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INVITED REVIEW

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ThermoTRP channels and cold sensing: what are they really up to?

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Abstract Cooling is sensed by peripheral thermoreceptors, the main transduction mechanism of which is probably a cold- and menthol-activated ion channel, transient receptor potential (melastatin)-8 (TRPM8). Stronger cooling also activates another TRP channel, TRP (ankyrin-like)-1, (TRPA1), which has been suggested to underlie cold nociception. This review examines the roles of these two channels and other mechanisms in thermal transduction. TRPM8 is activated directly by gentle cooling and depolarises sensory neurones; its threshold temperature (normally ~26–31°C in native neurones) is very flexible and it can adapt to long-term variations in baseline temperature to sensitively detect small temperature changes. This modulation is enabled by TRPM8's low intrinsic thermal sensitivity: it is sensitised to varying degrees by its cellular context. TRPM8 is not the only thermosensitive element in cold receptors and interacts with other ionic currents to shape cold receptor activity. Cold can also cause pain: the transduction mechanism is uncertain, possibly involving TRPM8 in some neurones, but another candidate is TRPA1 which is activated in expression systems by strong cooling. However, native neurones that appear to express TRPA1 respond very slowly to cold, and TRPA1 alone cannot account readily for cold nociceptor activity or cold pain in humans. Other, as yet unknown, mechanisms of cold nociception are likely.

Keywords Cold · Menthol · Thermoreceptors · Transduction · Adaptation · Nociception · TRPM8 · TRPA1

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The past four years have transformed our understanding of how temperature is sensed by thermoreceptors and nociceptors. Moving on from the isolation of two noxious heat transducers, transient receptor potential (vanilloid)-1 and -2 (TRPV1 and TRPV2), four other thermally activated TRP channels have been identified and the whole family dubbed "thermoTRPs" [44, 63]. This review will focus on the two cold-activated thermoTRP channels, TRP (melastatin)-8 (TRPM8) [57, 64] and TRP (ankyrin-like)-1 (TRPA1) [87]. The biophysics, pharmacology and modulation of these channels in expression systems is becoming understood in increasingly fine detail [1, 6, 7, 10, 20, 43, 53, 95], but work in the field has drifted away somewhat from its roots in native cold receptors. At the same time, an oversimplified model of cold sensing is gaining ground, as a look at some teaching and popular science websites will reveal, with TRPM8 sometimes presented as "the" receptor for innocuous cooling, and TRPA1 as "the" noxious cold receptor (the original thermoTRP literature paints a more subtle picture). For both reasons, it is now a good time to return to the native systems and take a close look at the functions of these cold-activated TRP channels.

In the following, I will survey what we know about transduction in native cold thermoreceptors, largely from patch-clamp recordings and intracellular calcium measurements on cultured sensory neurones, and try to relate this to work on cloned TRPM8 and TRPA1 as well as to mammalian cold receptors in vivo and human psychophysics. My conclusion will be that TRPM8 explains much of what we know about innocuous cold sensing, but not all of it, while the role of TRPA1 in sensing noxious cold is far from certain.

The many uses of cold sensing

Thermal information from our skin surface is used in many ways. One of them is object identification: when we pick something up, it feels cool or neutral (less often warm), and this tells us whether it is made of wood, metal,

plastic, glass or stone, and whether it is dry or wet. Cold can hurt, too, and cold-induced pain has several different qualities: a deep ache experienced in cold, wet weather or very cold water; the burning pain of playing with snowballs; and the sudden sharp pain of skin freezing.

Thermoregulation is another essential function of both central and peripheral thermoreceptors. Skin thermoreceptors have a "feed-forward" role in behavioural thermoregulation: when ambient temperature drops, we put on extra clothes or close a window to keep warm before our core temperature begins to change. We do this because we feel the air to be unpleasantly cold: the affective aspect of thermoreception is involved both in our own subjective comfort or discomfort and in our feelings towards those with whom we come into contact, and is discussed lucidly by Craig [22]. Although not obvious at first sight, the emotional content of thermal sensations is of crucial importance in human interactions: leaving aside the more intimate situations where thermoreceptive input comes into play, recall your impressions on first meeting an individual whose handshake is warm or cool.

Peripheral cold receptors in mammals

The first clear suggestion that these sensations originate in discrete receptors in our skin came from 19th century work on "sensory spots" (reviewed in [59]). Sensations can be elicited only from discrete points on the skin, usually specific for one modality ("warm spots", "cold spots" and so on). Importantly, strong heat applied to a cold spot elicits a sensation of cold, not heat (the "paradoxical cold" sensation). These observations led naturally to the idea that discrete receptors, each specific to one modality, may underlie the spots.

Action potentials from cold receptors were first recorded in the 1930s by Zotterman [99] and first studied in detail by Hensel and Zotterman [39, 40] (see [65] for a review of early work on skin receptors). Early work was mostly in cats and was held back by their relatively poor thermoreceptive innervation; substantial quantitative information on thermoreceptors came only when work began in primates in the early 1970s [25, 37, 45] (reviewed in [24]). Little information is available on human cold receptors: one heroic study using teased-fibre recording managed to record from Aδ cold receptors [36], while innocuous human cold receptors with C fibres have been studied in some detail using microneurography [15, 80]. Psychophysical evidence indicates that Aδ cold receptors are probably involved in the conscious perception of coolness [31, 32, 42, 55, 97] while innocuous C-cooling receptors do not appear to evoke a conscious sensation [15], and may well have an affective role in bodily comfort and emotional touch [22].

Cutaneous thermoreceptors fire action potentials continuously at comfortable skin temperatures; cooling increases cold receptor firing, while warming causes them to shut down (Fig. 1a). In natural situations, this is the activity that would be evoked by picking up a cold or warm object. At constant temperatures, cold and warm receptors have characteristic temperatures for maximum static discharge frequency, distributed over a range from about 20–30°C for cold receptors and close to 40°C for warm receptors (Fig. 1b) [24, 35, 86].

Both cold and warm receptors show pronounced adaptation when temperature is held constant after a sudden change (the kind of activity that would be elicited by going outside from a warm room, or jumping into the sea). In primate cold receptors, a brief (~10 s) cooling pulse initially elicits high-frequency action potentials; firing frequency decays rapidly with a time constant of a few seconds (Fig. 2a) [15, 25]. Over long periods, a slower time constant of receptor adaptation (1–2 min) is apparent in primates (Fig. 2b) [45]; this is roughly comparable to the rate of adaptation of human temperature perception [46].

Cold receptors do not respond only to cooling. A number of them (about half) fire action potentials on strong heating, presumably the correlation of the

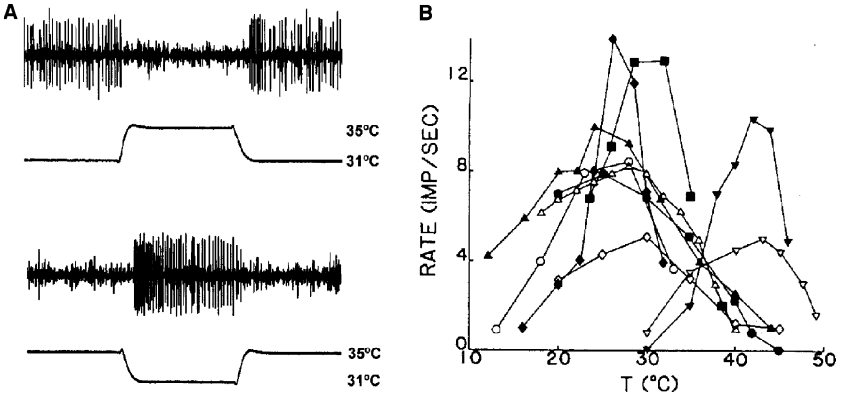

Fig. 1a,b Spike activity in cold receptors. **a** Resting discharge in a human C-fibre innocuous cold receptor at a skin temperature of 31°C is suppressed temporarily by warming (~10 s) to 35°C (*top*); when held at 35°C the unit is silent, but is activated by cooling to 31°C (*bottom*; from [15]). **b** Steady-state activity of cold and warm receptors in several preparations: cold receptors are from rat scrotum (*open circles*); cat nose (*open triangles*); monkey hand (*filled circles*); frog skin (*filled diamonds*); monkey hairy skin (*open diamonds*); dog lip (*filled squares*) and monkey hand (*filled triangles*). Warm receptors from rat scrotum (*filled triangles*) and monkey hairy skin (*open triangles*) (from [86])

