CGRP)- and Substance P (SP)-positive neurons (Figures 2D–2F, and data not shown). CGRP and SP are secreted inflammatory peptides expressed in a subset of nociceptive neurons (Scott, 1992). Roughly 97% (69/71) of ANKTM1-positive neurons were CGRP-positive. We next examined the expression of ANKTM1 relative to known thermo-activated TRP channels. TRPV1 (VR1) is a well-characterized receptor for noxious heat, pH, and capsaicin. Double in situ experiments with ANKTM1 and TRPV1 probes (Figures 2G–2I) revealed that 97% (100/103) of ANKTM1-positive neurons also express TRPV1, while 30% (100/336) of TRPV1-positive neurons express ANKTM1. We have previously shown that TRPM8, the cold and menthol receptor, is not coexpressed with CGRP and TRPV1 in DRG neurons (Peier et al., 2002a). Quantitative analysis of double in situ hybridizations confirmed our initial observation: we found no TRPV1 expression in 95 TRPM8-positive neurons (data not shown). Since ANKTM1 expression overlaps with CGRP and TRPV1, we would expect ANKTM1 and TRPM8 not to be coexpressed. Indeed, in double in situ hybridizations, we observed no overlap of expression between ANKTM1 and TRPM8 (n = 113 for ANKTM1 and 137 for TRPM8) (Figures 2J–2L). Taken together, these results indicate that ANKTM1 is expressed in a subpopulation of nociceptive/thermoceptive neurons that coincides with noxious heat-activated TRPV1 but not with the cool/cold-activated TRPM8 (Figure 2M).

Since ANKTM1 is a putative TRP-like channel expressed in sensory neurons, we tested whether sensory stimuli activate ANKTM1. Full-length mouse ANKTM1 was stably transfected in Chinese hamster ovary (CHO) cells containing FRT sites (CHO-K1/FRT). We generated cell lines either constitutively expressing ANKTM1, or under the control of a tetracycline (Tet)-inducible promoter via Flp recombinase-mediated recombination. Cells constitutively expressing ANKTM1 appeared unhealthy and downregulated ANKTM1 expression after
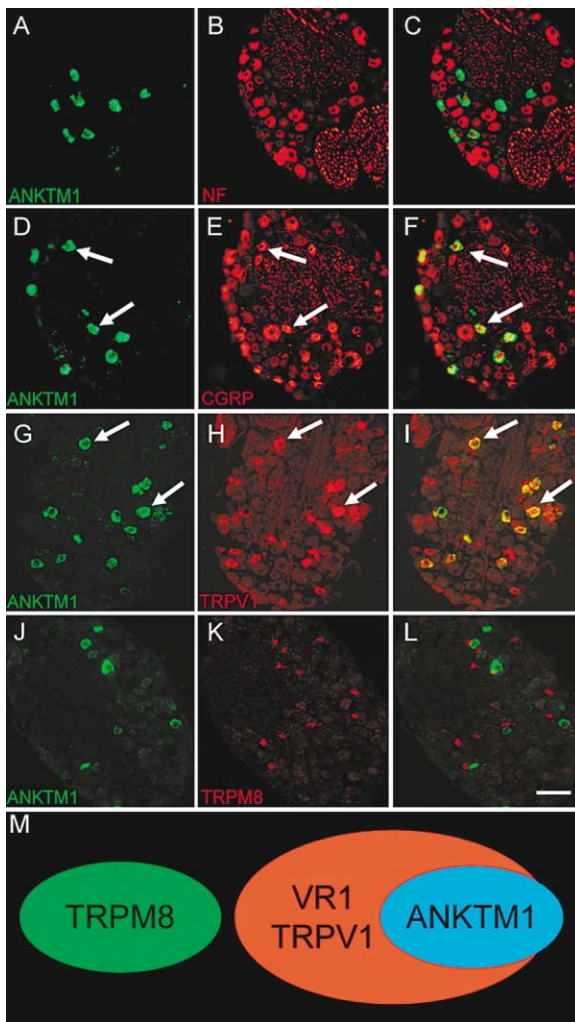
Figure 2. In Situ Hybridization and Immunostaining Analyses Demonstrate ANKTM1 Expression in a Subset of TRPV1-Positive Nociceptive DRG Neurons

(A–C) ANKTM1 mRNA is not present in heavily myelinated neurons marked by neurofilament (NF 150) antibody.
(D–F) ANKTM1 and calcitonin-gene-related peptide (CGRP) are coexpressed (arrows D–F).
(G–L) Double in situ hybridization shows ANKTM1 is present in a sub-population of thermosensitive neurons expressing TRPV1 (arrows G–I), but not TRPM8. Size bar is 50 μm.
(M) A schematic of overlap in expression among thermo-activated TRP channels according to expression analysis in adult DRGs.

several passages in culture (data not shown). Therefore, we performed all functional analyses in the Tet-inducible system. Most TRP channels are non-selective cation channels and readily allow calcium into cells when activated, permitting both electrophysiological studies and intracellular calcium imaging. We used fluorescence microscopy to monitor increases in intracellular calcium concentration in response to sensory stimuli by loading ANKTM1-expressing CHO cells with the calcium indicator dye, Fura-2. Hypotonic solutions, known to elicit calcium influx in TRPV4-expressing cells, did not activate ANKTM1 (data not shown) (Liedtke et al., 2000; Strotmann et al., 2000). Heat stimuli of up to 42°C and

52°C, strong activators of TRPV1 and TRPV2 respectively, did not activate ANKTM1-expressing CHO cells (data not shown) (Caterina et al., 1999, 1997). Menthol or cool stimuli in the range of 23–28°C, the threshold of activation of TRPM8, failed to activate ANKTM1 (McKemy et al., 2002; Peier et al., 2002a). However, lowering temperature to 10°C caused a large influx of calcium in ANKTM1-expressing cells, but not in Tet-treated CHO cell controls (Figure 3A). The response to cold stimulus by ANKTM1 was abolished upon removal of extracellular calcium, suggesting that ANKTM1 is acting as a calcium permeable channel rather than releasing calcium from intracellular stores (Figure 3A). Similar responses to cold were obtained from CHO cells transiently transfected with ANKTM1 and from Tet-inducible human embryonic kidney (HEK 293) cells stably expressing ANKTM1 (data not shown). Ruthenium red, a blocker of thermosensitive TRPV channels, also blocked cold responses by ANKTM1. Pre-incubation of ANKTM1-expressing cells in 5 μM ruthenium red followed by application of cold completely blocked responses, which were not fully restored after a ten minute washout (Figure 3, and data not shown). Similar experiments performed with 1 μM ruthenium red caused complete block of the cold response in 90% of cells from multiple experiments (data not shown).

