Chemical and Cold Sensitivity of Two Distinct Populations of TRPM8-Expressing Somatosensory Neurons

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Xing, Hong, Jennifer Ling, Meng Chen, and Jianguo G. Gu. Chemical and cold sensitivity of two distinct populations of TRPM8-expressing somatosensory neurons. J Neurophysiol 95: 1221–1230, 2006; doi:10.1152/jn.01035.2005. The cold- and menthol-sensing TRPM8 receptor has been proposed to have both nonnociceptive and nociceptive functions. However, one puzzle is how this single type of receptor may be used by somatosensory neurons to code for two distinct sensory modalities. Using acutely dissociated rat dorsal root ganglion (DRG) neurons without culture, we show that TRPM8 receptors are expressed on two distinct classes of somatosensory neurons. One class is sensitive to menthol and features nonnociceptive neuron properties, including capsaicin-insensitive, ATP-insensitive, transient acid response, and expression of TTX-sensitive sodium channels only. This class is termed the menthol-sensitive/capsaicin-insensitive neuron class (MS/CS). The other class is also sensitive to menthol but has characteristics of nociceptive neurons including capsaicin-sensitive, ATP-sensitive, prolonged acid response, and expression of both TTX-sensitive and TTX-resistant sodium channels. This class is termed the menthol-sensitive/capsaicin-sensitive neuron class (MS/CS). The presence of these two neuron classes in acutely dissociated DRG neurons support the idea that TRPM8 receptors can produce strong selection of the MS/CS neuron population over the other populations, and this difference may provide a mean of selective activation of MS/CS neurons at low stimulation intensity.

INTRODUCTION

Cold temperatures produce either innocuous or noxious sensations, depending on stimulation intensity, i.e., the degree of cold temperatures. Noxious cold can be experienced as a burning pain sensation (Morin and Bushnell 1998). Similar to cold temperatures, topical menthol produces an innocuous cooling sensation at low concentrations and a burning pain sensation at high concentrations (Green 1992; Wasner et al. 2004). Studies indicate that cold and menthol could lead to membrane depolarization and action potential firing on a subpopulation of primary afferent neurons (Okazawa et al. 2000; Reid and Flonta 2001; Reid et al. 2002; Viana et al. 2002). Searching for molecular identities of cold-induced sensory responses has resulted in molecular cloning of TRMP8 receptor (McKemy et al. 2002; Peier et al. 2002). Similar to TRPV1, a noxious heat receptor (Caterina et al. 1997), TRPM8 belongs to the transient receptor potential (TRP) family (Clapham et al. 2001; Minke and Cook 2002). While TRPV1-expressing sensory neurons are nociceptive neurons sensitive to both noxious heat and the TRPV1 agonist capsaicin (Caterina et al. 1997, 2000), it remains controversial whether TRPM8-expressing neurons have nociceptive functions (McKemy 2005). TRPA1, another TRP member, has been suggested to be a noxious cold thermoreceptor (Story et al. 2003). However, TRPA1 as a thermoreceptor is still debatable (Jordt et al. 2004; McKemy 2005). Other mechanisms that may contribute to cold sensitivity include inhibition of Na+/K+/ATPases (Pierau et al. 1975) and of “background” K+ conductances (Viana et al. 2002).

TRPM8 detects cooling temperatures in a broad range. When expressed on a heterologous cell system, temperatures <24–28°C start to evoke membrane currents, and the currents reach maximum near 10°C (McKemy et al. 2002; Peier et al. 2002; Voets et al. 2004). The temperature thresholds for activating TRPM8 receptors have been found to be voltage dependent (Voets et al. 2004). The currents have biophysical properties indistinguishable from those observed in cool-sensitive afferent neurons under similar conditions (Okazawa et al. 2000; Reid and Flonta 2001; Reid et al. 2002), suggesting that TRPM8 serves as a cold sensor at peripheral nerve endings (McKemy et al. 2002; Peier et al. 2002). TRPM8 can also be activated by menthol, an active ingredient of peppermint (McKemy et al. 2002; Peier et al. 2002). Studies have indicated that TRPM8 is highly permeable to Ca2+ (McKemy et al. 2002; Peier et al. 2002), and its activation results in increases of intracellular Ca2+ levels (McKemy et al. 2002; Okazawa et al. 2000; Peier et al. 2002; Reid et al. 2002; Tsuzuki et al. 2004). About 5%–10% of rat dorsal root ganglion (DRG) neurons expressed TRPM8 (McKemy et al. 2002; Peier et al. 2002). TRPM8-expressing neurons are small-sized neurons that do not express classical markers of nociceptive neurons such as calcitonin gene-related peptide, isolectin B4, and TRPV1 in mouse DRGs (Peier et al. 2002).