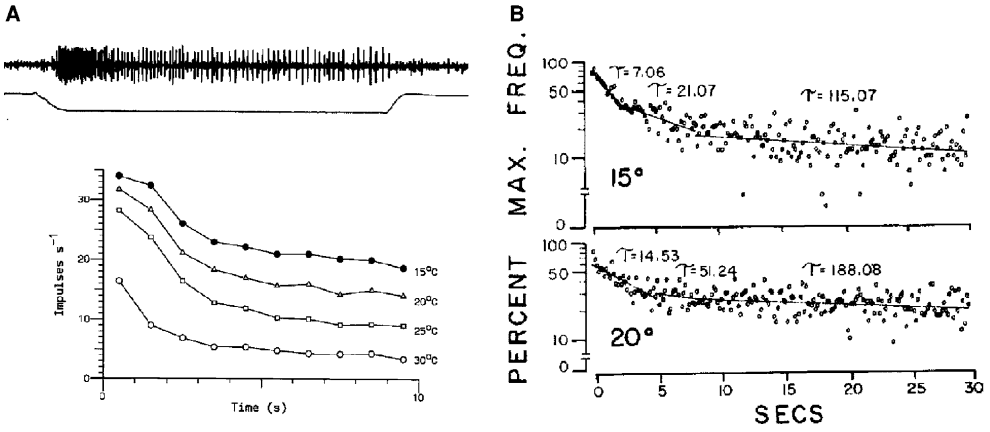

Fig. 2a,b Rapid and slow adaptation in cold receptors. **a** Rapid adaptation during a 10-s pulse from 35°C to 30°C in a human C-fibre innocuous cold receptor (*top*) and spike frequencies during 10-s pulses to 15–30°C (adapted from [15]). **b** Slow adaptation: spike frequencies in rhesus monkey cold receptors during prolonged cold stimuli to the temperatures indicated (from [45])

paradoxical cold sensation [15, 29, 54]. Menthol has long been known to induce a cool sensation; one early study showed that menthol stimulates and sensitises cold receptors, while warming inhibits its action, suggesting it might be acting directly on the cold transduction mechanism [39]. This was confirmed by later work, of course, as will be described below.

While gentle cooling induces cool sensations, strong cooling can be perceived as "icy" or can induce pain. A small population of innocuous cold receptors responding only to strong cooling has been identified in primates [50], and these may contribute to the "icy" sensation. A much larger number of cold-sensitive nociceptors can be excited by cooling to levels that cause pain (10–15°C and below; Fig. 6a). In rat, different studies report ~20–30 % of nociceptors to be excited at 0–10°C, while further cooling excites all nociceptors [49, 81, 82]; in primates, around 10 % of A- and C-fibre nociceptors respond to cold [33]. However, some cold nociceptors respond even to gentle, innocuous cooling (see below).

Early work on thermal transduction

Attempts to understand transduction in thermoreceptors have been hampered by the difficulty in recording directly from the sensory terminal. Recently, this has become possible, at least for corneal cold receptors [13, 16, 17], but early attempts to understand thermal transduction relied on action potential activity recorded from teased nerve fibres, using interspike intervals to make inferences about changes in membrane potential at the terminal [11, 12]. These experiments were interpreted in terms of known ion transport mechanisms: all ion channels and ionic pumps are temperature dependent and, thus, in principle, any of them could play a role in thermal transduction. The Na^+/K^+ ATPase is an attractive candidate because, like all enzymes, it is inhibited by cold, which would be expected to depolarise the receptor. Blocking the Na^+/K^+ ATPase with ouabain does indeed have pronounced effects on cold receptor firing [67, 85], consistent with a role in cold transduction, although later work has shown that its role is probably a minor one [74].

The search for specific transduction mechanisms

A major breakthrough in understanding thermal transduction came with the identification of a heat-activated ion channel in sensory neurones [19]. This study used the somata of dorsal root ganglion (DRG) neurones in culture as a model of their otherwise inaccessible receptor terminals [5]: following excision of the DRG and loss of the axon, proteins normally destined for the receptor terminal begin to appear in the soma and can be studied with patch-clamp recording or intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) imaging. The heat-activated channel is a non-selective cation channel activating at temperatures above ~42°C, and is thus a natural candidate for a transducer of noxious heat in nociceptors. Its probable molecular substrate was identified soon after the native current was isolated: the capsaicin receptor, now known as TRPV1, an ion channel directly activated by noxious heat and low pH as well as capsaicin [18].

In the late 1990s, several groups began to search for cold transduction mechanisms in cultured DRG or trigeminal ganglion (TG) neurones. Cold receptors are rare in comparison with heat-sensitive nociceptors, so all groups pre-selected cold-sensitive neurones by imaging $[Ca^{2+}]_i$. Early work identified small numbers of DRG neurones (10% or fewer) responding either to cold [88] or to menthol [62], but neither study tested both stimuli and neither investigated the transduction mechanism.

When we applied cold and menthol to the same DRG neurones, about 7% responded to both stimuli with a large and rapid increase in $[Ca^{2+}]_i$; co-expression of a rapid cold response with menthol sensitivity was 100% [73]. Patch-clamping these neurones revealed a large cold-induced depolarisation and action potentials. The frequency of action potentials increases with cooling near the beginning of the ramp stimulus, and tails off as depolarisation proceeds, possibly due to Na^+ channel inactivation (Fig. 3a); this is reminiscent of the bell-shaped curve relating temperature to firing frequency in intact cold receptors (Fig. 1b). The depolarisation depends on a substantial inward current, activated by cooling and sensitised by menthol, which is specific to cold-sensitive neurones (Fig. 3b, c) [73]. Warming to 37°C switches off the menthol-induced current (Fig. 3b).

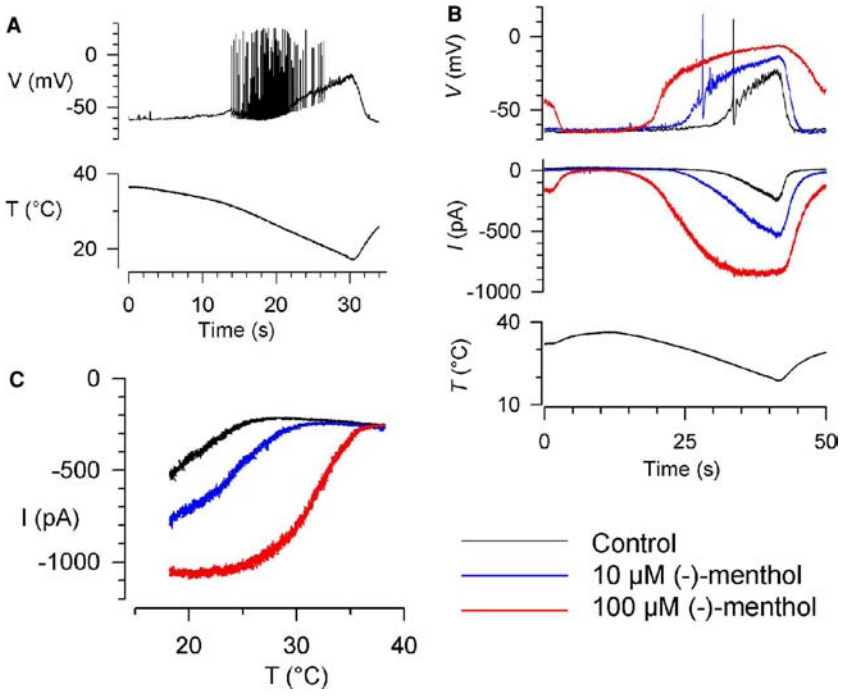

Fig. 3a–c Cold- and menthol-activated current in cold-sensitive rat DRG neurones. **a** Depolarisation and action potentials induced by the cooling ramp shown in the lower panel. Base temperature was 32°C and the stimulus was the same as in **b**; only the descending part of the ramp is shown (adapted from [73]). **b** Cooling-induced depolarisation and current in 1, 10 and 100 μM (−)-menthol. Recording was in the presence of 100 nM tetrodotoxin to reduce spike activity and expose the underlying depolarisation more clearly (adapted from [72]). **c** Current/temperature relation of the cold-activated current and its potentiation by menthol. Adapted from [73]