We were intrigued by the possibility that cooling agents other than menthol might trigger calcium influx in ANKTM1-expressing cells. High concentrations of camphor (400 μM), which fail to activate TRPM8, also had no effect on ANKTM1 (data not shown). Icilin, a cooling agent and potent activator of TRPM8, also activated ANKTM1-expressing cells (Figure 3B) (McKemy et al., 2002; Wei and Seid, 1983). Icilin did not activate TRPV1-expressing cells. Capsaicin, a potent activator of TRPV1, did not activate TRPM8- or ANKTM1-expressing cells, as expected (Figure 3B). It is interesting that icilin (which is structurally distinct from menthol) activates both cold receptors, despite the fact that TRPM8 and ANKTM1 have no significant similarity at the amino acid level. However, higher concentrations of icilin were required for ANKTM1 activation compared to TRPM8, and the response was relatively delayed in ANKTM1 cells (15–60 s delay for ANKTM1 versus approximately 5 s for TRPM8; data not shown). Therefore, it is not clear if icilin directly binds to and activates ANKTM1.

Both TRPM8 and ANKTM1 respond to cold. However, initial experiments suggested the threshold for activation of ANKTM1 is lower than that of TRPM8. We have previously reported that TRPM8 is activated at approximately 23°C (Peier et al., 2002a). To further investigate this issue, we performed calcium-imaging experiments where cells were incubated at 33°C for several minutes before the bath temperature was lowered gradually to 10°C. The majority of TRPM8-expressing cells exhibited increased intracellular calcium concentration when the temperature was lowered to 20°C (Figure 4A). However, the majority of ANKTM1-expressing cells were not activated until the temperature was cooled below 20°C (Figure 4A). Quantitative analysis showed that TRPM8-expressing cells exhibited an activation threshold (defined as 20% increase from baseline fluorescence) at temperatures ranging from 19–24°C with a mean activation temperature of 22.5 ± 1°C (mean ± SD, n = 60). ANKTM1-

A
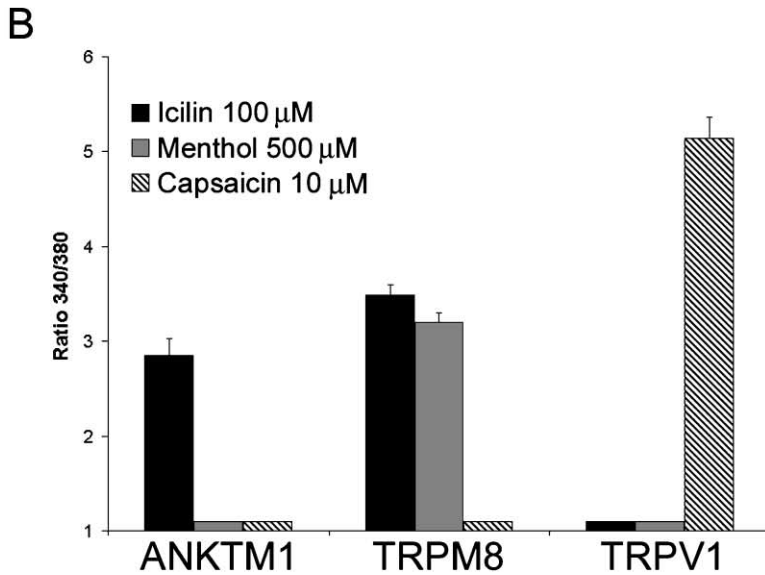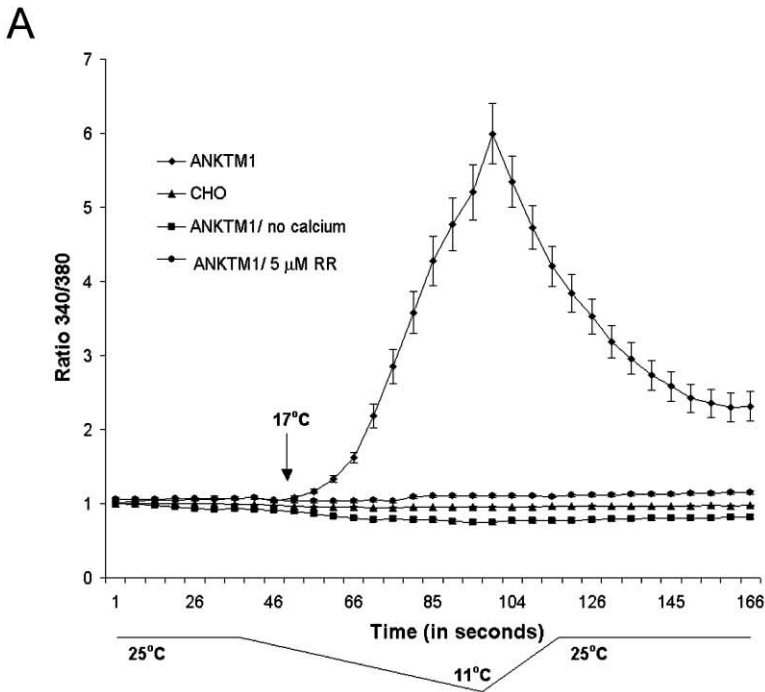
Figure 3. ANKTM1 Is a Calcium Permeable Cation Channel that Responds to Cold and Icilin, and Is Blocked by Ruthenium Red

(A) A cold stimulus elicits a rise in $[Ca^{2+}]_i$ in ANKTM1-expressing CHO cells. A schematic representation of the stimulus is indicated below the graph. When buffer is cooled, $[Ca^{2+}]_i$ increases rapidly in ANKTM1-expressing CHO cells, but not in untransfected CHO cells. Average activation temperature (measured at the inlet of the cell chamber by a miniature thermocouple) is approximately 17°C. Cooling in the presence of 5 μM ruthenium red and in the absence of extracellular Ca^{2+} eliminates cold-evoked responses. Values shown are average increase in ratio 340/380 $\pm$ SEM of 30–40 cells from representative experiments.

