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A novel type of cold-sensitive neuron in rat dorsal root ganglia with rapid adaptation to cooling stimuli

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Abstract

Cold sensing in mammals is heterogeneous and more than one type of receptor molecule is likely to be involved in the transduction process. Most features of innocuous cold receptors have been explained by TRPM8, the cold and menthol receptor, but their fast adaptation to cooling has not yet been reproduced in cellular systems. In this study we have used a newly developed system for applying fast thermal stimuli to dissociated dorsal root ganglia (DRG) neurons from young rats (150–200 g) in primary culture. We describe a novel type of cold-sensitive rat DRG neuron with rapid adaptation to cooling. These cells (4.3% of the total DRG population) do not express either TRPM8 or the other cold-activated TRP channel, TRPA1, and the epithelial sodium channel (ENaC) is not involved in their transduction. Increases in intracellular calcium induced by cooling in rapidly adapting neurons are caused by calcium entry. These neurons express a large and rapidly adapting cold-induced inward current with a time constant of adaptation in the seconds range, and may correspond to the rapidly adapting cold receptors described *in vivo*.

Introduction

Rapid progress has been made recently in understanding thermal transduction with the cloning of a number of thermosensitive ion channels from the TRP family, which span the whole range of physiologically relevant temperatures, from noxious cold (TRPA1) and gentle cooling (TRPM8), through warm (TRPV3 and TRPV4) and into the noxious heat range (TRPV1 and TRPV2; Patapoutian *et al*., 2003). It has been proposed that TRPM8, the cold and menthol receptor, is primarily involved in innocuous cold sensing (McKemy *et al*., 2002; Peier *et al*., 2002) while TRPA1, activated by stronger cooling and by pungent compounds such as cinnamaldehyde (CA), mustard oil and garlic oil, senses noxious cold (Story *et al*., 2003; Bandell *et al*., 2004). However, while the cold- and menthol-activated current mediated by TRPM8 can explain most of the known characteristics of innocuous cold receptors (Reid & Flonta, 2001a; Reid *et al*., 2002), the role of TRPA1 as a cold sensor is uncertain (Babes *et al*., 2004; Jordt *et al*., 2004; Nagata *et al*., 2005). Two other ion channels have been suggested to play a role in cold sensing, based on their temperature dependence in heterologous systems; TREK-1 (Maingret *et al*., 2000) and the epithelial sodium channel (ENaC; Askwith *et al*., 2001). Evidence from native systems supports an involvement of background potassium channels in cold sensing (Reid & Flonta, 2001b; Viana *et al*., 2002). It is as yet unclear how each of these molecules contributes to transduction in intact innocuous cold receptors and cold nociceptors (reviewed in Reid, 2005).

In native dorsal root ganglion (DRG) neurons, TRPM8 adapts with a time constant of approximately 1 min (Reid & Flonta, 2001a; Reid *et al*., 2002), which may account for the slow adaptation of cold receptors *in vivo* (Kenshalo & Duclaux, 1977). However, recordings made up to now in cultured DRG neurons have been unable to account for the rapid adaptation of intact cold receptors on applying a fast cold stimulus (within a few seconds; Darian-Smith *et al*., 1973; Kenshalo & Duclaux, 1977; Campero *et al*., 2001). This may simply be due to the relatively slow cooling stimuli that have been applied up to now in cultured neurons, with time constants of several seconds.

Using a newly developed system for fast application of thermal stimuli (applying temperature jumps within ~20 ms), we have observed a new population of cold-sensitive neurons in rat DRG, which adapt rapidly during step cooling stimuli. According to their pharmacological profile, these rapidly adapting neurons do not express either TRPM8 or TRPA1 and we also provide evidence against a role of the ENaC in their response to cooling. These neurons express a cold-induced inward current, which adapts with a time course of seconds during a cold step, consistent with the fast adaptation of intact cold receptors. This is the first report of a new type of cold-sensitive neuron in rat DRG, which most likely expresses a still unknown cold transducer molecule. A preliminary version of this report has been presented in abstract form (Babes *et al*., 2005).

Materials and methods

DRG culture

Adult Wistar rats (150–200 g) were killed by inhalation of 100% CO_2 followed by decapitation, following a UK Home Office approved method (Schedule 1) in the absence of local guidelines or national legislation. DRGs from spinal levels L1 to S1 were removed and

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dissociated as previously described (Reid *et al*., 2002). Briefly, they were incubated in IncMix solution (see below) containing 1 mg/mL collagenase (type XI) and 3 mg/mL dispase (nonspecific protease) for 1 h at 37 °C. Neurons were dissociated by trituration, plated onto glass coverslips, which had been treated with poly D-lysine (0.1 mg/mL, 30 min) and cultured in 5% CO_2 in air at 37 °C 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. Nerve growth factor (NGF) 7S was added to the medium before plating at a final concentration of 100 ng/mL. Recordings were made from 2 h after plating to up to 2 days in culture. All materials for cultures were from Sigma.

