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Two populations of cold-sensitive neurons in rat dorsal root ganglia and their modulation by nerve growth factor

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Abstract

Cold sensing in mammals is not completely understood, although significant progress has been made recently with the cloning of two cold-activated ion channels, TRPM8 and TRPA1. We have used rat DRG neurons in primary culture and calcium fluorimetry to identify distinct populations of cold-sensitive neurons, which may underlie different functions. Menthol sensitivity clearly separated two classes of cold-responding neurons. One group was menthol-sensitive (MS), was activated at warmer temperatures and responded faster and with a larger increase in intracellular calcium concentration during cooling; the fraction of MS neurons in culture and their cold sensitivity were both increased in the presence of nerve growth factor. Neurons in the menthol-insensitive (MI) group required stronger cooling for activation than MS cells and neither their proportion nor their cold sensitivity were significantly altered by nerve growth factor. The two groups of cold-sensitive neurons also had different pharmacology. A larger fraction of MS cells were capsaicin-sensitive and coexpression of menthol and capsaicin sensitivity was observed in the absence of NGF. MI neurons were not stimulated by the super-cooling agent icilin or by the irritant mustard oil. Taken together these findings support a picture in which TRPM8 is the major player in detecting gentle cooling, while TRPA1 does not seem to be involved in cold sensing by MI neurons, at least in the temperature range between 32 and 12 °C.

Introduction

Temperature sensing is critical for survival and adaptation to the environment in plants and animals. Peripheral thermoreceptors in mammals are neurons with cell bodies located in dorsal root ganglia (DRG) or trigeminal ganglia (TG), and long processes innervating body skin or facial skin and the oral cavity, respectively. Important progress has been made in the last few years in understanding the molecular logic of thermal transduction, especially with the cloning of VR-1 (more recently known as TRPV1), an ion channel activated by high temperatures in the noxious range (Caterina *et al*., 1997). Other channels activated by various degrees of heating have been cloned and characterized more recently: VRL-1 (or TRPV2 according to the TRP nomenclature), TRPV3 and TRPV4 (for a review, see Patapoutian *et al*., 2003). In spite of these advances, a clear picture of the molecular events involved in transduction of innocuous warming and of noxious heat has not yet emerged.

The situation is quite similar for cold sensing. A fundamental step was the discovery of an inward current activated by cold and menthol in DRG neurons in primary culture (Reid & Flonta, 2001a). Shortly after this current was reported, an ion channel activated by cooling below 27 °C and by menthol was cloned and characterized (McKemy *et al*., 2002; Peier *et al*., 2002). This channel, named TRPM8, was heterologously expressed and was shown to posses all the features of the native cold- and menthol-induced current. Recently, another ion channel activated by lowering the temperature has been identified: TRPA1 (or ANKTM1) is activated by strong cooling (below 18 °C) and is expressed in a subset of sensory neurons expressing nociceptive markers such as TRPV1, calcitonin gene related peptide (CGRP) and substance P (Story *et al*., 2003). Taken together, these findings make TRPA1 a strong candidate for the transducer of noxious cold.

An additional mechanism has been suggested for cold transduction which involves closing of a background potassium conductance (Reid & Flonta, 2001b; Viana *et al*., 2002). The molecule involved may be TREK1, a potassium channel strongly inhibited by cooling, member of the two-pore domain family (Maingret *et al*., 2000).

One class of cold- and menthol-responding neurons in DRG or TG cultures has already been described and characterized (Reid & Flonta, 2001a; McKemy *et al*., 2002; Viana *et al*., 2002). The most likely transduction mechanism is a cold- and menthol-activated inward current which can account for most properties of intact cold receptors: sensitization by menthol, adaptation and effects of extracellular calcium (Reid & Flonta, 2001a; Reid & Flonta, 2002; Reid *et al*., 2002).

In two recent reports, two populations of cold-sensitive neurons were described in rat TG, separated based on their temperature threshold for activation by cooling (Nealen *et al*., 2003; Thut *et al*., 2003), but a clear identification of the transducer molecules was not possible. Moreover, the cold-activated ion channel TRPA1 was reported after the publication of these studies, and therefore the authors could not test the involvement of TRPA1 in cold sensing.

Using rat DRG primary cultures and calcium fluorimetry we identified two distinct groups of cold-sensitive DRG neurons based on menthol sensitivity. These groups differ markedly in the characteristics of their cold responses, in their pharmacological profile and in their modulation by nerve growth factor. One of the main goals of our work

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was to test the hypothesis that TRPA1 may be involved in cold detection in menthol-insensitive neurons. We have not found evidence for a role of TRPA1 in detection of cooling above 12 °C.

Materials and methods

DRG culture

Adult rats (150–200 g) were killed by inhalation of 100% CO_2, followed by decapitation. DRG neurons were cultured as described elsewhere (Reid *et al*., 2002). DRGs from spinal levels L1–S1 inclusive were removed and incubated in 1 mg/mL collagenase (type XI from Sigma) and 3 mg/mL Dispase (nonspecific protease, Sigma) for 1 h at 37 °C in IncMix solution (see Solutions, below). After trituration the dissociated cells were plated onto borosilicate glass coverslips (0.17 mm thick) which had been treated with poly D-lysine (0.1 mg/mL for 30 min), and cultured (37 °C, 5% CO_2 in air) in a 1 : 1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 10% horse serum and 50 μg/mL gentamicin. The cultures were split and half of the coverslips in each culture were kept in the presence of 100 ng/mL nerve growth factor (NGF) 7S (Sigma) which was added to the culture medium 1 h after plating the cells. Recordings were made from 2 h after plating to up to 3 days in culture.