(B) Average fold increase in ratio 340/380 $\pm$ SEM of 20 ANKTM1-, TPRM8-, and TRPV1-expressing CHO cells in response to hot- (capsaicin) and cold- (menthol, icilin) inducing compounds.

B

expressing cells exhibited a broader range of activation temperatures (12–24°C) with an average activation temperature of 17.5 $\pm$ 3.5°C (mean $\pm$ SD, n = 100). Figure 4B illustrates traces of typical responses of individual ANKTM1- and TRPM8-expressing cells, as temperature is gradually decreased.

ANKTM1- and TRPM8-expressing CHO cells were assayed electrophysiologically using the whole-cell patch clamp technique. The cells were clamped at −60 mV and the temperature of the perfused bath solution was decreased from 32°C to 10°C. In ANKTM1-expressing cells, small and slowly developing inward currents were observed followed by rapid and larger phase currents with an average peak amplitude of 0.55 $\pm$ 0.07 nA (n =

35) (Figure 5A and 5B, and data not shown). When maintained at lower temperatures, the current inactivated quickly (Figure 5A). Similar to results obtained by calcium imaging, the activation temperature for the currents varied from cell to cell, ranging between 8–28°C (n = 35). A comparison of representative currents from ANKTM1- and TRPM8-expressing cells demonstrates a colder threshold of activation for ANKTM1 (Figure 5B).

Currents evoked by decreasing the temperature in ANKTM1-expressing cells show outward rectification, with substantial current in the inward direction (Figures 5C and 5D). A reversal potential of +7.7 $\pm$ 1.2 mV was observed in an external solution containing 140 mM NaCl (n = 6). Reducing the NaCl in the external solution
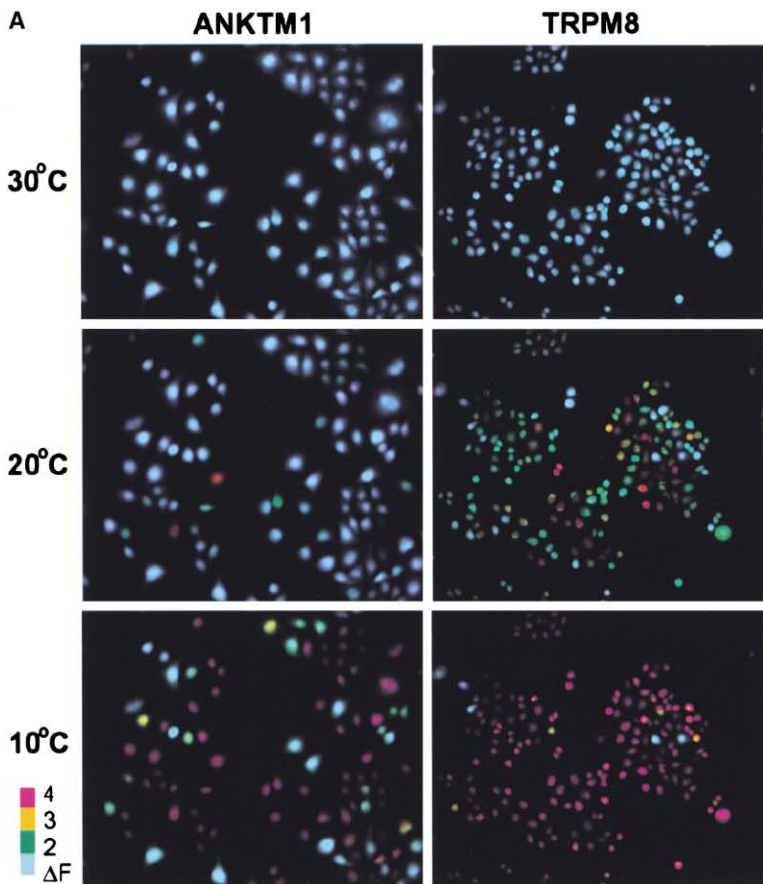
A

B

Figure 4. ANKTM1 Is Activated at Colder Temperatures Compared to TRPM8

(A) Representative images taken from calcium imaging experiments comparing responses of Fura-2 loaded ANKTM1- and TRPM8-expressing CHO cells to gradual cooling. Increases in fluorescence correspond to increases in intracellular calcium levels and are indicated by a change in color where red > yellow > green > blue. The color scale bar indicates deltaF values, defined as the average fold increase in the Fura-2 340/380 ratio in response to the stimulus. Ratios were normalized to baseline fluorescence. Note that the majority of ANKTM1-expressing cells (left) are not active until temperatures fall below 20°C, while many TRPM8-expressing cells (right) are already strongly activated at 20°C.

(B) Graphic presentation of responses of individual ANKTM1- and TRPM8-expressing CHO cells to gradual cooling from 31–9°C. Responses shown are from representative cells.
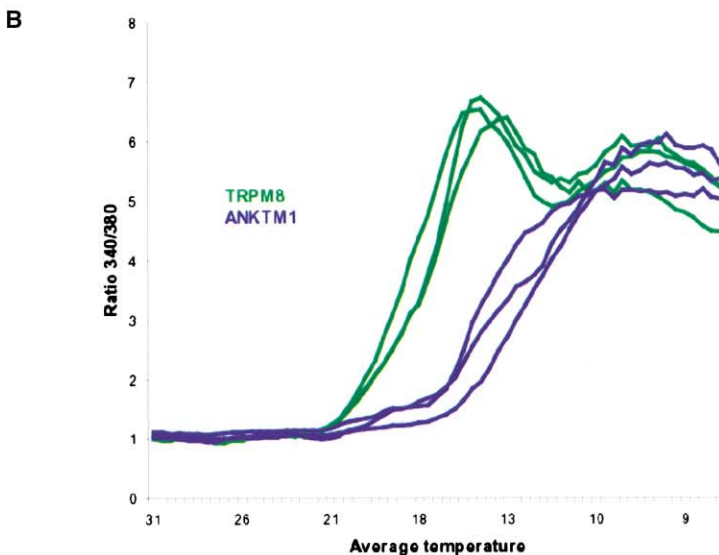
to 40 mM (by equimolar replacement with 100 mM choline chloride) caused a negative shift in the reversal potential, consistent with ANKTM1 being a cation channel. Differences in reversal potentials were used to determine the ionic selectivity of ANKTM1. The shift in reversal potential by −18 mV to −10.35 ± 1.6 mV (n = 7) seen upon replacing 100 mM NaCl with 100 mM choline Cl gives a relative permeability ratio of $P_{choline}/P_{Na} = 0.28$. The reversal potentials of the cold activated cur-