In cultured rat DRG and trigeminal ganglion (TG) neurons, many cold- and menthol-sensitive neurons were also capsaicin-sensitive (McKemy et al. 2002; Reid et al. 2002; Viana et al. 2002). If this is not completely caused by a phenotype change of neurons under culture conditions, TRPM8 receptors on...
capsaicin-sensitive neurons may be involved in burning pain induced by noxious cold and menthol. In this study, we attempted to address whether TRPM8 receptors are expressed on both nonnociceptive and nociceptive neurons, and if so, what are differences between these two TRPM8-expressing neuron populations.

METHODS

Principles of Laboratory Animal Care” (National Institutes of Health) were followed. DRGs were removed from adult rats weighing ~150–250 g and placed in a 36°C bath solution containing dispase (neutral protease, 5 mg/ml; Boehringer Mannheim) and collagenase (2 mg/ml; Sigma type 1). After 1-h incubation, DRGs were triturated to dissociate the neurons. The dissociated cells were plated on coverslips precoated with poly-d-lysine and allowed to adhere for 1 h in normal bath solution. The normal bath solution contained (in mM) 150 NaCl, 5 KCl, 2 MgCl2, 2 CaCl2, 10 glucose, and 10 HEPES, with pH 7.3 and osmolarity adjusted to 320 mOsm with sucrose. All experiments were performed within 4 h after cell plating.

For Ca2+ imaging experiments, the Ca2+ indicator Fluo-3 was loaded into DRG neurons on coverslips by incubation with 5 μM Fluo-3-AM in 20% pluronac acid (Molecular Probes, Eugene, OR) for 30 min at 37°C. After dye loading, the cells were perfused with normal bath solution in a 0.5-ml chamber on the microscope stage (Olympus IX70). Fluo-3 fluorescence in the cells was detected with a peltier-cooled charge-coupled device (CCD) camera (PentaMAX-III System, Roper Scientific, Trenton, NJ) under a ×10 objective. Excitation was at 450 nm and emission was at 550 nm, achieved by fluorescence filter sets. Images were taken at one frame per second. Effects of menthol (1, 10, and 100 μM), ATP (100 μM), acid (pH 5.0 bath solution), cold (cold bath solution), and capsaicin (0.5 μM) on intracellular Ca2+ levels were tested by bath application of these solutions for 20 s, and capsaicin was always tested last. Each application was followed by a 20-min washoff in normal bath solution, and a subsequent test was then performed. Acid solution of pH 5 was made by adding HCl to the normal bath solution until pH value was reduced to 5. Cold stimulation was achieved by application of cold bath solution in a manner described in our previous study (Tsuzuki et al. 2004), which yielded a temperature trop from 26 to 19°C within 20 s. Testing solutions were rapidly applied to neurons through a glass tube (~500 μm ID) positioned 1.0 mm away from cells. All experiments were carried out at the ambient temperature of 26°C (slightly heated room). For Ca2+ imaging data analysis, relative fluorescence intensity ΔF/Fo was used, and neurons with ΔF/Fo values of ≥0.2 (i.e., equal or above 20% of increase) were assigned as responsive cells (Reid et al. 2002).