Intracellular Ca^{2+} imaging

Coverslips with attached neurons were incubated for 30 min at 37 °C in standard extracellular solution (see below) containing 2 μM Calcium Green-1 AM and 0.02% Pluronic (both from Molecular Probes, Leiden, the Netherlands). After this period, the cells were left to recover in the incubator for at least 30 min before use. Coverslips were then mounted in a Teflon chamber (MSC-TD, Digitimer, Welwyn Garden City, UK) on the stage of an Olympus IX70 inverted microscope. Temperature was controlled by local superfusion with a Peltier-based system described elsewhere (Reid *et al*., 2001). The temperatures experienced by the neurons were measured after the experiment by placing a miniature T-type thermocouple (1T-1E, Physitemp, Clifton, NJ, USA) where the cell had been and repeating the thermal stimuli. Reproducibility of successive thermal stimuli is very good. The stimulus consisted of a 10-s warming step to ≈36 °C, followed by a cooling ramp to ≈12 °C at a rate of ≈0.8 °C/s. Between successive stimulations the cells were kept at a holding temperature of 32 °C.

Fluorescence changes were recorded with a CCD camera (Cohu 4910, Pieper GmbH, Schwerte, Germany). Neurons were illuminated with a 100-W halogen lamp and filter wheel (Cairn Research, Faversham, UK) controlled by the Axon Imaging Workbench 2.2 software (Axon Instruments, Union City, CA, USA) which was also used for image acquisition and analysis.

For each field of cells which was imaged a phase-contrast image was recorded in order to measure cell diameters. Mean diameter was estimated from cell area, which was measured using a program written by G.R. in the IDL language (version 5.3, Research Systems, Boulder, CO, USA) and assumed to be equal to πr^2. Diameters of neurons located at the edges of a field could not be measured.

Definition of cold sensitivity, temperature threshold and maximal slope of the cold response

For this study we have measured changes in fluorescence of the nonratiometric calcium indicator Calcium Green-1 during cooling for each cell in all fields recorded (a total of 779 cells without added NGF and 769 cells in the presence of NGF). Neurons were allowed to adapt for 5 min at 32 °C before stimulation. The cold response was measured as the change in fluorescence on cooling ΔF (from the moment the thermal stimulus was applied to the time point when the lowest temperature was reached) as a fraction of the initial fluorescence F_0. A histogram of cold responses ($\Delta F/F_0$) for all 779 neurons cultured without NGF was fitted with a two-peak gaussian. To define cold sensitivity, the cutoff value of $\Delta F/F_0$ was taken as the mean + 2 SD of the gaussian peak centred closest to zero. A cutoff value of 0.2 was thus obtained and those cells which responded to cooling with a $\Delta F/F_0 \geq 0.2$ were considered cold-sensitive. A similar procedure was used to define sensitivity to capsaicin (cutoff value of 0.1), icilin (0.055), menthol (0.06) and mustard oil (0.055).

Temperature threshold was measured at the frame before the one at which the fluorescence level was increased by >4 SD compared to baseline.

Maximal slope was defined as the largest fluorescence change between two successive frames during the rising phase of the calcium signal, as a fraction of the initial fluorescence, divided by the time interval between the corresponding frames.

Experimental protocols

A first group of 578 cells without NGF and 585 cells with added NGF were studied using the following sequence of stimuli: (i) cooling; (ii) application of 100 μM menthol at the holding temperature of 32 °C, followed by cooling in the presence of menthol; (iii) application of 50 μM icilin at 32 °C, followed by cooling in the presence of icilin; (iv) application of 2 μM capsaicin at 32 °C.

A second group of 106 neurons without NGF and 113 neurons with added NGF were stimulated with a different protocol: (i) cooling; (ii) 100 μM menthol application at 32 °C followed by cooling in menthol; (iii) application of 20 μM mustard oil at 32 °C; (iv) application of 2 μM capsaicin at 32 °C.

A third group of 95 cells without NGF and 71 cells with added NGF were stimulated using the following protocol: (i) cooling; (ii) 100 μM menthol application at 32 °C followed by cooling in menthol; (iii) cooling; (iv) cooling in 10 μM Ruthenium Red (RR); (v) cooling; (vi) application of 20 μM mustard oil at 32 °C; (vii) application of 2 μM capsaicin at 32 °C.

Neurons were allowed to recover for 5 min at the holding temperature between two successive stimulations.

Solutions

The IncMix solution for DRG incubation contained (in mM): NaCl, 155; K_2HPO_4, 1.5; HEPES, 5.6; NaHEPES, 4.8; glucose, 5. The antibiotic gentamicin was added to 50 μg/mL.

The standard extracellular solution used in all experiments contained (in mM): NaCl, 140; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; Hepes, 10; NaOH, 4.55; glucose, 5 (pH 7.4 at 25 °C).

