

Ion channels activated by cold and menthol in cultured rat dorsal root ganglion neurones

Gordon Reid^{*}, Maria-Luiza Flonta

Department of Animal Physiology and Biophysics, Faculty of Biology, University of Bucharest, Splaiul Independenței 91-95, 76201 Bucharest, Romania

Received 23 January 2002; received in revised form 18 February 2002; accepted 18 February 2002

Abstract

A cold- and menthol-activated ionic current has been described in sensory neurones, which probably has a role in temperature sensing. Here we describe the ion channels underlying this current. Cooling activated non-selective cation channels (conductance, about 22 pS; reversal potential, -4.2 mV) in outside-out patches from cold-sensitive rat dorsal root ganglion neurones, and their activity was strongly increased by menthol. The activation threshold was 17.9 °C, shifting to 24.3 °C in 100 μ M (-)-menthol, about 10 °C colder than observed in intact neurones. Channels in excised patches did not adapt to sustained cooling, unlike the current in intact neurones. We conclude that the ion channels underlying the cold- and menthol-induced current are directly activated by these stimuli, although other modulatory factors appear to be important in determining threshold and adaptation. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cold; Thermoreceptor; Sensory transduction; Adaptation; Dorsal root ganglion

Skin temperature is detected by specific warm and cold receptors. Although the responses of these receptors to thermal stimuli have been intensively studied [7,14], thermal transduction is still probably the least understood of any sensory modality. The main obstacle to progress has been the inaccessibility of cutaneous receptor terminals to direct electrophysiological measurement. The cell body of a sensory ganglion neurone maintained in culture is a good model of its receptor terminal [3], and it has been shown that cold transduction in this model depends on at least two mechanisms. Firstly, cooling activates an inward non-selective cation current [11,17], and secondly, it inhibits a background K^+ current [12] with similar properties to the TREK-1 channel which had already been suggested as a possible cold sensor [9]. The inward current is sensitized by the cold receptor stimulant (-)-menthol, and it shares several characteristics with intact cold receptors, including adaptation and modulation by Ca^{2+} [11]; it therefore seems likely that it plays the dominant role in cold sensing.

It is not yet clear whether this cold-activated inward current results from direct activation of an ion channel by

cold, analogous to the direct activation of some ion channels by heat [4,5], or whether temperature acts on an intracellular signalling pathway which modulates activity of a temperature-insensitive ion channel, as has been suggested for a warm-activated current in sensory neurones [10]. The present study was intended to answer this question by testing whether ion channels are activated by cold and menthol in excised membrane patches from cold-sensitive neurones, and to investigate to what extent these channels share the properties of the whole-cell current.

Cultures of adult rat dorsal root ganglion (DRG) neurones were prepared essentially as previously described [12], but without the use of capsaicin and with 10% horse serum (Sigma) in place of the serum-free supplement. Experiments were carried out after 1–4 days in culture. Basal temperature during recordings was maintained at 32 °C, close to mammalian skin temperature [7], and cooling stimuli were applied locally with a system already described [13]. The standard extracellular solution and K_2SO_4 -based pipette solution were the same as used previously [12]; experiments including voltage ramps were made with a CsCl-based pipette solution (140 mM CsCl, 6 mM NaCl, 10 mM HEPES, 2 mM EGTA, 7.6 mM NaOH, pH 7.2 at 25 °C) to inhibit outward K^+ currents. Pipette solutions did not contain adenosine triphosphate or guanosine triphosphate.

^{*} Corresponding author. Tel.: +40-93-188900; fax: +40-1-411-3933.

E-mail address: gordon@biologie.kappa.ro (G. Reid).

(-)-Menthol (Sigma) was added to the extracellular solution from a 200 mM stock solution in ethanol, which was stored in a glass bottle at -20°C to avoid evaporation; fresh working solutions were made every 2 h.