The cold- and menthol-activated channel immediately looked like a natural candidate for the primary cold transducer—but how to test this? A search for a blocker to enable functional studies in intact receptors initially revealed substances that either had no effect (e.g. amiloride) or turned out to activate the channel instead (e.g. ruthenium red [73]). Not till later did we find effective blockers [72]. So at first, it was possible only to establish whether the current accounted for known aspects of cold receptor activity. I have already mentioned stimulation by menthol and the reversal of the menthol effect by warming. Cold receptors are stimulated by low extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$), and the menthol effect is inhibited by high $[Ca^{2+}]_o$ [38, 78]: both effects are attributable to the cold- and menthol-activated current [72, 73]. Adaptation and recovery of the current during and after sustained cooling pulses (Fig. 4a) [73] (see also [61, 72]) is similar to the slow adaptation of primate cold receptors and of human cold sensation (Fig. 2b) [45, 46]. The cold- and menthol-activated current could thus account for most of what was known at the time about the behaviour of intact cold receptors, leading to the suggestion that it is probably the major mechanism of innocuous cold sensing [73].

A similar cold- and menthol-activated current was later found to be present in TG neurones [57] and a cold-activated current in another study in DRG neurones is probably the same [61]. Channel activation by cold and menthol is direct or membrane-delimited: excised patches from DRG neurones contain a cold-activated non-selective cation channel with about 20 pS conductance at negative potentials [61, 75], which is also activated by menthol [75].

In addition to the cold- and menthol-activated current, at least two other mechanisms contribute to cold transduction in cultured DRG or TG neurones. One is a background K^+ current that is inhibited by cooling, depolarising the neurone and increasing its input resistance [74, 92]. This amplifies the depolarisation produced by the relatively small cold-activated inward current (Fig. 3b), and this is aided by the fact that outwardly rectifying K^+ currents in cold-sensitive neurones are small [92] (see also [72]). Involvement of a background K^+ current is consistent with an earlier suggestion that the background K^+ channel TREK-1 may be involved in cold sensing [56], although there is no evidence that the channel in DRG and TG neurones is TREK-1 itself and not another member of the same family. The Na^+/K^+ ATPase, an early candidate cold transducer, is probably a minor mechanism modulating cold sensitivity and not a specific transduction mechanism: completely blocking it with ouabain depolarises cold-sensitive DRG neurones, but by only a fraction of the cold-induced depolarisation; and unlike cooling, ouabain never induces action potentials [74].

The cloning of TRPM8: is it the native cold and menthol receptor?

Shortly after the first work on the native cold- and menthol-activated current appeared, two groups independently cloned TRPM8, a cold and menthol receptor, the properties of which are very similar to those of the native channel. One group used expression cloning, with a high menthol concentration as the stimulus, to isolate TRPM8 from a rat TG library [57]. Another group searched for temperature-gated channels by looking for TRP-related sequences and testing them for thermal sensitivity: one of the channels they identified was TRPM8 [64], and another was TRPA1 [87], which will be considered later.

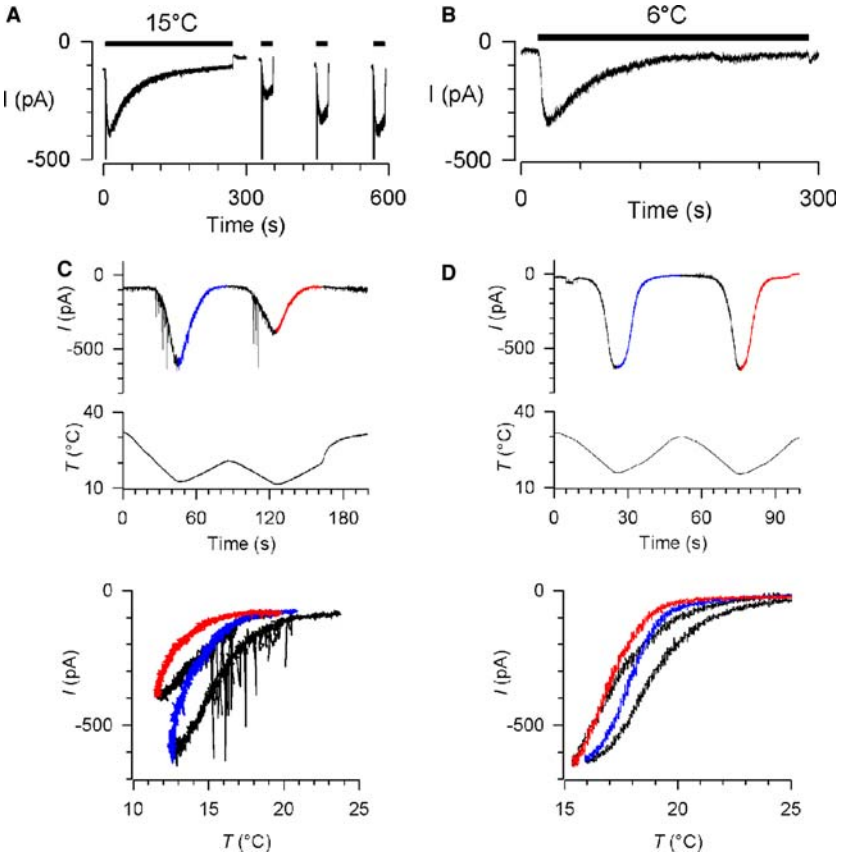

Fig. 4a–d Adaptation of the cold- and menthol-induced current (native TRPM8) and of cloned TRPM8 in HEK293 cells. **a** Time course of adaptation of the cold-activated current in a cold- and menthol-sensitive rat dorsal root ganglion (DRG) neurone during a prolonged 15°C stimulus (*long black bar*) from a base temperature of 32°C. Recovery from adaptation was followed using brief 15°C stimuli (*short black bars*) (adapted from [73]). **b** Adaptation of rat TRPM8 expressed in a HEK cell. Base temperature was 32°C; note the colder stimulus than in **a**, necessary because of the lower threshold temperature for activation of TRPM8 in HEK cells (G. Reid, unpublished; see [70]). **c** Shift in thermal sensitivity during adaptation of the native cold-activated current during a double-ramp cooling stimulus (*top panel*), and the current/temperature relation (*bottom panel*). The rising phase of the stimulus (blue after the first ramp, red after the second) was identical, to allow comparison of the cold sensitivity (adapted from [72]). **d** Shift in thermal sensitivity during adaptation of rat TRPM8 expressed in a HEK cell, during a double-ramp stimulus (colour coding as in **c**) (G. Reid, unpublished; see [70])

In mice and rats, TRPM8 is expressed in 5–10% of DRG neurones [64], similar to the number of cold- and menthol-sensitive DRG neurones (7%) [73]. TRPM8-expressing neurones are of small diameter [57, 64], similar to that of cold- and menthol-sensitive DRG and TG neurones [57, 62, 72, 88, 92]. TRPM8 was reported not to be co-expressed in vivo with several nociceptive markers: calcitonin gene-related peptide (CGRP), isolectin B4 (IB4) binding and TRPV1 [64], although there has recently been some dispute about the last of these findings [60]. A role for TRPM8 in innocuous cold sensing is supported by the action potential properties and voltage-gated currents of cold- and menthol-sensitive neurones [72], which are not consistent with those that have been described for nociceptors [28, 66]. However, a nociceptive function for some TRPM8-expressing neurones is a distinct possibility, as will be discussed below.