Drugs were added from the following stock solutions: (–)-menthol (Sigma), 200 mM in ethanol; capsaicin (Fluka), 2 mM in ethanol; mustard oil (Sigma), 1 : 1000 in H_2O; icilin (kind gift from Eddie Wei), 50 mM in DMSO, RR (RBI), 10 mM in H_2O. All drug dilutions were prepared on the day of the experiment and the menthol solution was renewed every 2 h because of evaporation.

Data are represented as mean ± SD. Student's *t*-test was performed using the Origin 6.0 software, while for the χ^2 test we have used a program written in the IDL language.

Results

Characterization of two types of cold-sensitive neurons in rat DRG

As described in the Materials and Methods section, we have considered cold-sensitive those cells which responded to cooling from 36 °C to 12 °C with an increase of fluorescence over F_0 ($\Delta F/F_0$) ≥ 0.2. Based on this criterion, 173 cells were cold-sensitive in a total of 779 in the absence of NGF (22%).

We have classified cold-sensitive neurons into menthol-sensitive (the MS group) and menthol-insensitive (the MI group). Menthol-sensitive neurons were considered those which either (a) responded to menthol at 32 °C or (b) whose cold responses were sensitized by menthol (activation threshold shifted to warmer temperatures by > 3 °C). Based on these criteria, > 70% of the cold-sensitive neurons were classified into the MS group (124/173). The remaining 49 cells were considered to belong to the MI group. Figure 1A shows a typical response of an MS cell to stimulation by cooling and its sensitization by menthol. Figure 1B shows the cold response of an MI cell and its lack of menthol sensitivity.

The two groups of cells were compared using the characteristics of their cold responses (amplitude, temperature threshold and maximal slope) and their pharmacology (sensitivity to icilin, mustard oil, RR and capsaicin). Marked differences have been found, which suggest that we have indeed identified two functionally distinct subclasses of cold-sensitive neurons.

MS cells responded to cooling more strongly, more rapidly and at warmer temperatures than MI cells

MS cells were substantially more cold-sensitive than MI cells. The amplitude of the fluorescence signal ($\Delta F/F_0$) was significantly higher for the MS neurons than the MI neurons (Table 1 and Figs 1 and 2A and B) and the temperature threshold of the cold response was also warmer for the MS group (Table 1). The maximal rate of change of fluorescence during cooling (maximal slope) was significantly higher for the MS neurons than for the MI group (Table 1). In conclusion, menthol-sensitive cells respond to cooling more strongly, faster and at a higher temperature than menthol-insensitive ones. These pronounced differences are emphasized in Fig. 2, where randomly chosen cold responses of 10 MS cells (Fig. 2A) and 10 MI cells (Fig. 2B) were superimposed.

In terms of size the two groups of neurons did not differ significantly (diameters 21.6 ± 7.0 μm for MS cells, $n = 107$ and 21.0 ± 6.9 μm for MI neurons, $n = 42$). However, both groups were significantly smaller than cold-insensitive neurons (23.7 ± 6.3 μm, $n = 515$, $P = 0.003$ vs. MS cells, and $P = 0.008$ vs. MI cells).

MS and MI neurons had markedly different pharmacology

Icilin is an artificial compound which is known to activate both mammalian cold-activated ion channels identified so far: TRPM8 and TRPA1 (McKemy *et al*., 2002; Story *et al*., 2003). We have recorded the response of the two groups of cold-sensitive neurons to 50 μM icilin, a concentration which should induce strong activation of both ion channels. Icilin sensitivity was almost exclusively restricted to the MS group. More than 30% of MS cells responded to icilin at 32 °C (24/79) compared to only one icilin-sensitive cell in 25 for the MI group (Fig. 3).

One interesting feature of the calcium signal elicited by icilin in MS neurons was its transient nature (Fig. 2C). In the same cells, menthol induced a sustained increase in calcium concentration (Fig. 2D).