We identified cold-sensitive DRG neurones by measuring the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) during cooling ramps to $18\text{--}20^{\circ}\text{C}$ [15]. Cells were loaded at 37°C for 30 min with $2\ \mu\text{M}$ Calcium Green-AM in standard extracellular solution containing 0.02% Pluronic (both from Molecular Probes, Leiden, the Netherlands). Fluorescent images were collected with a $\times 40/\text{NA } 0.95$ dry objective (Nikon) and a COHU 4910 integrating camera (COHU, San Diego, CA), using software written by G.R., and analyzed using programs written in the IDL language (Research Systems, Boulder, CO). The change in fluorescence (ΔF) relative to its initial level (F_0) was used as an index of $[\text{Ca}^{2+}]_i$ [16]. Neurones showing an abrupt increase in fluorescence at a distinct threshold temperature and a peak $\Delta F/F_0$ of at least 0.15 were selected for electrophysiological recording; these constitute about 7% of the total [11].

Outside-out patches from cold-sensitive neurones were recorded at $-80\ \text{mV}$ with an EPC-7 amplifier (HEKA, Lambrecht, Germany) using borosilicate glass pipettes (GC150T, Harvard Apparatus), heat-polished to a resistance of $6\text{--}10\ \text{M}\Omega$ and coated with Sylgard 184 (World Precision Instruments, Berlin, Germany). Currents were filtered at 10 kHz (three-pole Bessel) and recorded on video tape using a modified Sony pulse code modulation audio processor, then sampled offline at 40 kHz with GATHER software on a Labmaster 160 kHz DMA interface (both from Scientific Solutions, Mentor, OH). Data were analyzed using programs written in IDL and with pClamp 6 (Axon Instruments, Union City, CA). All values are expressed as means \pm SD. The present report is based on 32 outside-out patches from 21 cold-sensitive neurones.

From a base temperature of 32°C , cooling elicited channel openings in 28 of 32 patches (Fig. 1A). The threshold temperature for channel activation during 30-s ramps from 32 to 13°C was $17.9 \pm 1.7^{\circ}\text{C}$ (range, $15.4\text{--}19.8^{\circ}\text{C}$, $n = 10$). Application of (-)-menthol ($100\ \mu\text{M}$) induced a dramatic increase in channel activity at low temperatures in all of these 28 patches, with no activity at the base temperature of 32°C (Fig. 1A), and shifted the activation threshold to $24.3 \pm 2.0^{\circ}\text{C}$ ($20.7\text{--}29.3^{\circ}\text{C}$, $n = 18$). The mean threshold shift induced by menthol was $6.1 \pm 1.8^{\circ}\text{C}$ ($4.2\text{--}9.1^{\circ}\text{C}$, $n = 9$). Menthol did not induce any channel activity in the four patches that contained no cold-activated channels. Cyclohexanol, the cyclic alcohol on which menthol is based, did not induce channel openings or alter cold-induced channel activity ($n = 4$).

Cold-activated channels showed sporadic brief openings, with a very low open probability even at 5°C ; open probability was very much higher in the presence of menthol (Fig. 1B). The single-channel current at $-80\ \text{mV}$ was measured using all-points histograms based on channel openings of sufficient duration to reach a well-defined

open level. Without menthol, the single-channel current was $1.67 \pm 0.26\ \text{pA}$ ($1.43\text{--}2.23\ \text{pA}$, $n = 8$) and in $100\ \mu\text{M}$ (-)-menthol it was $1.73 \pm 0.25\ \text{pA}$ ($1.44\text{--}2.02\ \text{pA}$, $n = 7$); this difference is not significant ($P = 0.67$, two-tailed paired t -test, $n = 7$). Some channel openings, especially in menthol, had an amplitude about twice the main level (see Fig. 1B, right panel), with a clear transition from the main level to the higher level or vice versa. During periods of low open probability, such transitions were more frequent than would be expected from the opening of two independent channels, raising the possibility either of an additional conductance level larger than the main one, or of cooperativity between neighbouring channels. These possibilities were not investigated further in the present study. The reversal potential of the single-channel current was $-4.2 \pm 3.4\ \text{mV}$ with CsCl-based pipette solution (-7.0 to $+0.5\ \text{mV}$, $n = 4$; Fig. 2A), indicating a low degree of selectivity between monovalent cations. The single-channel conductance calculated from this current amplitude and reversal potential is approximately $22\ \text{pS}$ for inward current, but outward rectification was strong (Fig. 2A).