Intracellular calcium imaging and application of fast thermal stimuli

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 0.002% Pluronic (both from Molecular Probes, Leiden, The Netherlands) and then the cells were allowed to recover for at least 30 min at 37 °C before recording. Coverslips were mounted on a Teflon chamber (MSC-TD, Digitimer, Welwyn Garden City, UK) on the stage of an inverted microscope (Olympus IX70). The temperature was controlled by local superfusion with a feedback controlled Peltier system based on that previously described (Reid *et al*., 2001). Cooling steps were performed by switching (~20 ms) between two solution flows with independently controllable temperatures (Reid & Zorzon, 2005) using the SF-77B Perfusion Fast-Step (Warner Instrument Corporation, Hamden, CT, USA). The actual temperatures experienced by the neurons were measured after the experiment by repeating the thermal stimuli and placing a miniature T-type thermocouple (1T-1E, Physitemp, Clifton, NJ, USA) where the cell had been. Reproducibility of successive thermal stimuli is very good. Two types of thermal stimuli were used in this study; 10-s cooling steps to ~18 °C from a base temperature of 32 °C, and 30 s cooling ramps to ~20 °C preceded by a 10-s warming step from 32 to ~38 °C. Between successive stimuli cells were kept for 5 min at a holding temperature of 32 °C, which allowed a complete recovery of the cold-evoked calcium signal. 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 (Takahashi *et al*., 1999). Sensitivity to various chemical agents and to cooling was defined as in our previous study (Babes *et al*., 2004), namely the histogram of $\Delta F/F_0$ for each substance was fitted with a two-peak gaussian, and the cutoff value was taken as the mean + 2SD of the peak closest to zero. The following values were obtained: cold 0.2; menthol 0.06; capsaicin 0.1; icilin 0.05; cinnamaldehyde 0.065; pH 6 0.1; camphor 0.05; pH 8 0.06; ruthenium red (RuR) 0.04.

Patch-clamp recordings

Patch-clamp recordings were made using borosilicate glass pipettes (GC150T, Harvard Apparatus), heat polished to a resistance of 2–4 MΩ. Currents were recorded with a WPC-100 amplifier (ESF Electronic Göttingen, Germany), filtered at 3 kHz and digitized with a Labmaster 160 kHz DMA interface (Scientific Solutions, Mentor, Ohio, USA), using software written by G.R., which was also used to control the thermal stimulator. Analysis was performed using the Origin 6.0 software (OriginLab Corporation, Northampton, MA, USA). All whole cell recordings were made with the amphotericin perforated patch configuration (Rae *et al*., 1991).

Solutions and chemicals

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 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). For the acid solutions (pH 6), HEPES was replaced by MES (10 mM) and the pH was adjusted by adding 4.42 mM NaOH. Alkaline solutions (pH 7.6 and pH 8 at 25 °C) were made in HEPES buffer, using the appropriate amount of NaOH (5.68 and 7.69 mM, respectively).

The pipette solution for amphotericin perforated patch configuration contained (in mM): K_2SO_4 60; KCl 35; NaCl 10; $MgCl_2$ 1; sucrose 20; HEPES 10; EGTA 1; NaOH 3.45; KOH 2.35; (pH 7.2 at 25 °C). Amphotericin B (240 μg/mL; Sigma) and 0.02% Pluronic were added to the pipette solution before the recording. The pipette was backfilled with the amphotericin B-containing solution, after filling the tip with amphotericin-free solution.

Drugs were added from the following stock solutions: (–)-menthol (Sigma), 100 mM in ethanol; capsaicin (Fluka), 2 mM in ethanol; icilin (gift from Eddie Wei), 50 mM in DMSO; RuR (RBI), 10 mM in H_2O; cinnamon aldehyde (Sigma), 200 mM in ethanol; camphor (Sigma), 2 M in ethanol; amiloride (Sigma), 100 mM in DMSO.

Data are presented as mean ± SD. Two-tailed Student's *t*-test (paired and unpaired) was performed using the Origin 6.0 software. A value of $P < 0.05$ was considered to be statistically significant.

Results

Classification of cold-sensitive neurons according to the kinetics of their response to a fast cooling step

The stimulus used in this study consisted of a 10-s cooling step from a base temperature of ~32 °C to ~18 °C at the cell (lower traces in Fig. 1A and B). Based on previous work (Babes *et al*., 2004) we have considered cells that responded with $\Delta F/F_0 \geq 0.2$ to be cold-sensitive. In a first series of experiments, 2743 cells were imaged and the cold response of each individual cell was recorded and analysed. A total of 488 (17.8%) proved to be cold-sensitive.

Based on the kinetics of the rise in $[Ca^{2+}]_i$ during the cooling step, we identified four broad groups of neurons (Fig. 1): (i) rapidly adapting (in which $[Ca^{2+}]_i$ returned to values close to the baseline within 10 s; Fig. 1A, trace a); (ii) nonadapting (with a sustained response to cooling, in which the increase in $[Ca^{2+}]_i$ was maintained for the whole duration of the cooling step; Fig. 1A, trace b); (iii) intermediate (with intermediate kinetics between the previous two types; Fig. 1B, trace c) and (iv) slow (in which $[Ca^{2+}]_i$ increased continuously during cooling; Fig. 1B, trace d).

We considered that menthol sensitivity, a hallmark of an important fraction of cold-sensitive neurons (Reid & Flonta, 2001a; McKemy *et al*., 2002; Viana *et al*., 2002), would provide us with a useful quantitative parameter for an unambiguous classification of our cold-sensitive DRG neurons. A total of 72 cold-sensitive neurons were submitted to a cooling ramp from 38 °C to ~20 °C (Fig. 2A and B, lower part), followed by the same ramp in 100 μM (–)-menthol. Menthol-sensitive neurons were considered not only those that