Patch-clamp recordings were performed on neurons preidentified as menthol-sensitive neurons described above. Recordings were made with a Multiclamp 700A (Axon Instruments) set at 2 MHz and sampled at 5 kHz using pCLAMP 9.0 (Axon Instruments). Cells were voltage clamped at −70 mV in the whole cell configuration. The recording electrode internal solution contained (in mM) 135 K-gluconate, 0.5 CaCl2, 2 MgCl2, 5 KCl, 5 EGTA, 10 HEPES, 2 Na2-ATP, and 0.5 Na2-GTP, with a pH of 7.2 adjusted by KOH and osmolarity of 315–325 mOsm. Total KCl after pH adjustment was 5 mM. Recording electrode resistance was between 3.0 and 5.0 MΩ. Testing compounds were rapidly applied to neurons through a glass tube (~500 μm ID) positioned 1.0 mm away from cells. Intervals between drug applications were 20 min.

For testing reversal potential of menthol-evoked current, a voltage ramp was applied during the peak of menthol-evoked current. The voltage ramp was from −90 to +70 mV within 140 ms. Current change during the voltage ramp in the absence of menthol was subtracted from the voltage ramp test in the presence of menthol.

Lidocaine (10 mM) was present to block sodium channels and most of calcium channels (Gu and MacDermott 1997) during the voltage ramp tests.

To examine electrophysiological properties of neurons, voltage-activated currents were revealed by three protocols as described in a previous study (Petruska et al. 2000). The three protocols are shown in Fig. 8, A–C. Protocol 1 was used to examine the pattern of hyperpolarization-activated currents. With protocol 1, currents were evoked by a series of hyperpolarizing pulses presented from a V0 of −60 mV (10 mV/step to a final potential of −110 mV; 500-ms, 4-s interstimulus interval). Protocol 2 was used to produce outward current patterns. From a V0 of −60 mV, a 500-ms conditioning pulse to −100 mV was followed by 200-ms depolarizing command steps (20 mV) to a final potential of +40 mV. Protocol 3 was used to produce inward current patterns. With the cell held at −60 mV, a 500-ms conditioning pulse to −80 mV was followed by a series of depolarizing command steps (10-mV steps, 2.0-ms duration) to a final potential of +10 mV. Currents evoked by protocol 3 were tested in the absence and presence of 500 nM TTX. In addition to the above three voltage protocols, a 3-ms, 1,500-pA current step was used to determine afterhyperpolarization (AHP) and action potential duration at the base (APDb). To quantify AHP, we used a criterion of 80% recovery to baseline (AHP80) (Petruska et al. 2000).

Cell size was determined by calculating the average of shortest and longest diameters. For cell membrane surface areas, each cell is considered to be a sphere, and its area is calculated based on the averaged diameter. Data are represented as means ± SE. Student’s t-test was used for statistical comparison, and significance was considered at the P < 0.05 level.

RESULTS

Responsiveness of menthol and cold in two distinct populations of menthol-sensitive neurons acutely dissociated from rat DRGs

To minimize a potential change of sensory neuron phenotypes, we used acutely dissociated DRG neurons throughout this study. These cells were plated in dishes and maintained in normal bath solution rather than cell culture medium, and the bath solution had the components identical to the one used for recordings. All experiments were completed within 4 h of plating. The basal temperature of bath solution for which cells were maintained was 26°C. Higher basal temperature was not necessary for this study because TRPM8 receptors are not significantly activated at temperature >25°C when cells are near resting membrane potentials (Voets et al. 2004).

We first addressed whether menthol-sensitive cells in acutely dissociated DRG neurons consist of two different classes: capsaicin-insensitive class and capsaicin-sensitive class. Menthol-sensitive neurons were identified by Ca2+ imaging after bath application of 100 μM menthol for 20 s. Of 3,324 cells tested, menthol increased Fluo-3 fluorescence intensity, i.e., increased intracellular Ca2+, in 202 neurons (6.1%; Fig. 1A). The change of Fluo-3 fluorescence intensity showed large variations among menthol-sensitive neurons (Fig. 1A). After recovery in normal bath solution for 20 min, capsaicin sensitivity was subsequently tested by bath application of 0.5 μM capsaicin (Fig. 1, B–D). Of the 202 menthol-sensitive neurons, 113 had no response, and 89 had responses to capsaicin. Thus similar to cultured DRG and trigeminal ganglion neurons (McKemy et al. 2002; Reid et al. 2002; Viana et al. 2002), menthol-sensitive cells of acutely dissociated DRG neurons also could be divided into two classes: menthol-sensitive neurons that responded to cold and menthol and those that responded only to menthol.
sensitive/capsaicin-insensitive neurons (MS/CIS) and menthol-sensitive/capsaicin-sensitive neurons (MS/CS). MS/CIS neurons were 3.4% (113/3,324) and MS/CS neurons were 2.7% (89/3,324) of the total population of acutely dissociated DRG neurons.