Although the channels did not require any component outside the membrane for their activation by cold and menthol, we observed pronounced differences between their behaviour in excised patches and in the intact neurone. Firstly, there was virtually no accommodation in excised patches: during long periods of cooling ($1\text{--}3\ \text{min}$, to 6°C ($n = 12$) and $11\text{--}12^{\circ}\text{C}$ ($n = 5$) in the absence of menthol, and to 6°C ($n = 19$), $11\text{--}12^{\circ}\text{C}$ ($n = 9$) and $16\text{--}18^{\circ}\text{C}$ ($n = 6$) in $100\ \mu\text{M}$ (-)-menthol), channel activity remained constant or declined only slightly (Fig. 1A). This suggests that the near-complete accommodation we observed over a similar period in intact neurones [11] is not a property of the ion channels themselves, but depends on a modulatory factor that is lost on patch excision. Secondly, the temperature thresholds mentioned above in excised patches are $10\text{--}15^{\circ}\text{C}$ lower than those in intact neurones [11] (Fig. 2B). To exclude the possibility that we underestimated the activation threshold by measuring it from the first channel opening during a cooling ramp, we confirmed the thresholds in $100\ \mu\text{M}$ menthol in five patches that were stable for long enough periods to allow activity during several cooling ramps to be averaged (trace (a) in Fig. 2B). The same threshold values were obtained from averaged currents as from single-channel activity in the same patch (mean difference, $0.4 \pm 1.8^{\circ}\text{C}$; range, -1.7 to $+2.6^{\circ}\text{C}$, $n = 5$). To exclude a major change in the properties of the whole-cell current between the previous study and this one, thresholds were compared directly in five intact neurones (using perforated-patch recording; see Ref. [12] for methods) and in five outside-out patches from the same neurones. In $100\ \mu\text{M}$ (-)-menthol, the threshold in the patches was $13.4 \pm 2.5^{\circ}\text{C}$ lower than in their parent neurones (range, $10.7\text{--}16.3^{\circ}\text{C}$, $n = 5$). The shift in temperature sensitivity on patch excision is therefore real and substantial.

In this study, we have described cold- and menthol-acti-

vated ion channels that are likely to underlie the cold- and menthol-induced current previously reported [11,17]. The channels are directly sensitive to temperature; cold is therefore not sensed by an intracellular modulatory pathway acting on a temperature-insensitive ion channel. Menthol

activation is also independent of soluble intracellular factors. However, channels in excised patches do show two striking differences from those in the intact cell: they are substantially less sensitive to cold, and they do not adapt on sustained cooling. This suggests that activation threshold