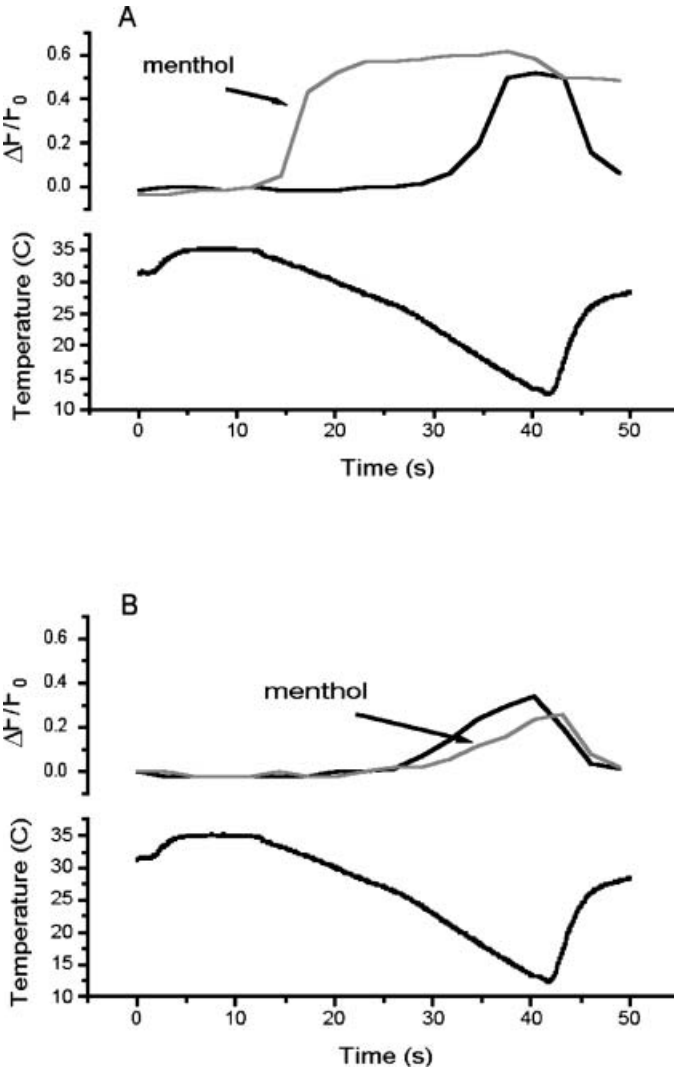

FIG. 1. Cold-sensitive neurons were classified into menthol-sensitive (MS) and menthol-insensitive (MI). Typical fluorescence changes induced by cooling in (A) an MS neuron and (B) an MI neuron, and the effect of menthol on the cold response. Upper traces, $\triangle F/F_0$ is plotted against time. Lower traces represent the temperature stimulus against time. Both upper and lower traces share the same time axis. The fluorescence traces recorded in 100 μM menthol are indicated and displayed in grey. Note the shift in threshold to warmer temperatures induced by menthol in the MS cell (A).

TABLE 1. Characteristics of the cold-induced increase in fluorescence for MS cells compared to MI cells

	MS neurons	*n*	MI neurons	*n*	*P*-value[†]
Amplitudes ($\Delta F/F_0$)	0.44 ± 0.24	124	0.28 ± 0.10	49	< 0.001
Thresholds (°C)	24.9 ± 3.7	120	22.9 ± 2.5	46	< 0.001
Maximal slopes (s^{-1})	0.07 ± 0.05	120	0.05 ± 0.05	46	0.02

[†]Student's unpaired *t*-test. The *n*-values in the same column are different because some cold-sensitive neurons also responded to the warming step preceding the cooling ramp, and that made the measurement of the threshold and slope impossible.

Mustard oil (allyl isothiocyanate) has been shown to activate TRPA1 when expressed in HEK293 cells and in *Xenopus* oocytes (Jordt *et al*., 2004). Mustard oil (20 μM) activated 16% of all neurons

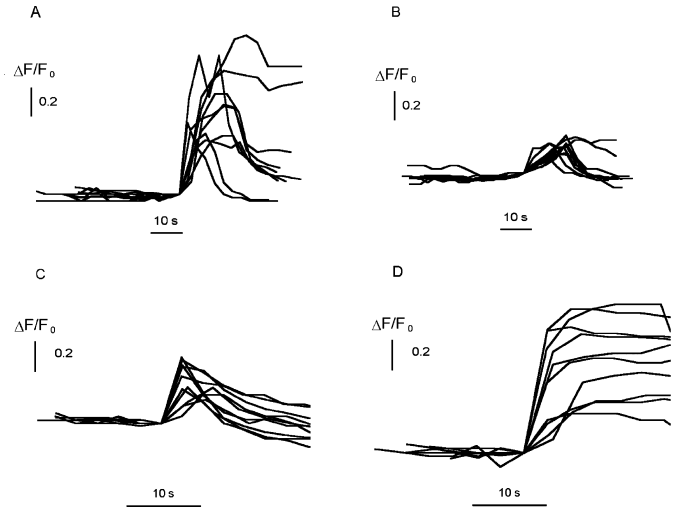

FIG. 2. (A and B) MS neurons responded more strongly and more rapidly to cooling than MI neurons. Superposition of 10 randomly taken cold responses ($\triangle F/F_0$) from (A) MS cells and (B) MI cells. For a clearer picture the fluorescence traces were shifted along the time axis and also vertically so that they would coincide at the threshold. (C and D) The response of MS neurons to icilin was transient. Comparison of the fluorescence change ($\triangle F/F_0$) induced by (C) 50 μM icilin and (D) 100 μM menthol in the same 10 MS neurons. Just as in panels A and B, the traces were shifted along the time axis and also vertically to make them coincide at the moment when the drug was applied. Note the transient nature of the fluorescent signal induced by icilin. In all panels (A–D), $\triangle F/F_0$ is plotted against time.