rents were similar in simplified external solutions containing 100 mM choline Cl and 40 mM NaCl, KCl, or CsCl. The measured reversal potentials yield relative permeability ratios of $P_K/P_{Na} = 1.19$ and $P_{Cs}/P_{Na} = 1.42$ (NaCl, $E_{rev} = −10.35 ± 1.6$ mV, n = 7; KCl, $E_{rev} = −7.65 ± 1.5$ mV, n = 8; CsCl, $E_{rev} = −4.75 ± 0.75$ mV, n = 7). The relative permeability of Ca^{2+} and Mg^{2+} were estimated from the shift in reversal potentials when their concentrations were raised from 1 to 30 mM in a 40 mM
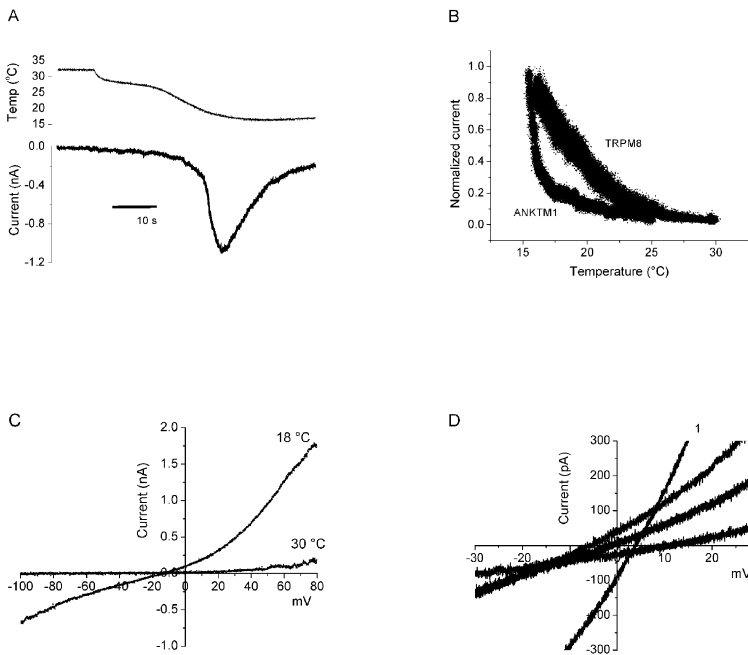
Figure 5. Currents Evoked by Decreasing Temperatures in CHO Cells Expressing ANKTM1

(A) Example of an inward current evoked by cooling of a cell voltage clamped at −60 mV.
(B) Comparison of temperature current relationship for ANKTM1 and TRPM8.
(C) Current voltage relationship for ANKTM1 (voltage ramp is from −100 mV to +80 mV with two second ramps). The current is outwardly rectifying and there is an increase in both inward and outward currents when the temperature is lowered (30°C–18°C).
(D) Comparisons of current-voltage relationships for ANKTM1 in various external solutions. The main charge carrying ions are: (1) 140 mM NaCl; (2) 40 mM NaCl/100 mM choline; (3) 1 mM CaCl₂ (with 40 mM NaCl/100 mM choline); and (4) 30 mM CaCl₂ (with 40 mM NaCl/100 mM choline). E_{revs} for these examples are, +4, −6, −2, and +11 mV, respectively.

NaCl/100 mM choline Cl solution containing the divalent cation under investigation. The reversal potential shifted from +0.54 ± 3.3 (1 mM CaCl₂, n = 6) to +14.16 ± 3.9 mV (30 mM CaCl₂, n = 6) for Ca^{2+} and from −14.36 ± 1.1 (1 mM MgCl₂, n = 6) to +7.01 ± 2.9 mV (30 mM MgCl₂, n = 6) for Mg^{2+}, corresponding to P_{Ca}/P_{Na} = 0.84 and P_{Mg}/P_{Na} = 1.23 (Figure 5D, and data not shown). These results indicate that ANKTM1 is a non-selective cation permeable channel, similar to many previously described TRP channels.

To investigate the properties of ANKTM1 in another heterologous system, we injected *Xenopus* oocytes with ANKTM1 cRNA. We observed large currents in response to cold temperatures, similar to the activity of ANKTM1-expressing CHO cells (Figure 6). In both *Xenopus* oocytes and CHO cells, a strong desensitization of

ANKTM1 to cold stimuli was observed (Figure 6, and data not shown). Cold activation of ANKTM1 showed a marked desensitization during a first cold pulse and desensitization to repeated cold pulses (Figure 6). On average, the second cold pulse resulted in a current that was 26% of the first pulse in ANKTM1-injected oocytes (SD = 6.5, n = 5), compared to 78% for TRPM8 (SD = 5.9, n = 4). We also tested whether icilin activated *Xenopus* oocytes injected with ANKTM1 cRNA. Currents were elicited by 25 μM and 200 μM of icilin, but not with 1 μM. All three concentrations activated TRPM8 injected oocytes but not uninjected ones (data not shown).

The characterization of ANKTM1 as a cold-sensitive channel expressed in nociceptive neurons distinct from TRPM8-positive neurons immediately raises the interesting question as to whether ANKTM1-like characteris-
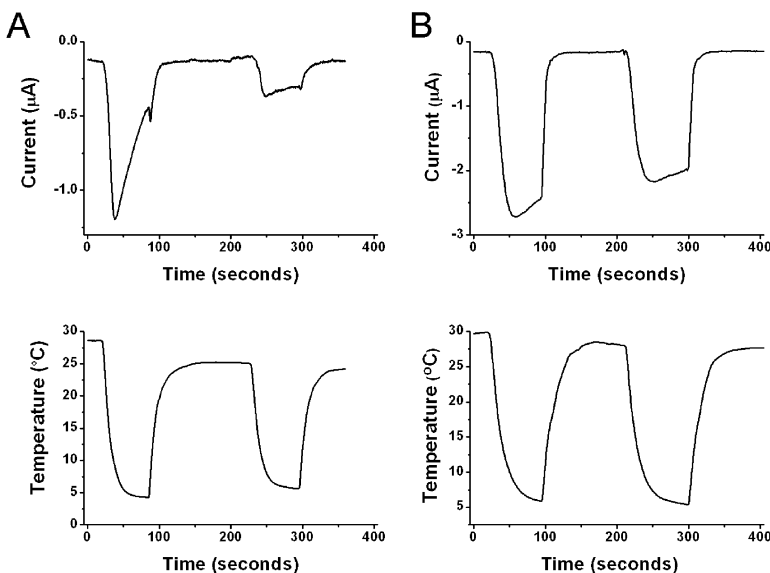