We analyzed menthol responses of both MS/CIS neurons and MS/CS neurons (Fig. 1, C and D). It was found that, on average, menthol responses were significantly larger in MS/CIS neurons than in MS/CS neurons. The averaged peak response to 100 μM menthol, expressed as increases of Fluo-3 fluorescence intensity ($F/F_0$), was 1.31 ± 0.08 (n = 113) for MS/CIS and 0.88 ± 0.04 (n = 89, P < 0.05) for MS/CS neurons. Thus as a population, the MS/CIS neuron class had a greater response to menthol than the MS/CS neuron class (Fig. 1D, left). Many neurons were menthol-insensitive but capsaicin-sensitive (MIS/CS) cells. We randomly sampled 248 cells that were tested for both menthol and capsaicin and found that ~33% (81/248) were MIS/CS cells. We compared capsaicin responses between MS/CS and MIS/CS neurons. It was found that capsaicin responses were significantly smaller in MS/CS neurons than in MIS/CS neurons (Fig. 1D, right). The averaged peak responses ($\Delta F/F_0$) to 0.5 μM capsaicin were 0.89 ± 0.08 (n = 89) for MS/CS neurons and 1.64 ± 0.09 (n = 81, P < 0.05) for MIS/CS neurons.

Cold responsiveness of the two menthol-sensitive neuron populations was examined by application of cold bath solution that produced a temperature drop from 26 to 19°C in 20 s. Of the 22 MS/CIS neurons and 20 MS/CS neurons tested, all of them responded to the cooling stimulation. Cold responsiveness was significantly higher in MS/CIS neurons than in MS/CS neurons (Fig. 2, A–C). The averaged peak response ($\Delta F/F_0$) to the cooling stimulation was 1.57 ± 0.15 (n = 22) for MS/CIS neurons and 0.61 ± 0.09 (n = 20) for MS/CS neurons (P < 0.05; Fig. 2C, right). Consistently, menthol responsiveness was also significantly higher in MS/CIS neurons than in MS/CS neurons (Fig. 2, A–C). The averaged peak response ($\Delta F/F_0$) to 100 μM menthol stimulation was 1.74 ± 0.12 (n = 22) for MS/CIS neurons and 0.89 ± 0.15 (n = 20) for MS/CS neurons (P < 0.05; Fig. 2C, left).
ATP sensitivity and acid sensitivity of the two menthol-sensitive neuron populations

We asked whether, in addition to capsaicin sensitivity, there were other differences in chemical sensitivity between the two classes of menthol-sensitive neurons. To answer this question, we characterized ATP sensitivity of MS/CIS and MS/CS neurons (Fig. 3, A–C) because ATP and its receptors play important sensory roles including nociception. None of the MS/CIS neurons showed any response to 100 μM ATP (0/29 cells). In contrast, most MS/CS cells responded to ATP (24/39 cells). In addition to MS/CS neurons, many MIS/CS neurons also responded to 100 μM ATP. The magnitude of peak ATP responses was comparable between MS/CS cells (ΔF/F0 = 0.82 ± 0.12, n = 24) and MIS/CS cells (ΔF/F0 = 0.80 ± 0.07, n = 81). However, the decay phases of ATP responses were different between MS/CS and MIS/CS neurons. For MS/CS neurons, an initial decay was followed by a plateau, but ATP responses decayed near baseline for MIS/CS neurons. Nevertheless, the decay time constants for MS/CS neurons (15 ± 1.7 s) and for MIS/CS neurons (16 ± 1.6 s) were not significantly different (Fig. 3C).