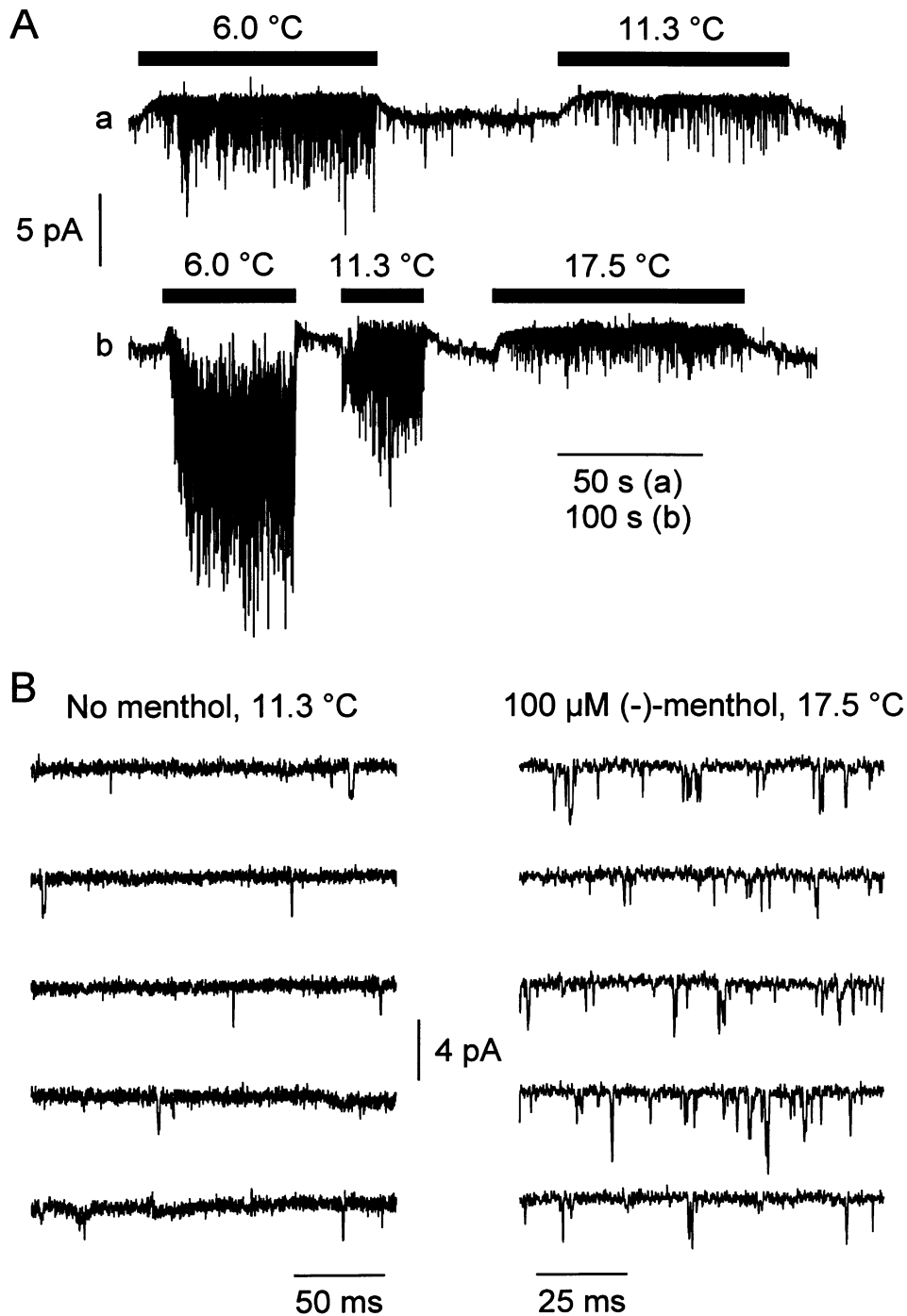


Fig. 1. Ion channel activity induced by cold and menthol in outside-out patches. (A) Activation by cooling (bars) from a base temperature of 32 °C to the temperatures shown, in the absence (a) and presence (b) of 100 μM (-)-menthol, in the same outside-out patch. Note the lack of adaptation on prolonged cooling. (B) Brief periods of channel activity from this recording on an expanded time scale, showing five consecutive periods of 200 (cold) or 100 ms (menthol), running from top to bottom. Holding potential, -80 mV; K₂SO₄-based pipette solution.

and adaptation depend to an important degree on intracellular mechanisms, as yet unidentified, which are lost on patch excision.

The channels activated by menthol are strongly cold-sensitive: menthol increased channel activity dramatically at low temperatures but induced no channel activity at the