Figure 6. ANKTM1 Currents Are Desensitized to Repeated Cold Stimuli

Inward currents recorded in response to cold steps from *Xenopus* oocytes expressing ANKTM1 (A) and TRPM8 (B). The oocytes were held at −70 mV during the recordings. ANKTM1 current responses were markedly reduced in the second cold step.
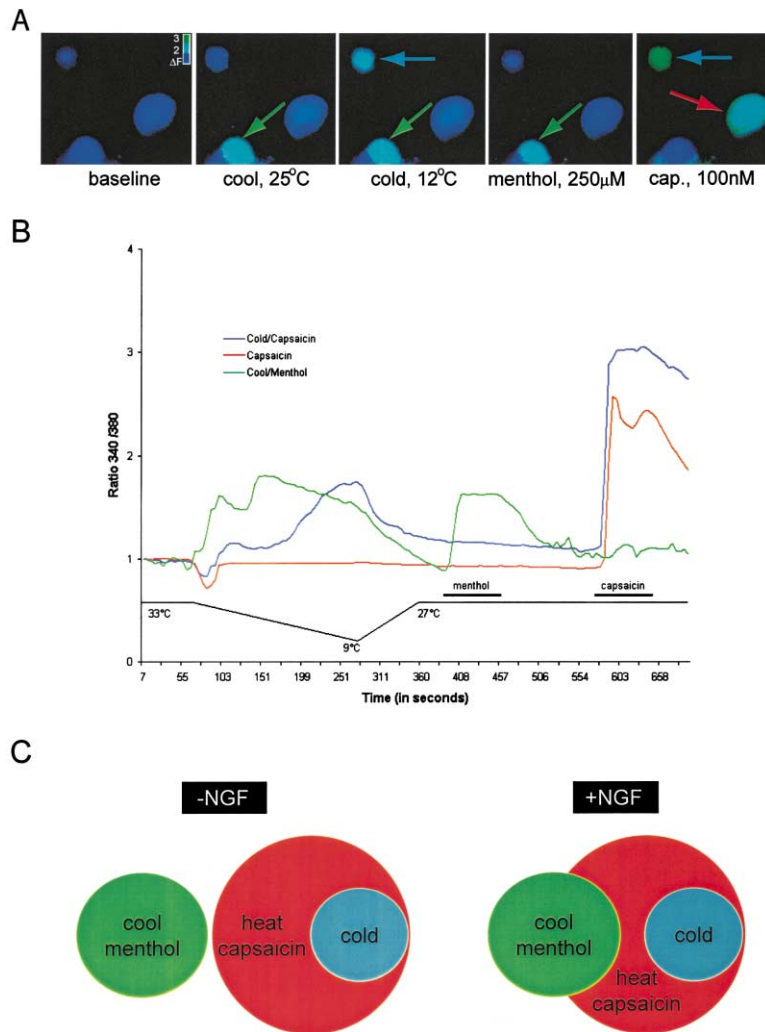
Figure 7. A Sub-Population of DRG Neurons Have ANKTM1-Like Characteristics

(A) Representative images taken from calcium imaging experiments of Fura-2 loaded adult DRG neurons cultured overnight without NGF. Cells are subjected to a cold stimulus (33–9°C), warmed to room temperature (9–27°C), followed by perfusion with menthol (250 μM), and capsaicin (100 nM). The field shown contains an ANKTM1/TRPV1-like (blue arrows), a TRPM8-like (green arrows), and a TRPV1-like (red arrow) neurons. The color scale bar indicates deltaF values, defined as the average fold increase in the Fura-2 340/380 ratio in response to the stimulus.

(B) Graphic presentation of the responses of the neurons shown in (A). The color-coding is preserved from the previous image.

(C) A schematic summary of results from adult DRG imaging experiments. The overlap between cold-, cool/menthol-, and heat/capsaicin-responders from cultures with or without NGF are shown. The response profiles from cultures with no NGF completely agree with the in vivo expression of ANKTM1/TRPM8/TRPV1 as summarized in Figure 2M.

tics are found in DRG neurons in vivo. To answer this question, we analyzed dissociated adult DRGs 18–36 hr after culture in the presence or absence of 100 ng/ml nerve growth factor (NGF). Adult DRG neurons are routinely cultured with NGF, but can survive for weeks without it (Aguayo and White, 1992). We assayed calcium influx in response to a variety of relevant thermal and chemical stimuli. We perfused these neurons with cold buffer (33–9°C), followed by menthol (250 μM), capsaicin (100 nm), and heat (45°C; heat activated the same neurons that responded to capsaicin over 95% of the time, and we focused mainly on the capsaicin response). Menthol represents a very powerful tool for these experiments, since it activates TRPM8-expressing cells, but not those that express ANTKM1. We observed two separate populations of cold-sensitive neurons: one group of neurons responded to cool temperatures (average threshold of activation 24°C) and to menthol, while another group responded to colder temperatures (average threshold of activation 15°C) but not to menthol (Figure 7 and Table 1). This is consistent with the expression profile and activation characteristics of TRPM8 and ANKTM1, respectively.

Since ANKTM1 is expressed in a subset of TRPV1-expressing neurons (and TRPM8 is not), we further characterized the response of the two populations of cold-sensing neurons to capsaicin and heat (TRPV1 activation). Interestingly, in the cultures with no NGF, all of the menthol-insensitive but none of the menthol-sensitive cold neurons showed activation by capsaicin (Figure 7 and Table 1). This agrees with our expression analysis and represents strong evidence that cold-sensitive neurons with ANTKM1-like characteristics are present within the DRGs. With NGF in the culture conditions, in addition to all of the menthol-insensitive cold neurons, about 50% of the menthol-sensitive neurons also responded to capsaicin (Table 1). DRG neurons have been previously reported to respond to both capsaicin and menthol in the presence of NGF (Mckemy et al., 2002; Reid et al., 2002). NGF is known to have an effect on capsaicin sensitivity (see Discussion). These results suggest that culture conditions without NGF better correspond to the in vivo expression profiles of the thermal-activated TRP channels (Figures 2M and 7C).