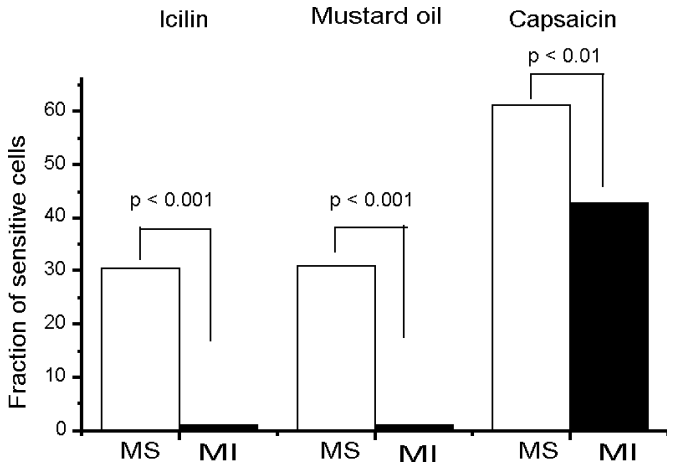

FIG. 3. MS and MI neurons had different pharmacology. The fraction of MS (open columns) and MI (filled columns) neurons responding to icilin, mustard oil (allyl isothiocyanate) and capsaicin are represented. The difference in sensitivity to all these agents between MS and MI neurons were all statistically significant. Probabilities were calculated using the χ^2 test.

in the absence of NGF (32/201). Only about half of these cells were cold-sensitive (15/32). Within the cold-sensitive group, sensitivity to mustard oil was almost entirely restricted to the MS group (14/45 MS cells responded to mustard oil, compared to 1 in 24 for the MI group; Fig. 3).

RR is a known blocker of TRPV channels (Patapoutian *et al*., 2003) and it has been shown to inhibit increases in intracellular calcium induced by cooling in TRPA1-expressing CHO cells (Story *et al*., 2003). We have tested the effect of 10 μM RR on the amplitudes of cold responses for a subset of 30 MS cells and 25 MI cells. The cells were stimulated by cooling, and then by cooling in the presence of menthol in order to assign them to either the MS or the MI group. A bracketed procedure was then used (stimulation by cooling, cooling in 10 μM RR and cooling after RR washout). Of the 30 cells identified as being MS based on the effect of menthol on their cold sensitivity, 24 responded to a subsequent (third) stimulation by cooling and only these cells were analysed further. The effects of RR on MS neurons were complex: in one third (8/24) of the cells cold responses were reversibly inhibited by RR (> 15% reduction in the amplitude), one third of the cells (8/24) were unaffected and in the last third cold responses were reversibly sensitized by RR (> 15% increase in amplitude). An interesting observation was that, of the eight cells in which the cold-induced increase in internal calcium concentration was reversibly blocked by RR, six were mustard oil-sensitive; this is compared to only three of the remaining 16 cells. The potentiation of the cold response in one third of the MS neurons tested can be accounted for by the sensitization of the cold- and menthol-induced inward current by RR (Reid & Flonta, 2001a).

In the case of MI neurons, only 13 of the 25 cells tested responded to a third cooling stimulus and were thus analysed further. A reversible inhibition of the cold response was measured in six of the 13 cells. In the remaining seven there was either an irreversible rundown of the cold response or no effect of RR that could be detected. A remarkable feature was that while four of the six neurons inhibited by RR responded to capsaicin at 32 °C, none of the seven cells in which an effect of RR could not be detected was capsaicin sensitive.

Capsaicin sensitivity is associated with expression of TRPV1, which is also activated by noxious heat in sensory neurons (Caterina *et al*., 1997). Co-expression of cold and capsaicin sensitivity has been reported by many groups (McKemy *et al*., 2002; Reid *et al*., 2002; Viana *et al*., 2002; Story *et al*., 2003). *In vivo* work has shown that, although TRPM8 is not coexpressed with TRPV1 at mRNA level (coexpression of the two receptors is induced by NGF), all TRPA1-positive neurons also expressed TRPV1 mRNA (Story *et al*., 2003). In our DRG cultures in which no NGF was added to the culture medium, > 60% of the MS neurons (likely to express TRPM8) were also capsaicin-sensitive at 32 °C (76/124, 61%). In the MI group significantly fewer neurons were capsaicin sensitive (21/49, 43%; Fig. 3).

Effects of nerve growth factor (NGF) on the two types of cold-sensitive neurons

Nociceptors and thermoreceptors experience increased levels of NGF at their terminals in pathological states such as inflammation and nerve injury. These conditions have been associated with hypersensitivity to cold (cold allodynia) (Choi *et al*., 1994; Takahashi *et al*., 2003). NGF is known to alter the expression pattern of a variety of ion channels and receptors involved in pain and thermal transduction. We have previously shown that NGF increases the cold sensitivity of cold- and menthol-responding DRG neurons (Reid *et al*., 2002). Our aim was to expand these observations by considering the effects of 100 ng/mL NGF added to the culture medium on the two distinct classes of cold-sensitive neurons described above.

NGF had pronounced effects on the overall cold-sensitivity of DRG neurons. The fraction of cold-sensitive neurons declined with time in culture, and this effect was antagonized by NGF (Table 2). The proportion of cold-sensitive neurons was significantly higher on days 1 and 2 after culture in the presence of NGF, and this was mainly due to an effect on MS cells (Table 2 and Fig. 4).