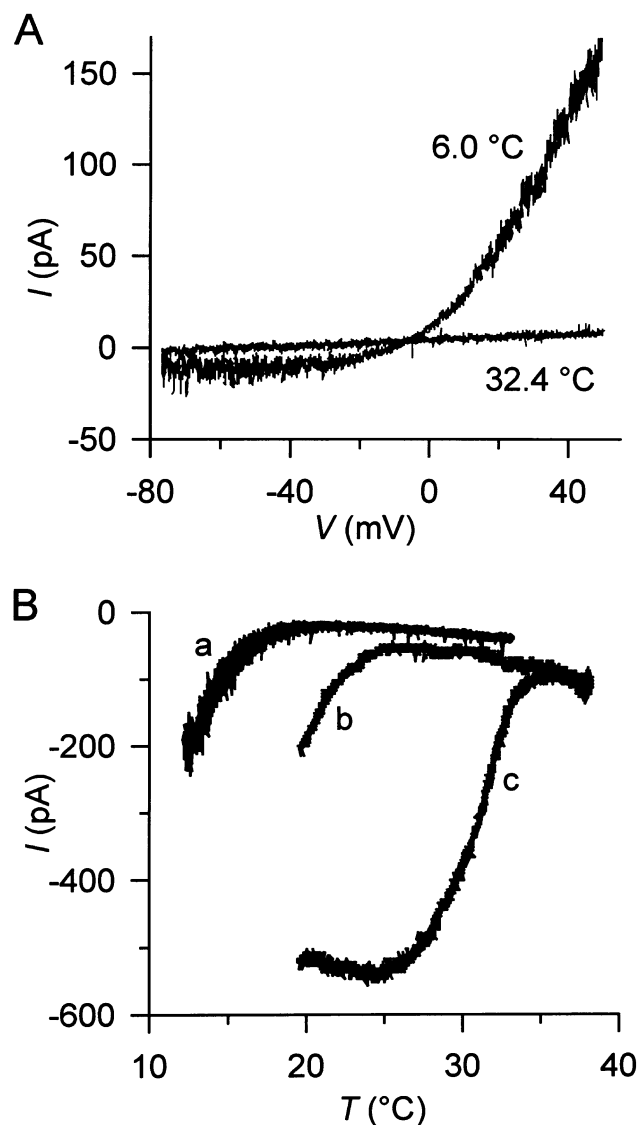


Fig. 2. Current–voltage and current–temperature relation of cold- and menthol-activated ion channels. (A) Current–voltage relation in a large outside-out patch in the presence of 100 μM (-)-menthol at 32.4 and 6.0 $^{\circ}\text{C}$, during a voltage ramp from -80 to $+50$ mV over 1.8 s. The reversal potential of the channels activated by cold in the presence of menthol was -4 mV in this patch. CsCl-based pipette solution. (B) Current–temperature relation of summed current from ten cooling ramps in another large outside-out patch in 100 μM (-)-menthol (a), compared with the whole-cell current (amphotericin perforated patch configuration, see Ref. [12] for method) without menthol (b) and in 100 μM (-)-menthol (c). The threshold in the excised patch in the presence of menthol was 15 $^{\circ}\text{C}$ lower than in the intact neurone. Holding potential, -80 mV; CsCl-based pipette solution in (a), K_2SO_4 -based pipette solution with amphotericin in (b) and (c).

base temperature of 32 $^{\circ}\text{C}$ (Fig. 1A). Cold and menthol therefore act on the same receptor, an ion channel that appears to be directly activated by these stimuli in a manner roughly analogous to the modulation of the vanilloid receptor VR1 by capsaicin, protons and heat [4,18] or cold [2]. The present study does not give a clear indication of what family of ion channels this receptor might belong to, although there are at least two clear candidates, the transient receptor potential (TRP) channels [6] and the degenerins [8]. These families account for most known transduction channels, and some of their members are gated or modulated by heat [4,5] or cold [1], respectively. The non-selectivity and rectification of the putative cold/menthol receptor described here are suggestive of the TRP family, whereas its small conductance is more consistent with the degenerins; its molecular identity will probably remain ambiguous until the sequence of the cloned channel is published.

Note added in proof

A few weeks after submission of this article, the cloning of a cold- and menthol-activated ion channel was reported (McKemy et al., *Nature* 416:52–58 (2000); Peier et al., *Cell* 108:705–715 (2000)). This channel, named respectively cold and menthol receptor-1 (CMR-1) or TRPM8, is a member of the TRP family. Its properties are fully consistent with those of the native cold- and menthol-activated current.

Funding was from the Romanian National Research Council CNCSIS through a loan from the International Bank for Reconstruction and Development (Grant C-326), from NATO and from the Physiological Society under the “Centres of Excellence” scheme. The authors would like to thank Dr Alexandru Babeș for comments on the manuscript, Dr Hans Braun and Dr Andreas Scholz for stimulating discussion, Dan Zorzon for expert technical assistance, Catriona Reid for helping with the image analysis and Victorița Teirău for valuable background support.