Many properties of the native cold and menthol receptor have also been demonstrated in cloned TRPM8 (Table 1). In one respect, however, there is a clear difference between the behaviour of TRPM8 in expression systems and native cold- and menthol-activated currents. The threshold temperature for current activation in native systems is 27–31°C [57, 61, 72, 73] but in expression systems, the activation threshold is several degrees colder, 21–26°C [57, 64]. Under the same conditions, the difference is reported to be 6°C [93], agreeing with our own observations of a 5–8°C difference (rat TRPM8 in HEK293 cells vs. rat DRG; perforated-patch whole-cell recording in both cases; G. Reid, A. Babes, unpublished). This points to a sensitisation mechanism in native receptors that is only partially present in expression systems, as will be considered in detail below in relation to cold receptor adaptation.

Apart from the difference in activation threshold temperature, the parallels between TRPM8 and the native cold and menthol receptor are remarkably close. Of course, heteromultimers or TRPM8 splice variants may be involved in forming the native receptor, and accessory subunits of some sort are highly likely, as the great majority of known ion channels are composed of several

Table 1 Similarities between native and cloned TRPM8. Properties shared between native and cloned TRPM8 are listed: *numbers* in *brackets* refer to published sources

Property	Native	Clone
Activation by cold in excised patches	[61, 72, 75]	[53, 95]
Activation by menthol in excised patches	[75]	[53, 95]
Strong outward rectification	[61, 75]	[57, 64]
Permeability Ca^{2+}/Na^{+}	3.2 [57]8.4 [61]	3.3 [57]0.97 [64]
Stereoselective action of menthol	[72]	[7]
Block by capsazepine	[72]	[7]
Adaptation: decline in current on prolonged cooling	[61, 72, 73]	[70]
Adaptation: shift in cold sensitivity on cooling	[72]	[70]

subunits [41]; it is thus dangerous to assume that the native cold and menthol receptor is a single molecular entity. Bearing this caveat in mind, TRPM8 can account for most of what we presently know about the native cold and menthol receptor, which I will refer to as "native TRPM8" or simply TRPM8 in the remainder of this review.

Cold transduction with native TRPM8: what happens?

The main effect of TRPM8 activation is to depolarise the receptor terminal and elicit action potentials. It also triggers Ca^{2+} influx into the terminal, underlying adaptation (see below), but direct Ca^{2+} entry through TRPM8 is a minor part of the total. In fact, most of the $[Ca^{2+}]_i$ increase on cooling depends on depolarisation, being nearly abolished by voltage clamping at a negative holding potential [72]. The $[Ca^{2+}]_i$ increase is reduced greatly by blocking voltage-gated Ca^{2+} channels (VGCCs) with Cd^{2+} [61, 89], indicating that they, and not TRPM8, conduct most of the Ca^{2+} into the neurone.

The depolarisation that opens the VGCCs depends mostly on the entry of Na^{+} and not Ca^{2+}, as removal of extracellular Na^{+} profoundly inhibits the $[Ca^{2+}]_i$ increase [72, 89, 92]. Action potential activity is an important part of this process: threshold temperatures for spike activity and Ca^{2+} influx are closely correlated [72, 92] and tetrodotoxin (TTX) causes a large shift in threshold temperature by abolishing action potentials [72]. Spike activity is not the whole story, however, because Ca^{2+} influx even in the presence of TTX is still much greater than under Na^{+}-free conditions. So what produces the remaining Na^{+}-dependent depolarisation? The answer turns out to be TRPM8 itself: replacing Na^{+} by choline or *N*-methyl-D-glucamine greatly reduces the current through native TRPM8 and consequently the cold-evoked depolarisation [72], indicating that the current is primarily carried by Na^{+} and not Ca^{2+} under physiological conditions. This is in no way inconsistent with the slight Ca^{2+} selectivity of TRPM8 (Table 1), when one takes into account the 100-fold higher extracellular concentration of Na^{+} than Ca^{2+}. During the depolarisation, there is an element of positive feedback: TRPM8 is voltage-dependent, and depolarisation increases its current [10, 95].

We can thus make a synthesis of events on cooling a cold-sensitive cultured DRG neurone: cooling opens TRPM8, causing a large Na^{+} influx and a relatively small Ca^{2+} influx. This depolarises the neurone, generating action potentials and opening voltage-gated Ca^{2+} channels which conduct most of the Ca^{2+} that enters the neurone. The TRPM8-dependent inward current is itself amplified by the depolarisation, and its effect is in turn enhanced by the reduction in outward current resulting from cold-induced inhibition of the background K^{+} current and of the Na^{+}/K^{+} ATPase.

Events in a cold receptor terminal are probably broadly similar. Direct recordings from corneal cold receptor terminals have given some information about transduction in intact cold receptors. Action potentials are generated in the axon, a little distance from the terminal itself [13], not because the terminal lacks Na^{+} channels but because it is normally depolarised and its Na^{+} channels are inactivated [16]. This resting depolarisation may well be what gives rise to the resting discharge of cold receptors, and probably originates from some degree of activation of TRPM8 at the base temperatures used in these studies.

The applicability of the cultured DRG or TG neurone as a model of the terminal should not be exaggerated: even if the same ionic mechanisms are expressed, they are probably expressed to different degrees and certainly in a structure with different geometry. Input resistances are higher in the growth cone than in the soma [96], suggesting that some channels normally expressed in the soma are lacking in the terminal. The growth cone in culture seems to be more excitable than the soma: in older cultures where neurite outgrowth is extensive, we frequently observe cold-induced action potentials before the somal depolarisation begins, and these presumably originate in the processes (G. Reid & A. Babes, unpublished). These differences suggest that it is over-optimistic to expect action potential activity in the cultured soma to reflect faithfully that in the terminal. Nevertheless, simulation studies show that only minor changes in current density and geometric parameters are needed to make a soma model behave like an intact cold receptor terminal (H. Braun, personal communication), suggesting that differences in excitability and electrical behaviour between the soma and the terminal are based on simple quantitative factors rather than fundamental differences.

Adaptation and modulation of native TRPM8

A near-universal property of sensory receptors is that they adapt to slowly shifting background conditions so that small rapid changes can be detected more sensitively. Thermoreceptor adaptation is a familiar experience. When we go swimming in the sea, the water feels

very cold at first but usually becomes comfortable after a few minutes; moving into deeper water, we again perceive it as cold. As mentioned above, this adaptation, happening over a period of minutes, is observable both in psychophysical measurements [46] and in studies on cold receptor activity [45]. It is well reproduced by the decline in native TRPM8 current during sustained cooling. Current declines with a time constant of ~1 min [61, 72, 73] and recovers with a similar time course (Fig. 4a) [73]. The decline in cold-activated current depends on influx of Ca^{2+}, as it is prevented by removing extracellular Ca^{2+} or by chelating intracellular Ca^{2+} with BAPTA [61, 72].