The coexpression of TRPV1 and ANKTM1 also raises the possibility that the two channels might interact by influencing the properties of one another. We addressed this issue directly by transiently transfecting human

Table 1. Cold Responses in Cultured Adult DRG Neurons

	−NGF	+NGF
Total neurons	477	458
Cold-sensitive neurons	44	37
Menthol-sensitive neurons	27	30
Capsaicin-sensitive	0/27	14/30
Average activation temperature	24.2 ± 4.1°C	23.4 ± 4.3°C
Menthol-insensitive neurons	17	7
Capsaicin-sensitive	17/17	7/7
Average activation temperature	14.8 ± 2.8°C	14.5 ± 2.5°C

TRPV1 in ANKTM1-positive CHO cells. Pulses of cold followed by heat, or heat followed by cold were applied to these cells and intracellular calcium imaging performed. The responses (including activation threshold) of ANKTM1 and TRPV1 were indistinguishable when compared to cells that expressed only one of the channels separately ($P > 0.05$, mean threshold values taken from 40 representative coexpressing cells from three experiments, data not shown).

Discussion

Most living creatures tolerate a very narrow temperature range. In humans, the necessity to maintain requisite physiological temperature is paired with an acute sensory detection system that discriminates temperature differences smaller than a single degree centigrade. The cloning and characterization of heat- and cold-activated channels establishes a molecular basis for understanding temperature detection and sensation.

Here, we describe the characterization of a cold-activated channel. ANKTM1 belongs to the superfamily of TRP channels, as does the menthol- and cold-activated receptor, TRPM8. Interestingly, icilin, a potent cooling compound, activates cells expressing either channel. However, at the amino-acid level, the two cold-receptors have no significant similarity. In contrast, the four heat-activated TRPV channels have at least 40% similarity. The transmembrane domain of ANKTM1 is more similar to TRPV members than to any other mammalian proteins. This amino acid similarity is likely significant at the structural level since ruthenium red, a potent blocker of TRPV channels, also blocks ANKTM1. However, in phylogenetic tree prediction programs, ANKTM1 is grouped with a different TRP-like family of channels, with ANKTM1 being the only mammalian member.

Functional studies of ANKTM1 agree with the phylogenetic characterization of this channel as TRP-like. There is now strong evidence that a group of TRP channels play a role in temperature detection in the mammalian peripheral nervous system. ANKTM1 belongs to the third TRP subfamily shown to play a role in temperature detection. Like other thermosensitive TRPs, ANKTM1 is a non-selective cation channel.

ANKTM1 is activated at lower temperatures than TRPM8, starting near 17°C, which approximates the threshold of noxious cold for humans (~15°C) (Davis and Pope, 2002). A role for ANKTM1 in noxious cold detection is also suggested by its expression pattern. Mouse ANKTM1 is specifically expressed in somatic

sensory neurons. Within this population, ANKTM1 is not expressed in neurons that express TRPM8. Instead, the vast majority of ANKTM1-positive cells also express TRPV1 and CGRP, markers for pain-sensing neurons. Our expression analysis reveals the presence of two distinct populations of cold-responding neurons: TRPM8-positive neurons (that lack TRPV1 expression) and ANKTM1-positive neurons (that also express TRPV1).

We have functionally validated our in vivo expression analysis by short-term cultures of adult DRG neurons. Two populations of cold-sensitive neurons are observed: one population responds to cool temperatures and menthol but not to capsaicin (presumptive TRPM8-positive neurons), and another population responds to cold and capsaicin but not to menthol (presumptive ANKTM1/TRPV1-positive neurons). Recent reports have also demonstrated the presence of two distinct groups of cold-sensitive neurons (M.L Nealen et al., 2002, and D.W. Beacham et al., 2002, Soc. Neurosci., abstract). However, most previous DRG culture studies have identified a significant percentage of neurons that respond both to menthol and capsaicin, implying coexpression of TRPM8 and TRPV1 (Mckemy et al., 2002; Reid et al., 2002). We believe that the response of single neurons to both menthol and capsaicin is due to a change in TRPV1 (or TRPM8) expression or activity levels induced by the culture conditions. Indeed, the presence of NGF (which is routinely used at high levels in these cultures) is known to have profound effects on capsaicin/heat-responsive DRG neurons in culture (Aguayo and White, 1992). NGF sensitizes TRPV1 activity, and has been shown to upregulate TRPV1 mRNA and protein in cultured DRG neurons (Chuang et al., 2001; Ji et al., 2002; Shu and Mendell, 1999; Winston et al., 2001). In our culture experiments, menthol/capsaicin coresponsive cells are readily detected in cultures with NGF, but no such coresponsive cells are observed in the absence of NGF. On the other hand, all of the menthol-insensitive cold neurons respond to capsaicin with or without NGF. These observations are consistent with our in vivo expression analysis and suggest that TRPM8-positive cells that might express low levels of TRPV1 mRNA in vivo (undetectable by in situ hybridizations) could gain capsaicin-responsiveness in culture with high NGF. High levels of NGF in culture may resemble an inflammatory state for these DRGs neurons. If coexpression of TRPM8 and TRPV1 indeed occurs in vivo during inflammation, it may have important physiological consequences.

Here, we show that a cold-activated channel (ANKTM1) is expressed in a subset of heat-sensitive (TRPV1-expressing) neurons. This suggests that such neurons

correspond to previously observed polymodal nociceptive DRG neurons that respond to both noxious hot and cold (Cain et al., 2001; Campero et al., 1996). Such neurons raise the question of how temperature information is then decoded in the spinal cord and brain. Electrophysiological characterization of DRG neurons has led to the labeled line hypothesis of sensory perception (Perl, 1998). According to this hypothesis, the nervous system interprets the environment by monitoring the electrical activity of distinct groups of sensory neurons that are specialized to detect a unique sensory modality: activity in "cold neurons" gives rise to the perception of cold and is distinguishable from activity in "hot neurons". What would this hypothesis predict in the case of polymodal nociceptors?