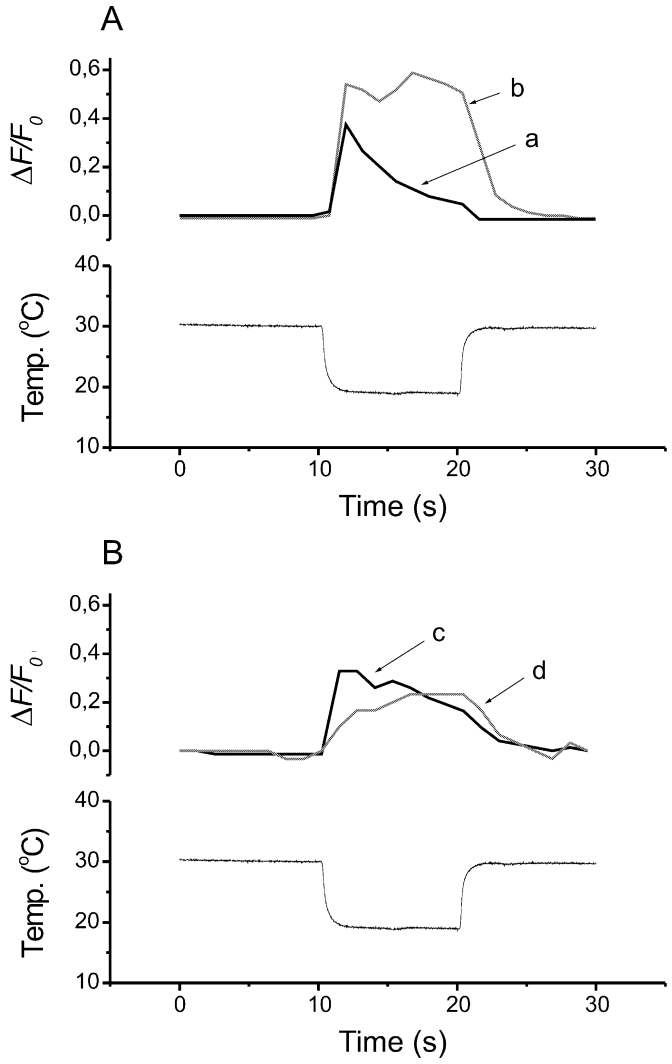

FIG. 1. Four types of responses to fast cooling in rat DRG. (A, upper traces) Illustrative responses of a rapidly adapting (a) and a nonadapting neuron (b). (B, upper traces) Illustrative responses of an intermediate (c) and a slow neuron (d). (A and B, lower traces) The cooling stimulus (~32 to ~18 °C) measured where the cell had been with a T-type thermocouple. The apparent time course of the cooling step is limited by the response time of the thermocouple; the actual time course is much faster (~20 ms; Reid & Zorzon, 2005).

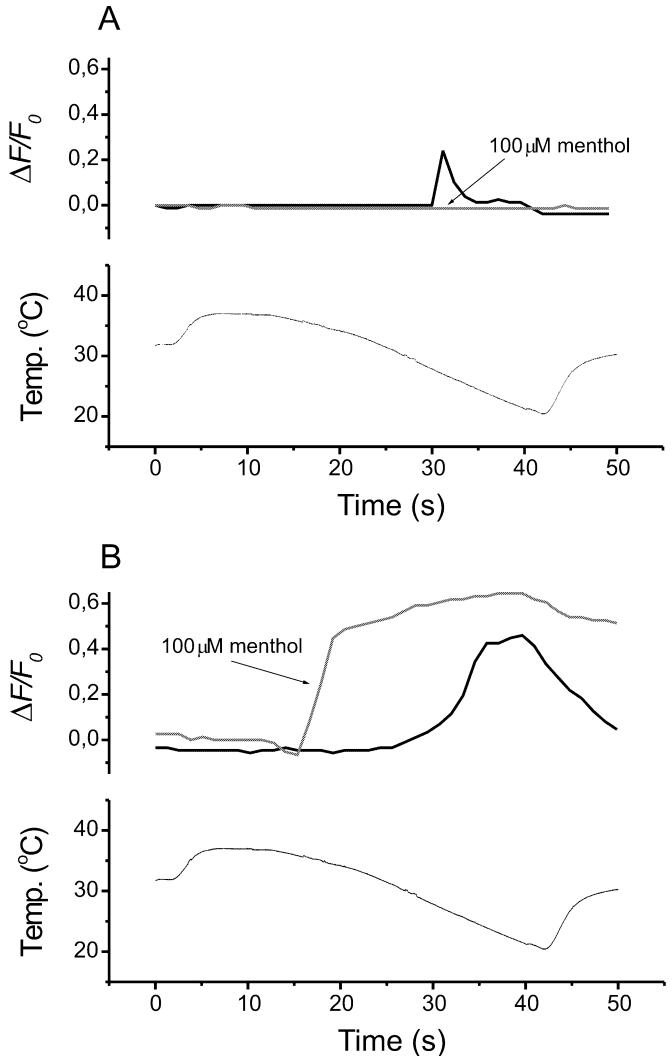

FIG. 2. Rapidly adapting neurons are not sensitized by menthol. (A, upper traces) Responses of the rapidly adapting neuron from Fig. 1A to a cooling ramp before (black trace) and during (grey trace) the application of 100 μM (–)-menthol. Note the inhibition of the cold response in menthol. (B, upper traces) Responses of the nonadapting neuron from Fig. 1A to a cooling ramp before (black trace) and during (grey trace) the application of 100 μM (–)-menthol. Note the strong sensitization induced by menthol. (A and B, lower traces) The cooling stimulus used in these experiments consisted of a 30-s cooling ramp from ~38 to ~20 °C, preceded by a 10-s warming step from the base temperature of 32 °C to 38 °C.

responded to menthol at the base temperature, but also those in which the response to cooling was sensitized (shifted to warmer temperatures) in the presence of menthol (Fig. 2B). Based on these criteria, exactly half of the cells (36/72) were menthol sensitive. Interestingly, we observed a correlation between the kinetics of the response to a cooling step and menthol sensitivity, in that almost all rapidly adapting neurons were not sensitized by menthol (Fig. 2A), while all nonadapting neurons were menthol sensitive (Fig. 2B).

A plot of $\Delta F/F_0$ in response to 100 μM menthol applied at 32 °C against the degree of adaptation (defined as the ratio between $\Delta F/F_0$ at the last frame, and $\Delta F/F_0$ at the first frame during the cooling step; Fig. 3) shows a cluster of rapidly adapting, menthol-insensitive neurons in the lower left corner and a region dominated by nonadapting, menthol-sensitive neurons.