Adaptation operates by shifting the temperature sensitivity of native TRPM8, so that the same maximum current can be elicited, but needs stronger cooling (Fig. 4c, d); raising $[Ca^{2+}]_i$ without cooling produces a similar shift in cold sensitivity [72]. The flexible threshold of TRPM8 probably underlies the wide range of activation thresholds observed in cold- and menthol-sensitive neurones [3, 61, 72, 89, 92]. Ca^{2+}-dependent modulation of TRPM8 provides the basis for a simple feedback mechanism: opening of TRPM8 by cooling causes Ca^{2+} influx, which reduces TRPM8's cold sensitivity and causes it to close. Stronger cooling opens it again. Feedback based on Ca^{2+} influx can thus explain how cold adaptation can keep TRPM8's threshold just below the adapting temperature, poised to open on slight cooling.

Patch excision also affects TRPM8's threshold profoundly, shifting it by ~13°C towards cooler temperatures compared with whole-cell recordings in the same neurones made with the perforated-patch technique (Fig. 5a) [75] (see also [61]); once this shift has taken place, adaptation is lost [75]. This suggests that the channel has a surprisingly low intrinsic temperature sensitivity and is sensitised strongly by its presence in an intact neurone; adaptation would thus correspond to a partial loss of this sensitisation, and channels in an excised patch behave as if they have lost all sensitisation and are thus unable to adapt further. Loss of sensitisation ("rundown") is not prevented by keeping the cytoplasm intact in an outside-out patch (Fig. 5a) [70], but modulation of cold sensitivity by intracellular Ca^{2+} is intact in some inside-out patches (Fig. 5b, c) [61]. Both observations suggest that membrane integrity is more important in sensitisation and cold adaptation of TRPM8 than is an intact cytoplasm, and raises the possibility that accessory subunits (including Ca^{2+}-binding proteins) are involved in the process.

The parallels between adaptation and rundown suggest that understanding channel rundown in excised patches may give clues to the adaptation mechanisms. One membrane-delimited mechanism that may contribute to adaptation involves phosphatidylinositol 4,5-bisphosphate (PIP_2). Applying exogenous PIP_2 to excised patches containing cloned TRPM8 restores channel activity after rundown, and PIP_2 also modulates TRPM8 in intact HEK cells [53]. PLC activation inhibits TRPM8, possibly by PIP_2 breakdown [6]. Intracellular acidification also inhibits TRPM8 currents in HEK cells by shifting their temperature sensitivity [1], but there is no indication of whether such a pH change may take place on cooling, or how it might depend on Ca^{2+} influx, so it is not clear whether this is a plausible adaptation mechanism. Involvement of either of these mechanisms in the Ca^{2+}-dependent cold adaptation of native TRPM8 remains to be investigated.

Given that normal thermal sensitivity of TRPM8 depends critically on cellular mechanisms, one might ask whether these mechanisms are specific to cold receptors, specific to neurones, or ubiquitous. Preservation of some aspects of adaptation in HEK cells (Fig 4b,d) indicates that part of the mechanism is ubiquitous. Patch excision from HEK cells also causes loss of sensitisation [53, 95], although the sensitisation in HEK cells is less than in native neurones [93]. However, some mechanisms

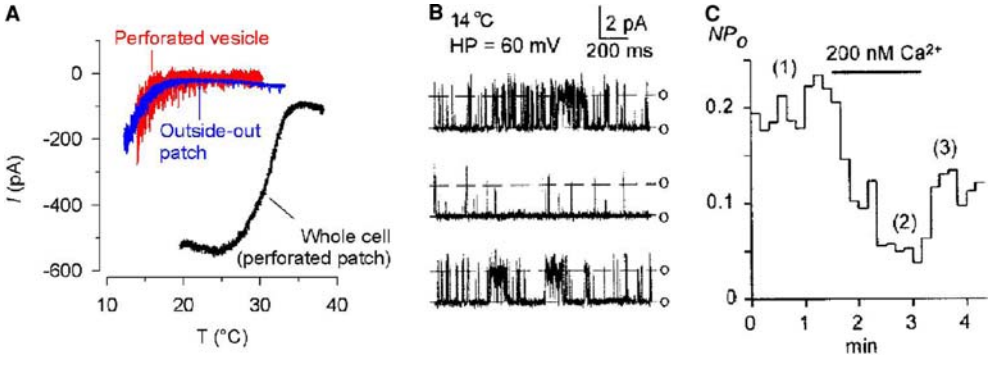

Fig. 5a–c Modulation of native TRPM8 depends on membrane integrity but not on the cytoplasm. **a** Current/temperature relation in excised patches compared with that in the whole cell. In conventional outside-out patches (*blue*), thermal sensitivity is shifted strongly in the cooling direction compared with a perforated-patch whole-cell recording (*black*). In the perforated vesicle mode (*red*), thermal sensitivity is the same as in conventional outside-out patches: the channel has lost sensitisation, despite the preservation of the cytoplasm. The perforated vesicle mode is produced by excising an outside-out patch starting with a perforated-patch whole-cell recording (method described in [51]). Whole-cell and outside-out patch from [75]; perforated vesicle (G. Reid, unpublished; see [70]) **b** Modulation of cold-activated channels (probably native TRPM8) by intracellular Ca^{2+} is intact in inside-out patches despite loss of the cytoplasm. Channel activity at 14°C (*top*) was inhibited by 200 nM $[Ca^{2+}]_i$ (*middle*); inhibition was reversible (*bottom*) (adapted from [61]). **c** Relative open probability (plotted as NP_o) during the recording in **b**. *Numbers* (*1*, *2*, *3*) indicate the times at which the recordings in **b** were made (adapted from [61])

appear to be specific to cold receptors and are not reproduced even in other neuronal types: threshold temperatures for TRPM8 expressed in hippocampal neurones are similar to those in HEK cells, but not to those of native cold- and menthol-activated currents in TG neurones [93].

Neurotrophin effects on native TRPM8

The first cultures we made in Bucharest were without nerve growth factor (NGF), simply because we did not have any: delivery of consumables to Romania is not a simple matter [69]. Arrival of a supply of NGF made work on native TRPM8 very much easier, by enhancing its cold sensitivity, and further work was done in the presence of NGF for this reason [72, 73].

Modulation of TRPM8 by NGF is not surprising given that TRPM8 is expressed in neurones that depend for survival or development on the high-affinity NGF receptor, tyrosine kinase A (TrkA) [64]. Comparing neurones cultured in the presence and absence of NGF, strong sensitisation by NGF of cold- and menthol-sensitive currents and $[Ca^{2+}]_i$ responses is apparent [3, 72]. NGF also maintains expression of TRPM8, preventing the number of cold- and menthol-sensitive neurones from declining in the first 3 days of culture [3]. NGF therefore appears to affect both TRPM8 gene expression and the properties of the individual molecules. Initial indications are that TrkA is involved in both effects, as both are elicited by as little as 1 ng/μl NGF [52]. One mechanism proposed for TrkA-mediated sensitisation of TRPV1 by NGF is activation of PLC, which reduces PIP_2 and releases TRPV1 from PIP_2-mediated inhibition [21, 68]. This is unlikely to underlie NGF effects on TRPM8, because a reduction in PIP_2 would make TRPM8 less and not more sensitive to cold [53]. There are several possible alternative mechanisms (e.g. [9]).

Cold pain: is it really the cold that hurts? How cold does it have to be?

As mentioned earlier, strong cooling of the skin induces various forms of pain: deep ache and superficial burning pain (Fig. 6a), as well as the sharp pain of freezing. Extreme cooling that activates all nociceptors [81, 82] may be acting by a mechanism that is not specifically cold-sensitive: such extreme cooling may well induce sufficient tissue damage to be an adequate stimulus for polymodal nociceptors that lack a specific cold sensor. But less extreme cooling activates more limited numbers of nociceptors [33, 49, 81, 82], suggesting cold-specific transduction mechanisms. On the other hand, strangely, cold-induced pain in normal subjects can also be associated with receptor temperatures in the innocuous range, implying a transducer that does not require strong cooling to activate it. This has been demonstrated in some intriguing human psychophysical experiments that illustrate important principles.