TABLE 2. Time dependence of cold sensitivity in primary cultures of rat DRG neurons

	Day 0			Day 1			Day 2		
	–NGF	+NGF	*P*-value†	–NGF	+NGF	*P*-value†	–NGF	+NGF	*P*-value†
n	154	186		366	342		180	168	
CS (%)	42	33	0.02	20	27	< 0.01	12	26	< 0.001
MS (%)	24	22	NS	16	21	< 0.01	8	19	< 0.001
MI (%)	18	11	0.01	4	6	0.03	3	7	0.02

†χ^2 test; CS, cold-sensitive.

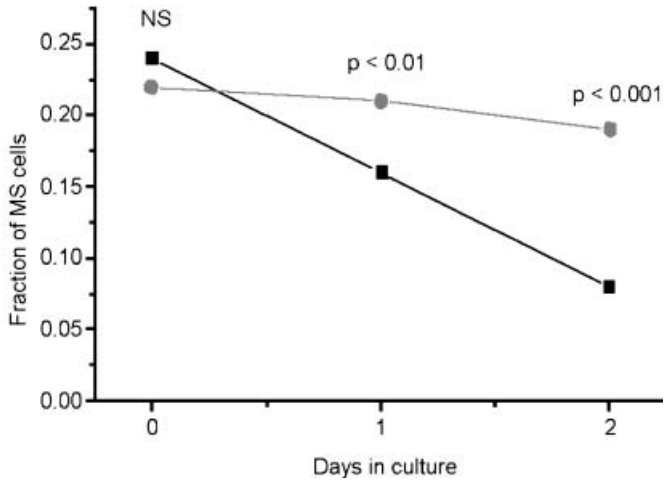

FIG. 4. NGF prevented the decline in the fraction of MS neurons with time in culture. The fraction of MS neurons in the total neuronal population is represented against time in culture in the absence (black squares) and in the presence (grey circles) of 100 ng/mL in the culture medium. Day 0 was the day when neurons were dissociated and plated. The difference in MS fraction in days 1 and 2 was statistically significant. Probabilities were calculated using the χ^2 test.

TABLE 3. Effects of NGF on MS and MI neurons

	–NGF	*n*	+NGF	*n*	*P*-value
MS neurons					
Fraction	124/779		159/769		< 0.001†
Amplitude ($\Delta F/F_0$)	0.44 ± 0.24	124	0.49 ± 0.30	159	0.08 (NS)‡
Threshold (°C)	24.9 ± 3.7	120	26.7 ± 4.2	154	< 0.001‡
Capsaicin-sensitive	76/124		115/159		0.01†
MI neurons					
Fraction	49/779		54/769		NS†
Amplitude ($\Delta F/F_0$)	0.28 ± 0.10	49	0.29 ± 0.10	54	NS‡
Threshold (°C)	22.9 ± 2.5	46	23.6 ± 2.7	51	NS‡
Capsaicin-sensitive	21/49		28/54		NS†

†χ^2 test; ‡Student's unpaired *t*-test.

NGF had significant effects on the MS group**.** The proportion of MS neurons was increased by about one third (159/769 compared to124/779; Table 3, upper part) and the thresholds of activation by cooling were shifted to warmer temperatures by NGF. The previously described increase in the amplitude of the response with NGF (Reid *et al*., 2002) failed to reach a significant level in this work. The fraction of capsaicin-sensitive cells in the MS group was increased from 61 (76/124) to 72% (115/159) in the presence of NGF (Table 3, upper part).

Interestingly, the MI group appears to be insensitive to NGF. Neither their cold sensitivity nor their pharmacology was changed by the addition of 100 ng/mL NGF to the culture medium (Table 3, lower part).

(3) MS neurons which responded to capsaicin were less cold-sensitive

Within the MS group, capsaicin sensitivity is associated with a reduced sensitivity to cold. In agreement with a previous report from our group (Reid *et al*., 2002), the amplitudes of cooling-induced fluorescence changes were significantly higher (0.51 ± 0.29, $n = 48$, compared to 0.39 ± 0.19, $n = 76$; Student's *t*-test, $P = 0.003$), and the calcium signals activated at a warmer temperature (26.0 ± 3.8 °C, $n = 46$, compared to 24.2 ± 3.4 °C, $n = 74$; $P = 0.008$) for capsaicin-insensitive MS neurons than for capsaicin-sensitive ones. A new observation is that capsaicin-insensitive MS neurons had significantly smaller diameters than capsaicin-sensitive ones (19.8 ± 7.1 μm, $n = 43$, compared to 22.9 ± 6.6 μm, $n = 64$, $P = 0.03$).

The pharmacology of the two subpopulations within the MS group was also different. More capsaicin-sensitive MS neurons responded to icilin (18/51, compared to 6/28) and all mustard oil-sensitive MS cells belonged to this group. Menthol sensitivity at 32 °C was more frequent in the capsaicin-insensitive fraction (23/28, compared to 37/51).

When the MI neurons were separated based on capsaicin sensitivity no significant differences were recorded in the amplitudes and temperature thresholds of their cold responses or in their size. The only difference we could observe was the inhibition by RR, which was more pronounced in capsaicin-sensitive MI cells (see paragraph 1b above).