The smallest end/initial ratio seen in any menthol-sensitive cell (i.e. the highest degree of adaptation) was 0.37 of the initial response (Fig. 3). We therefore used this value as a cut-off to define a rapidly adapting subpopulation of the menthol-insensitive group (RA neurons; those that adapt within 10 s to less than 37% of the initial response to cooling, contained in the grey oval in Fig. 3). The RA neurons comprised the great majority of the menthol-insensitive population (28/36, 71%). It is also evident from Fig. 3 that neurons in which $\Delta F/F_0$ in response to cooling either decreased or increased by less than 25% during the stimulus were all menthol sensitive (contained in the black oval in Fig. 3), so we consequently used these values to define a nonadapting subpopulation of the menthol-sensitive group (NA neurons; those whose response to cooling does not change from its initial value by more than 25% during the 10-s stimulus). In the remainder of this paper we shall concentrate on the comparison between these two relatively homogeneous groups of NA neurons (whose transducer is probably TRPM8) and RA neurons (apparently expressing a novel transducer molecule). As we did not apply menthol

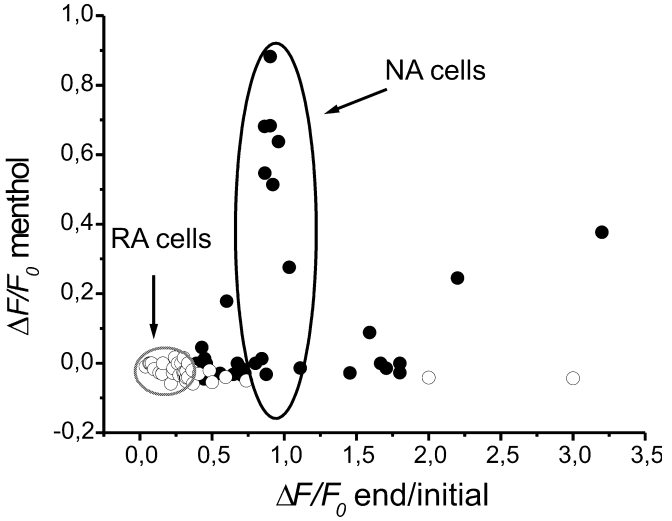

FIG. 3. Most neurons with rapid adaptation to cooling are menthol insensitive. Responses to 100 μM menthol applied at the base temperature of 32 °C are plotted against the degree of adaptation (end/initial ratio, i.e. the ratio of $\Delta F/F_0$ measured at the last frame to that at the first frame during the cooling step). Menthol-sensitive neurons (i.e. those that either responded to menthol at the base temperature of 32 °C, or whose response to cooling was sensitized by menthol) are represented with filled circles, and menthol insensitive ones with open circles. Notice that all neurons that adapt to less than 37% of the initial response are menthol insensitive (RA neurons), and all neurons whose response changes by less than 25% from its initial level are menthol sensitive (NA neurons).

in all experiments, we restricted our comparison to the NA group defined as above, as all these cells were menthol sensitive. The remaining intermediate and slow cold-sensitive neurons were also largely menthol sensitive (Fig. 3) but not 100%, and their pharmacology (i.e. sensitivity to agonists and antagonists) was very similar to that of NA neurons.

Of the total 2743 neurons imaged without selection, 488 were cold-sensitive and of these 118 were RA (4.3% of the total neuronal population) and 144 were NA (5.2%). The two groups of cells (RA and NA) were not significantly different in diameter (21.8 ± 2.9 μm, $n = 101$, for RA neurons; 21.9 ± 4.6 μm, $n = 114$ for NA cells), but both groups were significantly smaller than cold-insensitive neurons (27.7 ± 7.1 μm, $n = 1755$, $P < 0.0001$, Student's unpaired *t*-test). The remaining 156 (32% of cold-sensitive neurons) could not be assigned to either the RA or the NA group.

In order to identify the cold transducer in RA neurons we have treated these cells with agonists or antagonists for ion channels known to be activated or sensitized by cooling: menthol (agonist, TRPM8), cinnamon aldehyde (agonist, TRPA1), icilin (agonist, TRPM8, TRPA1), amiloride (antagonist, ENaC). Camphor is an interesting compound, known to increase the sensations evoked by both cooling and warming in humans (Green, 1990) and recently shown to activate two heat-activated ion channels TRPV1 and TRPV3 (Xu *et al.*, 2005).

Cold sensitivity of RA and NA cells; effects of menthol

The amplitude of the response to a 20 °C step was higher for the NA group (0.50 ± 0.23, $n = 144$; compared to 0.43 ± 0.14, $n = 118$; Student's unpaired *t*-test, $P < 0.01$). The effect of cold ramps and of cold ramps in the presence of 100 μM menthol was monitored in 28 RA cells and 11 NA cells.

Of the 28 RA cells only eight responded ($\Delta F/F_0 \geq 0.2$) during a cooling ramp from 32 °C to ~20 °C. Interestingly, when the cooling ramp was applied in the presence of menthol, the response of the RA cells to cooling was abolished, suggesting an inhibitory effect of menthol on these cells (see below and Fig. 2A). In order to exclude the possibility that RA neurons express a menthol-sensitive transducer but are not menthol sensitive during ramps because of strong adaptation, we have also applied 500 μM menthol with the fast application system; while seven of nine NA neurons were activated by rapid application of 500 μM menthol, none of nine RA cells responded to this stimulus.

Of 11 NA cells studied, eight responded during the cooling ramp, and the remaining three responded in the presence of menthol.

Comparing the responses of the two groups of cells during ramps, NA neurons responded with higher amplitudes (0.61 ± 0.23, $n = 8$; compared to 0.33 ± 0.09, $n = 8$; Student's unpaired *t*-test, $P < 0.01$) and had warmer temperature thresholds (33.5 ± 2.4 °C, $n = 8$; compared to 26.4 ± 2.6 °C, $n = 8$; $P < 0.001$).