Firstly, skin surface temperature is not the same as receptor temperature, and the difference may be large, depending on where the cold nociceptors are. There is evidence that at least one group of cold nociceptors are not near the skin surface, but instead in cutaneous veins. Application of water at 0°C to the skin quickly induces pain, which is abolished by intravenous, but not intracutaneous, application of local anaesthetics. When the time course of the development of subjective pain is compared with that of vein temperature, strong pain is experienced at vein temperatures (and thus receptor temperatures) of ~20°C, well above the range of temperatures usually considered to be noxious (Fig. 6b) [48].

Secondly, even gentle cooling can produce a sensation of burning pain, during pressure block of Aδ fibre conduction [26, 32, 97]. This is because cold (and other) nociceptive input is normally inhibited by Aδ

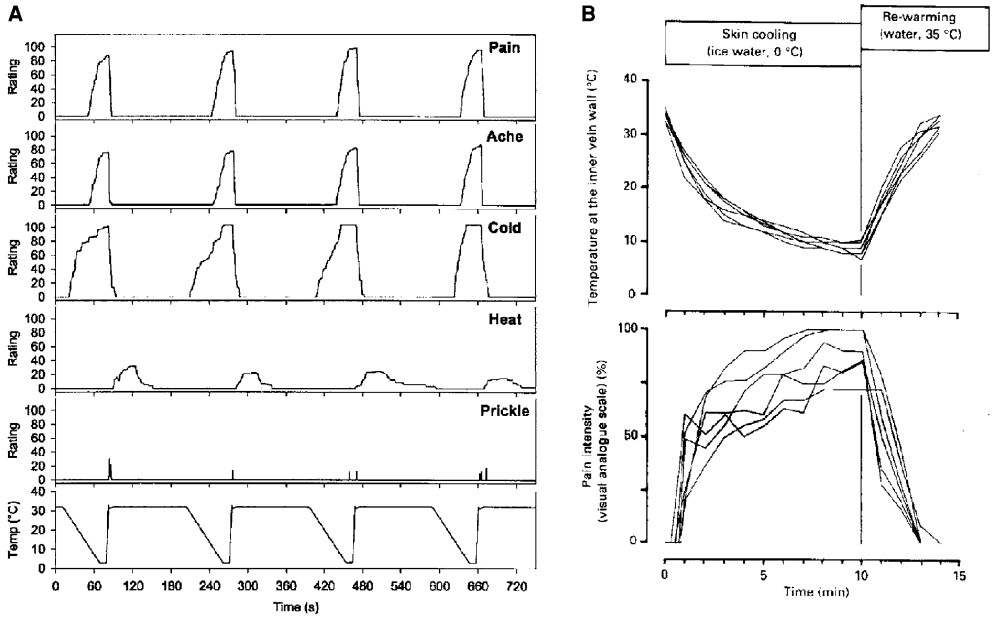

Fig. 6a,b Cold-induced pain experienced by human subjects. **a** Intensity ratings of five qualities of sensation (including pain and ache) induced by cooling to 3°C in a human subject. Pain and ache appear after short periods at temperatures below ~10°C (adapted from [27]). **b** Pain intensity (*lower panel*) and simultaneous measurement of vein wall temperature (*upper panel*) induced by water at 0°C on the skin in five human subjects. Note that considerable pain is felt even at vein (thus receptor) temperatures around 20°C (adapted from [48])

cool-specific fibres: release of this inhibition by Aδ block allows nociceptor activity to evoke a sensation of pain [23]. The related phenomenon of the "thermal grill" illusion illustrates the same effect. A series of bars or thermodes are set alternately to innocuous warm (e.g. 40°C) and innocuous cool (e.g. 20°C) temperatures. The sensation produced by this stimulus is one of burning pain, and the illusion can be explained in the same way as the nerve block experiments: the cool bars excite both cool-specific Aδ fibres and C-fibre cold nociceptors, but Aδ activity is reduced when some bars are warm, releasing the inhibition it normally exercises on C-fibre nociceptive activity and evoking pain [23].

These experiments tell us that some cold nociceptors are already excited at receptor temperatures well above the range usually considered to be noxious, and thus differ from "classical" nociceptors—receptors that are excited only by stimuli causing actual or threatened tissue damage [8, 14]. They are probably among the neurones that have been found to be excited by gentle cooling in DRG or TG cultures. Curiously, about half of cold- and menthol-sensitive DRG or TG neurones are excited by capsaicin and thus probably express TRPV1 [3, 57, 72, 92], suggesting that they might be cold nociceptors [57, 92]. However, their electrical properties are not at all like those of nociceptors [72], nor is TRPM8 co-expressed with any other nociceptive marker [64]. Capsaicin sensitivity is the only nociceptor-like property that these neurones possess. Co-expression of TRPM8 and TRPV1 may even be a culture artefact [87] resulting from inclusion of NGF in the culture medium [87]; on the other hand, some workers find it to be present in vivo [60], and we find it after as little as 2 h in culture, independently of NGF [3]. There may be a species difference: co-expression of TRPM8 and TRPV1 has consistently been reported to be absent in the mouse [64, 87], but present in the rat [3, 57, 60, 72, 92].

To conclude, it is at present an open question whether TRPM8 is involved in cold nociception: it is highly plausible, given that cold pain can be elicited by receptor temperatures in the innocuous range, but there is not yet clear evidence confirming it. Some cold nociceptors need stronger cooling to activate them than does TRPM8 [33, 49, 81, 82]; so, without forgetting the caveat about non-specific activation mentioned at the beginning of this section, we should probably also be looking for transduction mechanisms that operate only at colder temperatures.

Another cold-activated channel: TRPA1

As mentioned above, a database search for TRP-related sequences with ankyrin repeats in the N-terminus revealed a previously cloned gene, "ankyrin-like with transmembrane domains 1" (ANKTM1, now known as TRPA1), which turned out to be activated by strong cooling [87]. TRPA1 is activated in expression systems by cooling with a threshold around 17°C, and is co-expressed highly with the nociceptive markers TRPV1, substance P and CGRP (97% of TRPA1-expressing neurones also co-express TRPV1 and CGRP), leading to the suggestion that it is a noxious cold transducer [87]. Additional support for a nociceptive role comes from the fact that most native DRG neurones with TRPA1-like pharmacology (cinnamaldehyde-responsive; see below) are activated by bradykinin [6]. TRPA1 is expressed in a small fraction of mouse DRG neurones (3.6%), much smaller than the fraction of nociceptors that respond to strong cooling [33, 49, 81, 82].

As well as noxious cold, cloned TRPA1 is activated by pungent compounds (cinnamaldehyde, mustard oil and related compounds) and by the cooling compound icilin [6, 43, 87]. Specific TRPA1 agonists elicit a pungent or painful sensation in humans (not a cold sensation), as well as behaviour consistent with pain in mice, suggesting that TRPA1 is involved in nociception but not in the conscious perception of cold [6].

Some doubt has been expressed about whether TRPA1 can explain responses to noxious cold in DRG neurones [3], and even about whether it is activated by cold at all [43]. I will consider below whether the evidence supports a role for TRPA1 as a cold sensor in vivo, but first, we will look at non-TRPM8 cold sensing at lower temperatures in native neurones.

Colder than TRPM8: native cold receptor heterogeneity

Besides the neurones in DRG or TG cultures that show rapid responses to small degrees of cooling [57, 61, 72, 73, 92], others respond more slowly and require stronger cooling to excite them. If neurones are separated into two groups with threshold above or below 25°C [89], expression of TRPM8 detected with single cell RT-PCR is higher in the "low-threshold" group (LT; threshold >25°C) than the "high-threshold" group (HT; threshold <25°C) [58]. If TRPM8 is the transducer in only some LT neurones and fewer HT neurones, what other transducers may be involved?