- [1] Askwith, C.C., Benson, C.J., Welsh, M.J. and Snyder, P.M., DEG/ENaC ion channels involved in sensory transduction are modulated by cold temperature, *Proc. Natl. Acad. Sci. USA*, 98 (2001) 6459–6463.
- [2] Babes, A., Amuzescu, B., Krause, U., Scholz, A., Flonta, M.-L. and Reid, G., Cooling inhibits capsaicin-induced currents in cultured rat dorsal root ganglion neurones, *Neurosci. Lett.*, 317 (2002) 131–134.
- [3] Baccaglini, P.I. and Hogan, P.G., Some rat sensory neurons in culture express characteristics of differentiated pain sensory cells, *Proc. Natl. Acad. Sci. USA*, 80 (1983) 594–598.
- [4] Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D. and Julius, D., The capsaicin receptor: a heat-activated ion channel in the pain pathway, *Nature*, 389 (1997) 816–824.
- [5] Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J. and Julius, D., A capsaicin-receptor homologue with a high threshold for noxious heat, *Nature*, 398 (1999) 436–441.

- [6] Clapham, D.E., Runnels, L.W. and Strübing, C., The TRP ion channel family, *Nat. Rev. Neurosci.*, 2 (2001) 387–396.
- [7] Darian-Smith, I., Thermal sensibility, In I. Darian-Smith (Ed.), *Handbook of Physiology (Section 1 – The Nervous System)*, American Physiological Society, Bethesda, MD, 1984, pp. 879–913.
- [8] Gillespie, P.G. and Walker, R.G., Molecular basis of mechanosensory transduction, *Nature*, 413 (2001) 194–202.
- [9] Maingret, F., Lauritzen, I., Patel, A.J., Heurteaux, C., Reyes, R., Lesage, F., Lazdunski, M. and Honoré, E., TREK-1 is a heat-activated background K^+ channel, *EMBO J.*, 19 (2000) 2483–2491.
- [10] Reichling, D.B. and Levine, J.D., Heat transduction in rat sensory neurons by calcium-dependent activation of a cation channel, *Proc. Natl. Acad. Sci. USA*, 94 (1997) 7006–7011.
- [11] Reid, G. and Flonta, M.-L., Cold current in thermoreceptive neurons, *Nature*, 413 (2001) 480.
- [12] Reid, G. and Flonta, M.-L., Cold transduction by inhibition of a background potassium conductance in rat primary sensory neurones, *Neurosci. Lett.*, 297 (2001) 171–174.
- [13] Reid, G., Amuzescu, B., Zech, E. and Flonta, M.-L., A system for applying rapid warming or cooling stimuli to cells during patch clamp recording or ion imaging, *J. Neurosci. Methods*, 111 (2001) 1–8.
- [14] Spray, D.C., Cutaneous temperature receptors, *Annu. Rev. Physiol.*, 48 (1986) 625–638.
- [15] Suto, K. and Gotoh, H., Calcium signaling in cold cells studied in cultured dorsal root ganglion neurons, *Neuroscience*, 92 (1999) 1131–1135.
- [16] Svoboda, K., Denk, W., Kleinfeld, D. and Tank, D.W., In vivo dendritic calcium dynamics in neocortical pyramidal neurons, *Nature*, 385 (1997) 161–165.
- [17] Takao, K., Matsumura, K. and Kobayashi, S., Ionic basis of cold-sensitive neurons cultured primarily from dorsal root ganglia (DRG), *Soc. Neurosci. Abstr.*, 25(Part 1) (1999) 405.
- [18] Tominaga, M., Caterina, M.J., Malmberg, A.B., Rosen, T.A., Gilbert, H., Skinner, K., Raumann, B.E., Basbaum, A.I. and Julius, D., The cloned capsaicin receptor integrates multiple pain-producing stimuli, *Neuron*, 21 (1998) 531–543.