In order to measure the temperature threshold for activation of the two groups of cold-sensitive neurons by fast cooling, jumps were performed from a holding temperature of 32 °C to the following temperatures: 27.5, 25, 24, 22.5 and 19 °C. A total of 24 neurons (12 RA and 12 NA) were used for this experiment. While all NA neurons were already activated by a stimulus of 27.5 °C, RA cells crossed the 0.2 threshold only at temperatures below 24 °C (Fig. 4). Taken together these data show that RA neurons are less cold-sensitive than NA cells.

Cold responses in RA cells are blocked by menthol

In 19 RA neurons a cooling step was performed in the presence of 100 μM menthol, bracketed between two stimuli applied in normal extracellular solution. The response in menthol was significantly and reversibly decreased (to 61%; 0.28 ± 0.18, compared to 0.46 ± 0.13 before menthol, and 0.42 ± 0.2 after washout, $n = 19$; Student's paired *t*-test, $P < 0.001$). Ethanol alone (1 : 1000 dilution) had no significant effect on the cold responses in RA cells ($n = 5$).

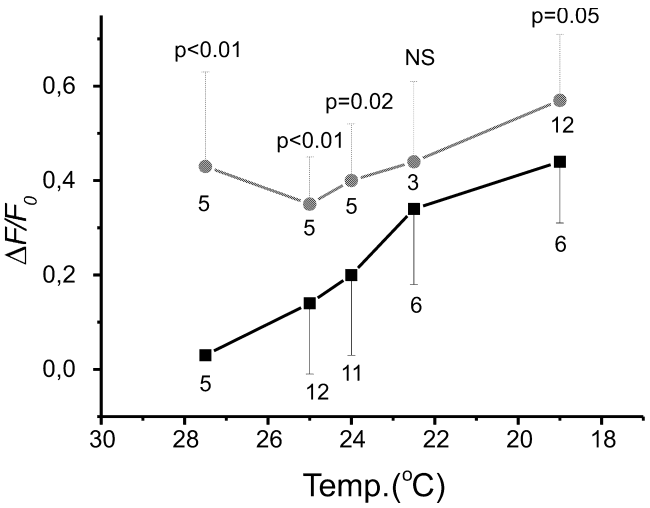

FIG. 4. RA neurons activate at lower temperatures than NA neurons. Each symbol represents the mean amplitude of responses to cooling steps from a base temperature of ~32 °C to the temperatures represented on the horizontal axis for RA (black symbols) and NA neurons (grey symbols). The number (n) for each mean is presented in the figure below each symbol. Error bars represent the SD. The results of the unpaired Student's *t*-test for comparison of the responses of RA and NA cells at each temperature are also given (NS, not significant).

Of 20 NA cells tested, 16 responded to the application of 100 μM menthol, and in the remaining four the cold response was increased in menthol, confirming that all cells in this group are menthol sensitive.

RA cells do not express TRPA1, but a fraction of them expresses TRPV1

Cinnamaldehyde (CA) is a TRPA1 agonist (Bandell *et al*., 2004). All 28 RA cells tested were insensitive to 200 μM CA, which extends the earlier finding of the lack of expression of TRPA1 in a menthol-insensitive population (Babes *et al*., 2004). CA activated 55% of NA cells (6/11; Fig. 5A and Table 1), in agreement with the observed functional coexpression of TRPM8 and TRPA1 (Babes *et al*., 2004).

Icilin was applied for ~90 s at 50 μM. While none of the 14 RA cells tested responded to icilin, almost all NA neurons were icilin-sensitive (13/14 in total, 93%; Table 1 and Fig. 5B).

Based on the lack of sensitivity of RA neurons to icilin and cinnamon aldehyde, we conclude that this group of cold-sensitive neurons does not express the cold-activated ion channel TRPA1.

Both groups were sensitive to 2 μM capsaicin applied at 32 °C; 62% (8/13) of the RA group and 31% (4/13) of NA cells (Table 1).

Calcium entry is responsible for the cold-induced elevation of intracellular calcium in rapidly adapting neurons

To see whether the increase in $[Ca^{2+}]_i$ induced by cooling is due to calcium entry or release, 11 RA neurons were stimulated by a fast cooling step in a calcium-free, magnesium-free solution (1 mM EGTA). The response in 0 calcium was strongly reduced, from 0.38 ± 0.1 to 0.03 ± 0.04 ($n = 11$, Student's paired t-test, $P < 0.001$), demonstrating that extracellular calcium is needed for the response recorded during cooling.

RA cells are more sensitive to alkaline pH than NA cells

Due to the temperature dependence of the pK of our HEPES buffer, a cold step from 32 to 20 °C would have caused a rise in pH from pH 7.4 to pH 7.6. When pH 7.6 was applied, three of five RA cells