This was investigated in the same two groups of neurones using pharmacological agents to target several putative transduction mechanisms [89]: menthol to activate TRPM8, amiloride to block the epithelial sodium channel ENaC [2] and gadolinium (Gd^{3+}) to block TREK-1 [56]. Menthol raised $[Ca^{2+}]_i$ in 65% of LT and 35% of HT neurones, roughly consistent with the expression pattern of TRPM8 seen with RT-PCR. However, the other agents used are non-specific, and conclusions based on their use would be safer if supported by direct recordings of membrane currents and not only of $[Ca^{2+}]_i$. Amiloride reduced the $[Ca^{2+}]_i$ signal, consistent with a role of ENaC, but it also blocks Ca^{2+} channels [47, 79, 90]; in our hands, amiloride does not affect cold-activated currents in DRG neurones [73]. Similarly, Gd^{3+} blocks voltage-gated Ca^{2+} channels as well as TREK-1, but these would have opposite effects on the $[Ca^{2+}]_i$ signal, making experiments with Gd^{3+} difficult to interpret.

The two studies just mentioned [58, 89] were useful in focussing attention on heterogeneity of cold responses in

cultured sensory neurones, but without a more precise pharmacological dissection, it is difficult to make firm conclusions about transduction mechanisms. The considerable overlap in pharmacology between the two groups suggests that the mechanisms are not clearly distinct.

Taking a different approach, a clearer separation results from dividing cultured DRG neurones into menthol-sensitive and menthol-insensitive groups, with some resulting overlap of thermal threshold [3]. During a cold stimulus into the noxious range (a 30-s ramp to 12°C), which begins to induce pain in human subjects (Fig. 6a) [27], the menthol-sensitive group behaved largely as already described, fully consistent with a dominant role for TRPM8 [3]; cf. [72]. The menthol-insensitive group required stronger cooling for activation than the menthol-sensitive group, and also produced a slower and smaller rise in $[Ca^{2+}]_i$ on cooling [3] (see also [83]). Responsiveness of the menthol-sensitive group to NGF has already been mentioned; in contrast, the menthol-insensitive group was completely unresponsive to NGF. The most striking distinction between the two groups was in their pharmacology. Agonists of TRPA1 excited some neurones in the menthol-sensitive group but virtually none in the menthol-insensitive group (Fig. 7a) [3], not the result that would have been expected from two mutually exclusive groups of neurones expressing either TRPM8 or TRPA1 [87]. Others have also found evidence of some TRPM8/TRPA1 co-expression in cultured neurones [6], but more interesting is the lack of any evidence of TRPA1 expression in the menthol-insensitive group [3]. We seem to have a group of neurones responding to strong cooling, but not using TRPA1 as their cold sensor.

Is TRPA1 a cold sensor at all in vivo?

This raises an apparent contradiction: others have observed cold activation of cloned TRPA1 and of DRG neurones with TRPA1-like pharmacology [6, 87], but the DRG neurones we were able to activate with strong cooling had pharmacology unlike TRPA1 [3]. Doubts had already been expressed about cold activation of TRPA1 in native neurones [43], but those authors were also unable to activate cloned TRPA1 with cold, suggesting that methodological factors may be involved. In contrast, we do find cold activation of cloned mouse TRPA1, consistent with earlier reports [6, 87]: a 12°C cooling ramp is able to activate mouse TRPA1 (G. Reid, A. Babes, unpublished), but unable to activate any "TRPA1-like" rat DRG neurones [3]. This begins to suggest a difference between TRPA1's behaviour in native neurones and expression systems. Perhaps it is more difficult to activate native than cloned TRPA1 by cooling it. Is there other evidence of this?

A comparison of published recordings of cold responses of cloned TRPA1 with those of "TRPA1-like" DRG neurones [6, 87] suggests that this is indeed the case. Human and mouse clones are activated rapidly and strongly by cold (Fig. 8a), but native rat DRG neurones are activated very slowly, with a delay of up to 1 min (Fig. 8b) [6, 87]. This difference in activation rate can explain why, in our hands, a briefer cold stimulus can activate cloned TRPA1 but not native "TRPA1-like" DRG neurones.

The evidence just cited raises the possibility that the cold sensitivity of TRPA1 is actively suppressed in native neurones, by a mechanism expressed in sensory neurones but not in HEK cells or oocytes and which can be overcome by extreme and prolonged cold stimuli. We would thus have a mechanism opposite to that described above for TRPM8, the cold sensitivity of which is amplified in vivo compared with that seen with the clone. This seems at first sight a surprising mechanism to find in a cold nociceptor, but if this inhibition can be released (e.g. by bradykinin [6]), it may be exactly what is needed to make the receptor respond to cold only in damaged or inflamed tissue. Further work in this direction may thus give insights into both cold pain and inflammation-induced cold hypersensitivity.

Turning now to the in vivo situation, the very slow activation of TRPA1 by cold in native neurones is consistent with a role for TRPA1 in pain during extreme and prolonged cold exposure, or possibly in damaged tissue. It does not explain observations from psychophysical experiments on healthy subjects who report sensations of "ache" and "pain" after only a few seconds at temperatures below 10°C (Fig. 6a) [27], nor the rapid onset of cold nociceptor firing recorded in intact receptors in undamaged skin [49, 81, 82]. TRPA1 is clearly also an unlikely candidate to explain cold pain elicited by innocuous receptor temperatures, such as the burning pain of the "thermal grill" illusion [23], the pain

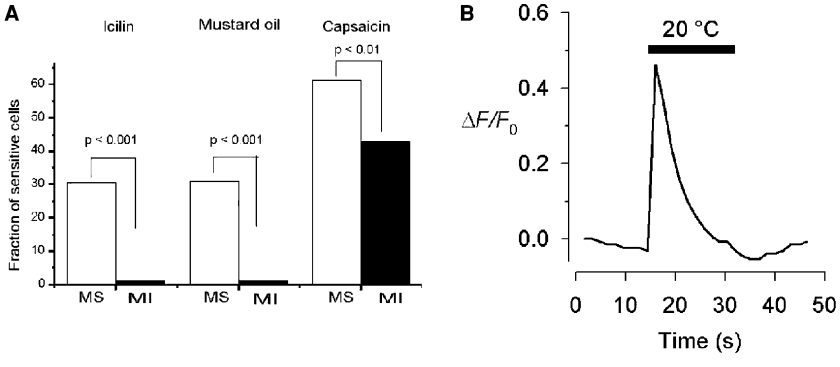

Fig. 7a,b Pharmacology and transient response of cold-sensitive and menthol-insensitive neurones in rat DRG. **a** Fraction of cold-sensitive rat DRG neurones in the menthol-sensitive (*MS*) and menthol-insensitive (*MI*) groups responding to icilin (50 μM), mustard oil (20 μM) and capsaicin (2 μM) (adapted from [3]). **b** Transient $[Ca^{2+}]_i$ increase in response to cooling in a rat DRG neurone in response to a 15-s step from 32 to 20°C (adapted from [4])

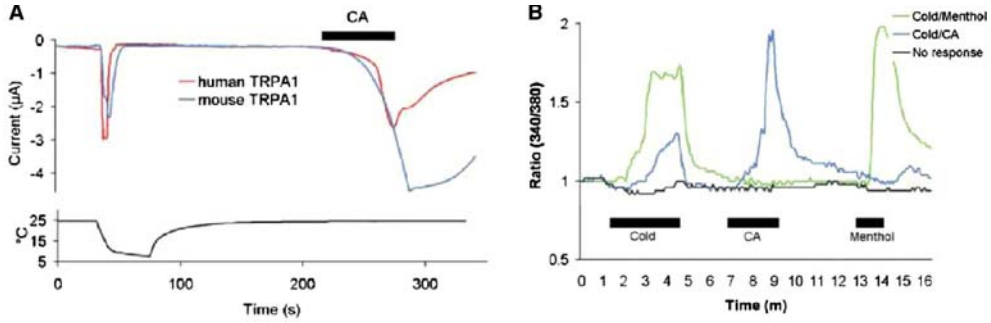

Fig. 8a,b Responses to cold of TRPA1 and of "TRPA1-like" rat DRG neurones. **a** Human and mouse TRPA1 activated rapidly by the cold stimulus shown, and by cinnamaldehyde (50 μM mouse, 100 μM human). **b** Responses of three rat DRG neurones to cold (9°C), 100 μM cinnamaldehyde and 250 μM menthol. One neurone responded rapidly to cold and menthol, another slowly to cold and cinnamaldehyde. Contrast the cold response with that in **a** (both **a** and **b** from [6])

on gentle cooling released by Aδ block [26, 32, 97], and the cold pain elicited by vein receptors at 15–20°C (Fig. 6b) [48].