Potentially, combinatorial activity in TRPV1-, ANKTM1/TRPV1-, and TRPM8-positive neurons could be integrated to interpret the actual temperature of a given stimulus. For example, activation of only ANKTM1/TRPV1-positive neurons would signal a noxious cold stimulus, while activation of both TRPV1- and ANKTM1/TRPV1-positive neurons would signal a noxious heat stimulus. The distinction of whether the noxious stimulus is hot or cold could also be dependent on the firing activity of TRPM8 neurons. The expression of ANKTM1 in a subset of TRPV1-positive neurons might also provide a molecular explanation of paradoxical cold (a local heat stimulus produces a cold sensation) (Dodt and Zotterman, 1952). Alternative to a labeled line theory, it is possible that neurons expressing both channels might distinguish the signals transduced from cold and hot stimuli. The strong desensitization of ANKTM1 in response to a persistent cold stimulus compared to the response of TRPV1 to noxious heat might contribute to such a distinction.

Collectively, our data suggests a model in which TRPM8-expressing neurons are involved in transmitting an innocuous and pleasant cooling sensation (due to its activation by menthol and its expression pattern that is mutually exclusive with any known nociceptive markers), while ANKTM1-expressing neurons are involved in transmitting a noxious cold stimulus (due to its lower threshold of activation and its expression pattern that strongly overlaps with nociceptive markers such as TRPV1 and CGRP). It is not clear if all cold sensation can be explained by the combined activity of ANKTM1 and TRPM8. Alternative mechanisms of cold sensitivity have been proposed, and these could function in conjunction with TRP channels (Askwith et al., 2001; Maingret et al., 2000; Reid and Flonta, 2001; Viana et al., 2002).

Experimental Procedures

Molecular Cloning of ANKTM1

Bioinformatic searches were done as described (Peier et al., 2002a, 2002b). Sequence analysis was performed using the Biology Workbench at the San Diego Supercomputing Center (http://workbench.sdsc.edu). A 902-base pair fragment of the mouse homolog of ANKTM1 was amplified from newborn mouse DRG cDNA using primers mANK-like F2 (5′-AGTGGGGAGACTACCCTGTG) and mANK-like R2 (5′-TTTATCATGCCCATTCTTTGC). From this initial sequence and subsequent hits to DNA databases, primers mANK-like start (5′TTTGGATCCGCCACCATGAAGCGCGGCTTGAGGAGG) and mANK-like stop (5′TTTGCGGCCGCCTAAAAGTCCGGGTGGCTAATAGAAC)

were designed to PCR-amplify full-length ANKTM1 from adult mouse trigeminal ganglia cDNA.

Expression Analysis

The rat tissue Northern blot was prepared as described (Peier et al., 2002a). Membranes were hybridized with a ^{32}P-labeled probe corresponding to nucleotides 590–1492 of mouse full-length ANKTM1.

For in situ hybridization and immunostaining of DRGs, adult mice were perfused with 4% paraformaldehyde and DRGs were rapidly dissected. Following post-fixation and cryo-protection in 30% sucrose in PBS, single DRGs were embedded in OCT, frozen in liquid nitrogen, and sectioned at 10 μm thickness. For double in situ hybridizations, sections were hybridized with in vitro transcribed digoxigenin- or fluorescein-labeled cRNA probes (Roche) corresponding to nucleotides 590–1492 of mANKTM1 and 1410–1980 of mTRPM8 (NM_029310). We used two cRNA probes corresponding to bases 1516–2065 or 1516–2482 of TRPV1 sequence (AF029310). Both probes showed consistent patterns of hybridization. Peroxidase-conjugated anti-digoxigenin-POD (1:500) and alkaline phosphatase-conjugated anti-fluorescein (1:2000) antibodies (Roche) were used to detect hybridized cRNA probes and visualized using tyramide signal amplification (TSA; NEN) and fast-red detection (Roche) systems, respectively. The immunostaining experiments followed hybridization of sections with digoxigenin-labeled cRNA probes and TSA fluorescent detection. Anti-NF150 kd (1:1000; Chemicon) and anti-CGRP (1:100; Biogenesis) primary antibodies and anti-rabbit Cy3 (1:200, Jackson Immunoresearch) secondary antibodies were used.

CHO Cell Expression System

ANKTM1-expressing CHO cells were generated as previously described (Peier et al., 2002a). ANKTM1-expressing stable CHO-K1/FRT lines appeared unhealthy (membrane blebbing, cytoplasmic granulations) in culture, and after several passages, a loss of ANKTM1 expression was observed (data not shown). Other investigators have previously claimed difficulty generating stable cell lines expressing human ANKTM1 (Jaquemar et al., 1999). To circumvent this problem, we generated cell lines in which we could control ANKTM1 expression. Tetracycline-inducible CHO-K1/FRT cells lines were generated by transfecting the CHO Flp-In host cell line with pcDNA6/TR according to manufacturer's instructions (Invitrogen). The expression vector pcDNA5FRT/TO containing full-length mouse ANKTM1 and the Flp recombinase expression plasmid pOG44 were cotransfected into the tetracycline-inducible CHO-K1/FRT cell line and selected via hygromycin resistance (200 μg/ml). Induction of ANKTM1 was accomplished by treating CHO cells with 1–2 μg/mL Tet 5–24 hr before experiments. Stable clones expressing ANKTM1 were identified by Northern blot analysis. Northern blot analysis of control and Tet-treated ANKTM1-inducible CHO cells showed high levels of ANKTM1 expression. A low-level of ANKTM1 expression in the absence of Tet was also present in these cells (data not shown). Therefore, CHO cells that stably expressed the Tet-repressor were used as controls in subsequent experiments. For some of the experiments described, we maintained cells in culture medium supplemented with 5 μm ruthenium red (RR, Fluka), which blocks ANKTM1 activity. This was done to overcome the slight leaky expression of ANKTM1 in the Tet-system. Cells cultured in RR looked healthier and a higher percentage of cells responded to cold compared to non-RR treated cells. The threshold of activation was not affected. For coexpression studies of ANKTM1 and TRPV1, Tet-inducible CHO cells stably expressing ANKTM1 were transiently cotransfected with human TRPV1 in the pcDNA5/FRT expression plasmid and GFP. ANKTM1 expression was induced by Tet treatment 24 hr after transfection.