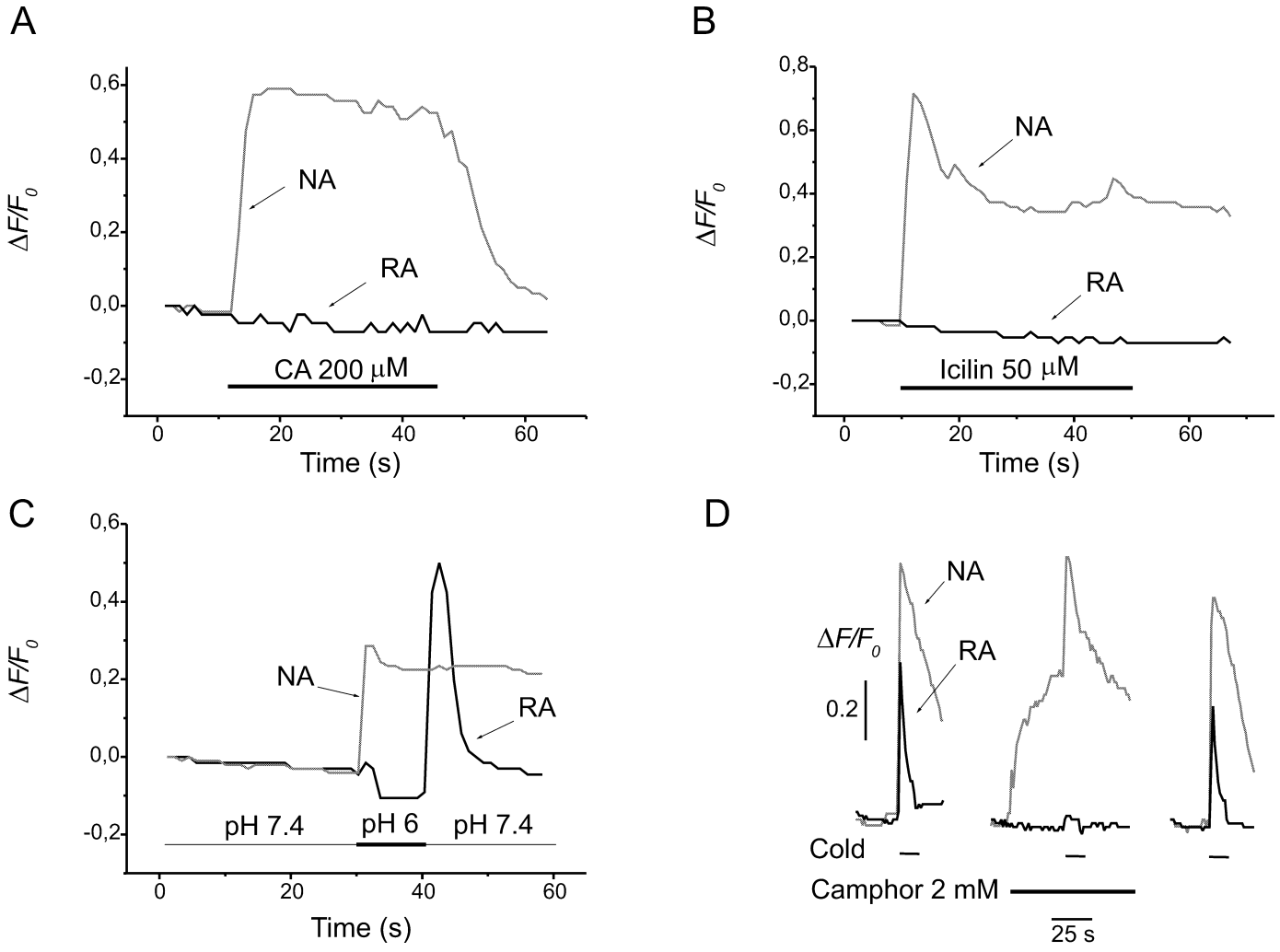

FIG. 5. RA and NA cells have different pharmacology. (A) Examples of a NA neuron (grey trace) responding and a RA neuron (black trace) insensitive to application of CA (200 μM). (B) Examples of a NA neuron (grey trace) responding and a RA neuron (black trace) insensitive to the application of icilin (50 μM). (C) Examples of a NA neuron (grey trace) responding to an acidic stimulus (pH 6) and a RA cell (black trace) insensitive to the acidic stimulus but responding to re-application of pH 7.4. (D) Effect of 2 mM camphor on a RA neuron (black trace) and a NA neuron (grey trace). Notice that the NA cell responds to the application of camphor at the base temperature and the inhibition of the cold response of the RA cell in camphor.

TABLE 1. Comparison of the pharmacological profile of RA and NA neurons

	RA neurons		NA neurons	
	Application at 32 °C	Effect on cold response	Application at 32 °C	Effect on cold response
Menthol	Insensitive (0/56)	Inhibition	Sensitive (30/40)	Sensitization
Alkaline pH (pH 8)	Sensitive (6/10)	No effect	Sensitive (8/11)	Sensitization
Acid pH (pH 6)	Slightly sensitive (4/15)		Sensitive (5/8)	
Ruthenium red	Sensitive (5/10)	Sensitization	Slightly sensitive (1/7)	Sensitization
Camphor	Insensitive (0/8)	Inhibition	Slightly sensitive (3/7)	No effect
DMSO	Insensitive (0/10)	Inhibition	Insensitive (0/10)	No effect
Amiloride	Insensitive (0/10)	No effect	Insensitive (0/10)	No effect
Cinnamon aldehyde	Insensitive (0/28)		Sensitive (6/11)	
Icilin	Insensitive (0/14)		Sensitive (13/14)	
Capsaicin	Sensitive (8/13)		Slightly sensitive (4/13)	

For each group the left column describes the effect of applying the drug at 32 °C (the population was considered sensitive if more than 50% of cells responded, slightly sensitive if less than 50% responded and insensitive if none responded). The column on the right describes the effect of the drug on the calcium signal during cooling. The effect was labelled as sensitization or inhibition if there was a statistically significant difference between the responses in the presence of the drug and control (Student's paired *t*-test). NA, cold-sensitive neurons with a nonadapting response to cooling; RA, cold-sensitive neurons with rapid adaptation to cooling.

tested responded with $\Delta F/F_0 > 0.1$ (0.11 ± 0.05, $n = 5$). None of the NA cells responded to this challenge. We then used a jump to pH 8, and six RA cells in ten responded, while the cold response itself was not affected by pH 8 in these cells. In a total of 11 NA cells, eight responded to pH 8, and the remaining three were sensitized, reminiscent of the effect of alkaline pH on TRPM8 (Andersson *et al.*, 2004) (Table 1).