Discussion

This work was aimed at understanding the molecular basis of cold sensing in an *in vitro* model of rat DRG neurons in primary culture. We have used a thermal stimulus consisting of a 30-s cooling ramp from ≈36 °C to ≈12 °C. Considering the rather short duration of our stimulus, and the fact that many cold-sensitive neurons responded with a slow increase in $[Ca^{2+}]_i$ with cooling, it is probable that our results are relevant for non-noxious cold sensing. It should also be pointed out that we are using DRG neurons in culture as a model of their terminals, and possible changes in phenotype in culture should be taken into consideration when interpreting the data.

A recent study reported heterogeneity in cold responses of neurons from rat TG cultures (Thut *et al*., 2003). Some neurons (the low threshold group) were activated by small decreases in temperature and responded with a fast increase in internal $[Ca^{2+}]$ followed by adaptation. In the second group, neurons were activated by stronger

cooling, had a slow rising calcium signal and very little desensitization over 4 min of stimulation by cooling. However, in terms of the transducer molecules involved, no clear distinction emerged between the two groups. Both were menthol-sensitive (although to a different extent, with a higher fraction of the low threshold group responding to menthol at 34 °C) and the cold-induced calcium signals were partially inhibited by amiloride in both groups. Therefore the authors concluded that there is a significant overlap in the properties of the two classes of cold-sensitive neurons and that there must be more than one transducer molecule for each group.

We have separated cold-sensitive neurons based on menthol sensitivity. This was motivated by the fact that the only menthol receptor identified up to now is TRPM8, which gives us the possibility to assign a probable molecular identity to at least a subpopulation of cold-sensing neurons (the alternative that there could be more than one menthol receptor cannot be completely excluded). We did not restrict the definition of menthol sensitivity to those cells which responded to 100 μM menthol at 32 °C, but also included those neurons in which menthol sensitized the response to cooling by shifting the activation threshold to warmer temperatures. Therefore we have separated a group of cold-sensitive neurons either responding to or being sensitized by menthol (the MS group), and which are very likely to be expressing TRPM8, the cold and menthol receptor. The remaining cold-responding cells were considered menthol-insensitive (MI).

Pronounced differences were found between MS and MI neurons in the characteristics of their cold response, pharmacology and modulation by NGF. MS cells responded with a larger and faster change in $[Ca^{2+}]_i$ with cooling and were activated at warmer temperatures. Within the population of cold-sensitive neurons, sensitivity to icilin and mustard oil (known activators of TRPA1) was restricted to the MS group. Sensitivity to capsaicin was significantly more frequent in MS neurons and their sensitivity to cold and capsaicin was increased by NGF, while none of the features of MI neurons appeared to be dependent on NGF.

While for the MS population we can identify with very high probability TRPM8 as the transducer of cold stimuli, in the case of MI neurons the identity of the transducer(s) is not yet clear. TRPA1 was a possible candidate for the role of cold sensor in the MI group, as it is activated by cooling with a threshold of ≈18 °C and a slow increase in $[Ca^{2+}]_i$ when expressed heterologously (Story *et al*., 2003). However, our data do not support the involvement of TRPA1 in mediating the cold responses in the MI group, as these neurons are insensitive to icilin and mustard oil, two agents known to activate TRPA1 (Story *et al*., 2003; Bandell *et al*., 2004; Jordt *et al*., 2004). Jordt *et al*. (2004) reported that only 4% of mustard oil-sensitive neurons in TG cultures responded to cooling to 5 °C and all these cells were menthol-sensitive, implying that their cold sensitivity can be attributed to the expression of TRPM8. Our data agree with this observation in that almost all mustard oil-sensitive cells which also respond to cooling belong to the MS group. However, the coexpression of mustard oil and menthol sensitivities is much more pronounced in our cultures. More than 75% of mustard oil-responding neurons were also menthol-sensitive, compared to the 4% overlap reported by Jordt *et al*. (2004). This may be due to a difference in ion channel expression in DRG neurons compared to TG neurons.

Moreover, the MI group as a whole is less sensitive to capsaicin, which has been shown to activate TRPA1-expressing DRG and TG neurons (Story *et al*., 2003; Jordt *et al*., 2004). Our observation that cold responses in capsaicin-sensitive MI neurons are inhibited by RR could be accommodated with a role of TRPA1 as a cold sensor in this subgroup of the MI population but, in view of their insensitivity to icilin and mustard oil, this seems unlikely.

Patapoutian and colleagues also make a distinction between menthol-sensitive and menthol-insensitive cold-sensing neurons from adult rat DRG (Story *et al*., 2003; Bandell *et al*., 2004). They propose a model in which the menthol-sensitive group responds to milder cooling and is capsaicin-insensitive, while the menthol-insensitive group is activated at lower temperatures and responds to capsaicin. It is suggested that the two groups are expressing TRPM8 and TRPA1, respectively. Our results do not support a role for TRPA1 in cold sensing by MI neurons. This discrepancy may be partly explained by the different definition of menthol sensitivity used in these two studies, compared with our work. While these authors consider only those neurons responding to menthol at the basal temperature as menthol-sensitive, we also include neurons in which the cold response is sensitized by menthol. The thermal stimulus used in these two papers is also quite different from ours, in that the basal temperature is different (25 °C compared to 32 °C in our case), the stimulus lasts longer (4 min compared to 30 s) and it reaches a lower temperature (9 °C compared to 12 °C). Although TRPA1 does not seem to be the cold sensor in our MI group, we do not exclude the possibility that this channel may be involved in cold transduction at lower temperatures than the ones reached in this study. The role of TRPA1 in cold transduction is still controversial, as the authors of a recent report (Jordt *et al*., 2004) found that TRPA1 expressed in HEK293 cells was not activated by cooling to 4 °C.