TRPA1 is thus not the only transducer involved in cold-induced pain; so what other mechanisms could play a role? As mentioned above, it is quite possible that TRPM8 is involved, although its expression is too restricted to account for more than a fraction of cold nociceptors. The role of ENaC needs to be re-examined: ENaC generates a slow cold-activated current in expression systems [2], which would be a plausible candidate for a noxious cold transducer, although it is not clear that all the components of functional ENaC channels are expressed in DRG [30]. An additional possibility would be the background K^+ current. Although this is clearly involved in modulating innocuous cold transduction [92], expression of a highly temperature-sensitive background K^+ current is not restricted to innocuous cold receptors: we found it in all neurones that were depolarised by cold, making up ~25% of total DRG neurones [74]. This is a large group that with hindsight probably included cold nociceptors as well as innocuous cold receptors, implying that the background K^+ current probably plays a similar role in both types of neurone.

Menthol-insensitive neurones revisited: some show rapidly adapting, transient responses on innocuous cooling

We should now return to the menthol-insensitive group of DRG neurones described above [3], which correspond in part to the HT group seen in earlier work [58, 89]. Their responses to ramp stimuli are small and slow, and they require strong cooling for activation; some of them are probably cold nociceptors. However, when exposed to a fast step-like stimulus (a 10-s step from 32°C to 20°C), the behaviour of a subset of these neurones becomes much more interesting: they show powerful and rapidly adapting responses to step cooling in the innocuous range (Fig. 7b) [4]. This could help to explain a puzzle that was unresolved in earlier work.

The first study on native TRPM8 identified an adaptation and recovery process that matched well to slow adaptation in intact cold receptors (Figs. 4a and 2b) [73], but this and subsequent work have revealed nothing like the rapid adaptation familiar from cold receptors in vivo (Fig. 2a) [15, 25]. This may be simply because the stimuli we and others have used up to now have been too slow; ours has a time constant of 5 s, and most workers have used similar or slower stimuli [71]. In search of a possible rapid phase of TRPM8 adaptation, we therefore designed and constructed a fast system with ~20 ms switching time between any two independently controllable temperatures [76]. Instead of rapid TRPM8 adaptation, we discovered, quite unexpectedly, a new population of innocuous cold-sensitive neurones with very rapid adaptation on step cooling, fully consistent with the rapid time course of adaptation in vivo (Fig. 7b) [4]. When exposed to a ramp cooling stimulus, it is their rapid adaptation that makes them produce small and slow responses [3], or sometimes no response at all.

This immediately raises the question of the mechanism underlying their transduction. Patch-clamp recordings clearly show a cold-activated transient inward current and not cold inhibition of an outward current. Transiently responding neurones are insensitive to menthol and to cinnamaldehyde and activated by alkaline pH, arguing against involvement of TRPM8, TRPA1 and acid-sensitive ion channels (ASIC) in their transduction. This led us to conclude that these neurones probably contain a novel cold-activated channel, and may be physiologically relevant in generating rapidly adapting responses to innocuous cooling [4].

A brief look outside the mammals

TRPA1 is an ancient molecule in evolutionary terms, being present in the nematode worm *Caenorhabditis elegans* and in *Drosophila* as well as in mammals; in contrast, TRPM8 seems to be a relative youngster, being traceable (by reciprocal BLAST search) only in tetrapod genomes: *Xenopus*, birds and mammals. The appearance of a thermosensor with a new range of temperature sensitivity thus appears to have accompanied the move from water to land and consequent exposure to wider temperature fluctuations; this may

not be a coincidence. Chick TRPM8 encodes a cold and menthol receptor with similar properties to mammalian TRPM8, except that it is insensitive to icilin [20]; in native chick DRG neurones, apart from icilin insensitivity, its behaviour is not dramatically different from that of native rat TRPM8 (G. Reid, unpublished). Innocuous cold receptors have been described in frogs [84] but little is known about their transduction or the extent to which TRPM8 or TRPA1 (both expressed in *Xenopus*) may be involved. Curiously, *Drosophila* TRPA1 is activated by warming and not by cooling [94], and unlike mammalian TRPA1, the *Drosophila* orthologue's role in thermal sensing is supported by behavioural evidence: TRPA1 knockdown eliminates thermotaxis in *Drosophila* larvae [77]. The related *Drosophila* TRP channel Painless [91] is involved in the detection of noxious heat. These findings open the possibility that the considerable body of work on innocuous warm and cold receptors in adult insects [34, 98] may soon be linked to the kind of molecular understanding that is emerging in mammals and birds.

Summary: thermoTRP channels and cold sensing: what are they really up to?

This review has attempted to illuminate some of the complexity of cold transduction and to show how limited is our present understanding of the process, despite the cloning of one likely and one possible cold transducer. To conclude, we can identify some simple take-home messages.

- Innocuous cold is probably detected in large part by TRPM8.
- The threshold of TRPM8 is very flexible and regulated by activity of TRPM8 itself, so that it adapts to a range of steady temperatures and can respond sensitively to small increments of cooling. This flexibility can also explain the wide range of thresholds of TRPM8-expressing neurones.
- Flexible adaptation is made possible by TRPM8's low intrinsic temperature sensitivity, apparent in excised patches: it needs sensitising mechanisms supplied by the cell for normal temperature sensitivity, which means that the cell can modulate it.
- Some cold nociceptors are excited by moderate cooling in vivo: their activity is normally blocked centrally so that moderate cooling does not cause pain, but this block can be overcome experimentally. These receptors may well use TRPM8 as their transducer and are unlikely to use TRPA1.
- Other cold nociceptors are excited only by strong cooling in vivo, but their responses to cold are more rapid than those of probable TRPA1-expressing sensory neurones. Possible cold nociceptors in DRG cultures do not have TRPA1-like pharmacology. There are a number of possible candidate transducers for painful cold apart from TRPA1, but no hard evidence for any of them.
- The role of TRPA1 as a transducer of noxious cold in vivo is thus uncertain, although it does appear to produce painful sensations. Its responses to painful cold may depend on sensitisation mechanisms.
- Very recent work has revealed a novel group of transiently responding innocuous cold receptors in rat DRG, which may be the correlation of the well-studied rapidly adapting cold receptors in humans and other primates; they do not appear to use TRPM8 (or another known cold-activated channel) as their transducer.

A final point: while using expression systems as a handy tool to develop our molecular understanding, we must bear in mind that a cold receptor is a neurone, not a molecule. A single molecule like TRPM8 or TRPA1 can give only limited insight into the behaviour of an intact cold receptor—especially when studied in an expression system and not in a native receptor. Receptor activity results from the interaction between a transducer and its context: the cellular mechanisms modulating it, other ionic currents cohabiting with it in the terminal, and the geometry of the terminal itself.

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Note added in the proof

A very recent study (Rohacs et al., Nature Neurosci 8:626-634, 2005) has considerably strengthened the evidence for involvement of PIP_2 in Ca^{2+}-dependent adaptation and modulation of TRPM8, making it likely that PIP2 has a dominant role in these processes.

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