Intracellular Calcium Imaging Experiments

Calcium imaging experiments were performed essentially as described (Peier et al., 2002a). Images of Fura-2 loaded cells with the excitation wavelength alternating between 340 nm and 380 nm were captured with a cooled LCD camera. The ratio of fluorescence intensity of the two wavelengths of groups of 30–50 cells in each experiment was analyzed using MetaFluor (Universal Imaging Corp). Ex-

periments were performed in triplicate. Unless otherwise indicated, graphs represent averaged responses of 20–40 cells from representative experiments. For direct comparison of results from different experiments, values were normalized to baseline of the ratio 340/380. For ANKTM1 and TRPM8 threshold experiments, the threshold temperature of activation of 60–100 cells each from three replicate experiments was analyzed. Threshold of activation was defined as 20% above baseline. For ANKTM1/TRPV1 coexpression experiments, GFP-positive cells in a field were chosen for analysis. Differences in threshold of activation were analyzed by using Student's t test. Hanks balanced salt solution (HBSS) and HEPES buffers were obtained from GibcoBRL. The compounds menthol, capsaicin, and icilin were obtained from Sigma, Fluka, and Tocris Woodson, respectively.

Electrophysiology

Recordings were performed essentially as described (Peier et al., 2002a). Recording solutions were as follows: pipette solution for all experiments was 140 MM CsCl; 10 mM BAPTA; 10 mM HEPES; 2 mM MgATP; titrated to pH 7.4 with CsOH. The external solution in the recording chamber was kept at 32°C and consisted of 100 mM cholineCl; 40 mM NaCl; 10 mM HEPES; 2 mM $CaCl_2$; 1 mM $MgCl_2$; titrated to pH 7.4 with NaOH. In some studies, the above solution was used with 140 mM NaCl and no choline. In monovalent permeability studies, equimolar KCl or CsCl replaced NaCl (40 mM). In divalent permeability studies, the solutions contained either 1 mM or 30 mM of test ion (Ca or Mg) and 100 mM cholineCl; 40 mM NaCl; and 10 mM HEPES. Osmolarity was maintained by addition of sucrose (80 mM) to the 1 mM divalent cation solution. The current-voltage relationships were determined using a 2 s ramp from −100 to +80 mV.

The permeability ratio of choline relative to Na^+ ($P_{choline}/P_{Na}$) was calculated, after partial replacement of the external Na with choline, from the following equation: $E_{shift} = \{RT/F\} \ln \{[Na]_{O(2)} + (P_{choline}/P_{Na} [choline]_{O(2)})/[Na]_{O(1)}\}$, where F is Faraday's constant, R is the universal gas constant, T is absolute temperature, $[Na]_{O(1)}$ is the initial concentration of external Na^+ and $[Na]_{O(2)}$ and $[choline]_{O(2)}$ refer to the concentration of these ions after substitution. Relative permeability ratios of other monovalent cations (X^+) were calculated using the equation $E_{shift} = \{RT/F\} \ln \{(P_X/P_{Na}[X]_{O(2)} + (P_{choline}/P_{Na} [choline]_{O(2)})/([Na]_{O(1)} + (P_{choline}/P_{Na} [choline]_{O(1)})\}$, where solution (1) contains Na^+ plus choline and solution (2) contains the substitution ion (X) plus choline. Permeability ratios relative to Na^+, for divalent cations were calculated as follows: $E_{shift} = \{RT/F\} \ln \{[Na]_O + P_{choline}/P_{Na}[choline]_O + 4B' [X]_{O(2)}\}/\{[Na]_O + P_{choline}/P_{Na}[choline]_O + 4B' [X]_{O(1)}\}$, where $B' = P'_X/P_{Na}$ and $P'_X = P_X/(1 + e^{EF/RT})$ and $[X]_{O(1)}$ and $[X]_{O(2)}$ refer to the two different concentrations of the divalent ions (Ca^{2+} or Mg^{2+}) tested.

ANKTM1 and TRPM8 were cloned into the pOX expression vector for expression in *Xenopus* oocytes (Jegla and Salkoff, 1997). Capped cRNAs were prepared by run-off transcription using the T3 mMessage mMachine kit (Ambion) and cleaned prior to injection using Qiaquick columns (Qiagen). Mature oocytes were isolated enzymatically as described (Wei et al., 1990). Oocytes were injected with 5–25 ng of cRNA in a 50 nl volume and incubated 2–5 days prior to recording at 18°C in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl, 1 mM MgCl, 5 mM HEPES, 2.5 mM Na-pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and [pH 7.5]). Recordings were made using standard two-electrode voltage clamp techniques with 3 M KCl electrodes (0.3–0.5 MΩ). The recording solution consisted of 96 mM NaCl, 4 mM KCl, 1 mM MgCl, 100 μM CaCl, and 5 mM HEPES, [pH 7.5]. Temperature control was achieved with a combination of a peltier-cooled stage and constant perfusion of cooled/heated solution. Temperatures were monitored using a miniature thermocouple placed adjacent to the oocyte in the recording chamber. Currents and temperatures were recorded using a TEV-200 amplifier (Dagan Instruments), an HCC-100A temperature controller (Dagan Instruments), and pCLAMP8 software suite (Axon Instruments).

DRG Cultures

Dorsal root ganglia from all spinal levels of adult Sprague Dawley rats were rapidly dissected and dissociated by incubation for 3 hr at 37°C in a solution of culture medium (Ham's F12/DMEM with 10% Horse Serum, 1% penicillin-streptomycin) containing 0.125% collagenase (Invitrogen). Cells were gently triturated using fire-pol-

ished Pasteur pipettes and centrifuged in culture medium containing 15% BSA to separate cells from debris. Cells were resuspended and plated onto coverslips coated with polyornithine (0.5 mg/mL; Sigma) and laminin (5 μg/mL; Invitrogen). Cells were cultured in medium either with or without 100 ng/mL NGF (Invitrogen). Calcium imaging experiments were performed 18–36 hr after plating. For calcium imaging experiments, DRG neurons were treated as described for CHO cells except that they were loaded with Fura-2 for 30 min at 37°C.

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