Work based on calcium fluorimetry in cultured sensory neurons (this study and also Jordt *et al*., 2004) suggests that TRPA1 may be expressed in a subset of menthol-sensitive (and thus very probably TRPM8-expressing) neurons. Our observation that RR blocks cold-induced increases in $[Ca^{2+}]_i$, mainly in mustard oil sensitive MS cells, suggests that in some cold-sensitive neurons both TRPM8 and TRPA1 contribute to the cold response. However, these results are not in agreement with the lack of coexpression of TRPM8 and TRPA1 at mRNA level *in vivo* (Story *et al*., 2003).

It is interesting to note that a subpopulation of the MS group expresses nociceptor-like sensitivity to the pain-inducing agents capsaicin and mustard oil. Moreover, this subgroup is characterized by a reduced sensitivity to cold, with smaller cold-induced changes in $[Ca^{2+}]_i$ and lower activating temperatures than capsaicin-insensitive MS neurons.

Co-expression of capsaicin and menthol sensitivity is still a controversial issue. While some authors reported that ≈50% of cold-sensitive sensory neurons in primary cultures also respond to capsaicin (McKemy *et al*., 2002; Reid *et al*., 2002; Viana *et al*., 2002), others propose that coexpression does not occur *in vivo*, and it is induced in cultures in the presence of NGF (Story *et al*., 2003). In agreement with this latter hypothesis, TRPM8 mRNA is not coexpressed with positive immunostaining for TRPV1 in freshly dissociated adult mouse DRGs (Peier *et al*., 2002). In contrast, we have recorded capsaicin responses from menthol-sensitive rat DRG neurons even in the absence of added NGF and, also, quite shortly (< 2 h) after plating the cells. Supporting our results, very recent evidence shows that TRPV1 is expressed in ≈30% of TRPM8-positive neurons *in vivo* (Okazawa *et al*., 2004).

This discrepancy may be explained by a species difference (mouse vs. rat). We can not exclude the possibility that coexpression of menthol and capsaicin sensitivity is a culture artefact, but if it is so it has a very fast onset. In any event, our data do not agree with a simple model according to which NGF induces expression of capsaicin sensitivity in cold- and menthol-responding DRG neurons (Story *et al*., 2003).

It should be added that the concept of neurons responding both to cooling and pain-inducing agents such as capsaicin is not completely devoid of physiological sense. It has been reported that, when Aδ fibres are blocked, gentle cooling evokes a sensation of burning pain (Yarnitsky & Ochoa, 1990) and this could be explained by a model in which stimulation of innocuous cold receptors inhibits neurons which respond to both cooling and painful stimuli, possibly at spinal cord level. Block of innocuous cold receptors (which are known to be Aδ fibres in primates) would unmask the activation of these cold-sensitive nociceptors by gentle cooling which would explain the sensation of burning pain and this phenomenon very probably explains the thermal grill illusion (Craig & Bushnell, 1994).

NGF increases cold sensitivity in rat DRG neurons in culture, primarily by enhancing cold responses of menthol-sensitive neurons. In the presence of 100 ng/mL NGF, MS neurons responded to cooling at warmer temperatures and with a larger increase in $[Ca^{2+}]_i$ (Reid *et al*., 2002 and this study). The proportion of MS neurons in the overall population of DRG neurons and the fraction of capsaicin-sensitive MS cells were also increased by NGF. These observations could explain cold allodynia, as they suggest ectopic expression of cold sensitivity (most probably through TRPM8) in capsaicin-sensitive nociceptors. However, a direct demonstration of this effect is still lacking. NGF is known to have long-term effects, involving altered gene expression (Kaplan & Miller, 2000), as well as acute effects on the biophysical properties of channels such as TRPV1 (Chuang *et al*., 2001). Both these effects may be involved in the increased sensitivity to cold induced by NGF in MS neurons. Our data also suggest expression of TRPM8 induced by NGF in cells previously not expressing the cold and menthol receptor.

In conclusion, our data favours the following picture of cold sensitivity in cultured DRG neurons in the rat: (i) gentle cooling is transduced in great part by the cold and menthol receptor TRPM8; (ii) a subset of TRPM8-expressing neurons may be serving a subtle nociceptive function; (iii) in the range of temperatures used (32–12 °C), TRPA1 may contribute to the cold response in a subset of TRPM8 expressing neurons but there is no evidence for a role of TRPA1 as a transducer in a separate group of neurons; and (iv) cold sensitivity is enhanced by the neurotrophin NGF through an effect on TRPM8-expressing neurons; the increase in the degree of overlap in TRPM8 and TRPV1 expression induced by NGF could account for the reported hypersensitivity to cold in pathological pain states.

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Abbreviations

DRG, dorsal root ganglia; MI, menthol-insensitive; MS, menthol-sensitive; NGF, nerve growth factor; RR, Ruthenium Red; TG, trigeminal ganglia.

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