To see whether the rise in $[Ca^{2+}]_i$ during cooling was due to the accompanying change in pH, we switched to the lower temperature using a solution whose pH at 25 °C was 7.2, instead of the normal 7.4. In this case, no pH change would have occurred during the temperature jump. In 14 RA cells tested the cold response was not affected by performing the temperature jump in pH 7.2, implying that the rise in $[Ca^{2+}]_i$ during cooling was not due to the change in pH.

A change in pH from pH 6 to pH 7.4 activates almost exclusively RA cells

An acid stimulus of pH 6 was used to examine the level of expression of acid-sensing ion channels (ASICs) that were suggested to have a strong temperature dependence and may play a role in cold-sensing (Askwith *et al.*, 2001). Only four RA cells in 15 responded to pH 6 challenge, which suggests a low level of expression of ASICs in these cells, in comparison to the NA group (five cells in eight responded to pH 6, Fig. 5C and Table 1). Application of pH 6 is a very weak stimulus for TRPV1 activation at room temperature, in that it evokes less than 10% of the maximal current (Tominaga *et al.*, 1998). However, 2 μM capsaicin is a saturating concentration for TRPV1. This may explain the discrepancy between the fractions of RA cells responding to pH 6 and capsaicin.

However, all RA cells were activated at the end of the stimulus, when the pH returned from 6 to 7.4 ($n = 15$, Fig. 5C). This type of response to alkaline pH was almost exclusively expressed by RA cells: altogether 20 cells in 122 imaged neurons were activated by this stimulus, and 15 of these were RA cells.

Ruthenium red (RuR) sensitizes both RA and NA cells

RuR is a TRPA1 antagonist (Story *et al.*, 2003). In a total of ten RA cells, five responded to RuR and four of the remaining five were sensitized. Similarly, of eight NA cells, one responded to RuR while the remaining seven were reversibly sensitized (Table 1). This may be related to the effect of RuR on the cold- and menthol-induced current in DRG neurons (Reid & Flonta, 2001a).

Camphor completely abolishes the cold response in RA cells

Camphor has been reported to activate the warm-sensitive ion channel TRPV3 (Moqrich *et al.*, 2005) but also TRPV1 (Xu *et al.*, 2005). None of the eight RA cells tested responded to 2 mM camphor, but, interestingly, at this concentration the cold response was almost completely and reversibly abolished (0.46 ± 0.11 inhibited to 0.10 ± 0.18, $n = 8$, $P < 0.001$, inhibition by 78%, Fig. 5D). Ethanol (which was used to make the camphor stock solution) at 1 : 1000 dilution had no effect.

Interestingly, of seven NA cells, three responded to 2 mM camphor (Fig. 5D and Table 1). Camphor had no effect on the cold response in these NA cells. Altogether we found eight camphor-sensitive cells in 60 neurons imaged (13%).

Amiloride has no effect on either RA or NA cells

In order to test the possible involvement of ENaC in cold sensing by RA cells we have applied the cooling step in the presence of 100 μM amiloride. The amiloride stock of 100 mM was prepared in DMSO, which yielded a final DMSO dilution of 1 : 1000. Interestingly, at this concentration DMSO inhibited RA cells by 22% (0.31 ± 0.10 in DMSO compared to 0.40 ± 0.09 in control conditions, $n = 10$, $P = 0.04$). Amiloride by itself caused no further inhibition of the cold response in RA cells. In nine NA cells neither DMSO nor amiloride had any significant effect (Table 1).

RA cells express a rapidly adapting cold-induced inward current

RA neurons were preselected using calcium imaging and then the patch-clamp technique was used in the amphotericin perforated patch mode to record the changes in membrane current during fast cooling steps. Cold evoked a large (659 ± 399 pA, $n = 4$) inward current in RA neurons clamped at −60 mV (Fig. 6). Adaptation during the cooling step was rapid, with a time constant of 1.9 ± 1.0 s, and almost complete (remaining current was $3.4 \pm 4.3\%$ of the peak value, $n = 4$). Menthol-sensitive neurons have a much slower adaptation, with a time constant of ∼60 s (Reid & Flonta, 2001a; Reid *et al.*, 2002), which is entirely missing in our RA neurons.

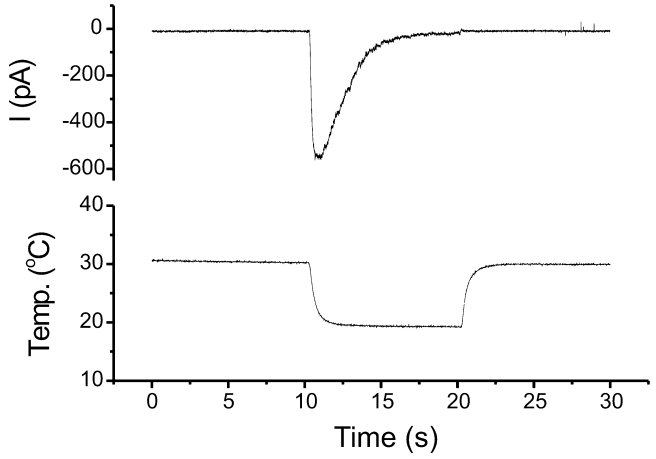

FIG. 6. RA neurons express a rapidly adapting cold-induced inward current. (Upper trace) Example of a cold-induced inward current in a RA neuron voltage-clamped at −60 mV. Note the almost complete adaptation of the response during the 10-s cold step. (Lower trace) The cooling stimulus (∼32 to ∼18 °C) measured where the cell had been with a T-type thermocouple; the true time course of the step is much faster (see legend to Fig. 1).

Discussion

We have studied cold sensitivity in primary DRG cultures using a system for applying fast (< 20 ms) 10-s cooling steps from ∼32 to ∼18 °C. As in a previous study (Babes *et al*., 2004), we have separated cold-sensitive neurons into menthol sensitive and menthol insensitive, based on the effect of 100 μM menthol on the relative change in $[Ca^{2+}]_i$ during cooling. We noticed that rapid adaptation was prominent in the menthol-insensitive population and that the most complete adaptation in the menthol-sensitive group was to 37% of the initial response ($\Delta F/F_0$ measured at the first frame after the cooling step). As a consequence, in order to define and characterize a homogeneous menthol-insensitive subpopulation, we have defined RA (rapidly adapting) cells as those that adapted to less than 37% of the initial response (Figs. 1A and 3). NA (nonadapting) neurons were considered those in which the calcium signal increased or decreased by less than 25% of the initial value, as all cells in this group proved to be menthol sensitive (Figs. 1A and 3).

Two other populations of cold-sensitive neurons were identified (intermediate and slow), but as they were largely menthol sensitive and their properties were very similar to those of the NA group and our aim was to describe the novel type of rapidly adapting cold-sensitive neurons by comparing them with a homogeneous subpopulation of cold- and menthol-sensitive neurons, in what follows we focused exclusively on RA and NA neurons.

RA cells were less cold-sensitive than NA neurons. While the temperature threshold for NA neurons activation by a cooling step is warmer than 27.5 °C, RA cells activate only at temperatures below 25 °C (Fig. 4). In addition, the amplitude of the response to cooling steps or cooling ramps was smaller for RA than for NA cells, and the temperature threshold for activation by cooling ramps was also colder for RA cells. Moreover, fewer RA neurons responded during a cooling ramp than NA cells. Thus, the NA population, most likely expressing TRPM8, is the major player in cold sensing in this range of temperatures (32–18 °C).

It is very likely that the RA population overlaps to a great extent with the menthol-insensitive (MI) population described in a previous study from our group (Babes *et al*., 2004). However, the ramp stimulus used in this study reached only ∼20 °C, compared to 12 °C in our earlier work, and the fact that only ∼30% of the RA cells responded during the cooling ramps may be due to this fact.

In our previous work we could find no evidence for a role of TRPA1 as a cold transducer in menthol-insensitive neurons for cooling to 12 °C (Babes *et al*., 2004). In the present study, none of the RA cells (which are all menthol insensitive) responded to the TRPA1 agonist, CA (200 μM), applied at the base temperature of 32 °C (Fig. 5A). These cells were also completely insensitive to 50 μM icilin, another TRPA1 agonist, which also activates TRPM8 (Fig. 5B). Together with the fact that menthol does not activate or sensitize RA cells (it actually has a significant inhibitory effect on them, Fig. 2A) and that the cold responses in RA neurons are not inhibited by the TRPA1 antagonist, RuR, these findings suggest that neither TRPM8 nor TRPA1 is expressed in these rapidly adapting neurons. The epithelial sodium channel ENaC was proposed to play a role in cold-sensing (Askwith *et al*., 2001), but our RA group failed to show any sensitivity to 100 μM amiloride, a concentration at which ENaC would have been significantly inhibited.

NA cells, on the other hand, were all menthol sensitive and a high proportion of them responded to icilin (93%) and CA (55%) (Fig. 5A and B), suggesting coexpression of TRPM8 and TRPA1 in cultured neurons, in agreement with previous work from our laboratory (Babes *et al*., 2004).

In order to obtain a clearer view of the pattern of ion channel expression in RA neurons, we have applied activators of a range of ion channels known to be present in cultured DRG neurons: capsaicin (TRPV1), camphor (TRPV1/TRPV3), CA (TRPA1), menthol (TRPM8), and pH 6 (ASIC and, to a lower degree, TRPV1). Based on our experiments, RA cells seemed to express only TRPV1 of the TRP channels listed above. Apart from the expression of TRPV1, which may be due to the presence of NGF (100 ng/mL) in our cultures, these results seem to indicate a non-nociceptive profile for the RA cells. On the other hand, NA neurons appear to express a remarkable mixture of TRP channels, TRPM8, TRPA1, TRPV1 and possibly TRPV3. To what extent is this aspect related to changes in cell phenotype in culture is not known. However, in a previous study we have shown that coexpression of menthol (TRPM8) and capsaicin (TRPV1) sensitivity, and of menthol and mustard oil (presumably TRPA1) sensitivity are present in the absence of added NGF and also very early after plating in primary DRG cultures (Babes *et al*., 2004).

Interestingly, the pharmacology of RA neurons shows both similarities to and differences from the pharmacology of the NA group, which is consistent with that of TRPM8. Like the NA group, the RA group is activated by alkaline pH and sensitized by RuR; RA neurons were more sensitive to high pH than NA cells, but as we have shown, their response to cold is not due to altered pH of the buffer on cooling. A sensitizing effect of high pH has been reported for TRPM8 (Andersson *et al*., 2004), while RuR is known to sensitize the cold- and menthol-induced current in DRG neurons (Reid & Flonta, 2001a). In contrast to the NA group and to TRPM8, neurons of the RA group are inhibited by camphor, DMSO and even menthol itself.

Cooling steps evoked a large desensitizing inward current in RA cells. This argues against a decisive role of background potassium channels in cold sensing in these neurons. The time course of adaptation of the current is of the order of seconds, and corresponds closely to the fast adaptation recorded in intact receptors *in vivo* (Darian-Smith *et al*., 1973; Kenshalo & Duclaux, 1977; Campero *et al*., 2001).

In conclusion, we have discovered a new type of cold-sensitive neuron, which does not express either of the two TRP channels activated by cold, TRPM8 and TRPA1. We propose that this novel

receptor is involved in innocuous cold sensing and is responsible for the fast adaptation during cold stimulation of intact receptors.

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Abbreviations

CA, cinnamaldehyde; DRG, dorsal root ganglia; NA, cold-sensitive neurons with a nonadapting response to cooling (in which the calcium signal increases or decreases by less than 25% of the initial response); RA, cold-sensitive neurons with rapid adaptation to cooling (adapting to less than 37% of the initial calcium response); RuR, ruthenium red.

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