Acid sensitivity is another often tested chemical sensitivity on primary afferent neurons, and acid response is shown to be different between nonnociceptive and nociceptive neurons. Acid sensitivity of MS/CIS and MS/CS neurons was examined by application of acidified bath solution with a pH value of 5. In both classes of neurons, many neurons responded to the acid solution. Of 30 MS/CIS neurons tested, 19 of them (63%) showed response to the acid solution. The peak responses (ΔF/F0) for these acid-sensitive MS/CIS neurons were 0.89 ± 0.18 (n = 19). Of 43 MS/CS cells tested, 34 of them (80%) had response to the acid solution. The peak responses (ΔF/F0) for these acid-sensitive MS/CS neurons were 0.32 ± 0.07 (n = 34), significantly lower than that of MS/CIS neurons (P < 0.05). Acid responses in these two classes of neurons were different kinetically. In MS/CIS neurons, acid responses were shown to have fast decay and short duration (Fig. 4, A and C). The decay time constants were 5.6 ± 0.6 s (n = 19). The responses returned to baseline within 30 s after a 20-s application of the acid solution. In contrast,
Acid response in MS/CS neurons was found to have very slow decay, with a plateau that lasted to the end of the 100-s recording period (Fig. 4, B and C). Prolonged acid responses were also found in many MIS/CS neurons (Fig. 4, B and C). The peak responses ($\Delta F/F_0$) for these acid-sensitive MIS/CS neurons were $0.75 \pm 0.08$ ($n = 77$), and the response did not return to baseline during the 100-s recording period.

We examined the cell size of MS/CS, MS/CIS, and MIS/CS cells (Fig. 5) to see if they were morphologically different. All MS/CIS neurons were found to be small-sized cells, and most of them were $<20 \mu m$ diam (Fig. 5A). On average, the cell diameters of MS/CIS cells were $18.8 \pm 0.3 \mu m$ ($n = 113$). MS/CS cells were also small-sized cells ($25.1 \pm 0.4 \mu m; n = 89$; Fig. 5B), but they were significantly larger than MS/CIS cells ($P < 0.05$). The cell size distribution of MS/CS was similar to that of MIS/CS neurons ($26.7 \pm 0.1 \mu m; n = 1,092$; Fig. 5C). Size distribution of total populations of acutely dissociated DRG neurons, including MS/CIS, MS/CS, MIS/CS, and other cells ($n = 3324$), are presented in Fig. 5D.

**Differential recruitment of the two menthol-sensitive neuron populations**

We examined how the two classes of menthol-sensitive neurons were recruited when menthol concentrations changed from low to high, i.e., increases of chemical stimulation intensity. In these experiments, neurons were tested with menthol at 1, 10, and 100 $\mu M$ and then tested with capsaicin at 0.5 $\mu M$. MS/CIS cells were defined as cells having responses to 100 $\mu M$ menthol but not to 0.5 $\mu M$ capsaicin. MS/CS cells were defined as cells having responses to both 100 $\mu M$ menthol and 0.5 $\mu M$ capsaicin. All the cells included in the analysis were sensitive to 100 $\mu M$ menthol, but they might have no detectable response to lower concentrations of menthol (Fig. 6). Figure 6, A–D, shows an example of preferential activation of MS/CIS neurons at lower stimulation intensity (1 and 10 $\mu M$ menthol; Fig. 6, A and B) and the recruitment of MS/CS neurons when high-intensity stimulation (100 $\mu M$ menthol; Fig. 6C) was applied. In this example, the MS/CS neurons had no response to 1 and 10 $\mu M$ menthol (Fig. 6, A and B) but were activated at 100 $\mu M$ menthol (Fig. 6C). Figure 6, E–G, shows at larger population scale the differential activation of the two neuron populations at different menthol concentrations. As tested with 100 $\mu M$ menthol and 0.5 $\mu M$ capsaicin, 70 cells were identified to be MS/CIS neurons and 65 cells were MS/CS neurons (Fig. 6G), and the ratio of these two neuron populations was near 1:1 (70/65). Relatively more MS/CIS neurons responded at lower concentrations of menthol. At 10 $\mu M$ menthol, 51 of 70 MS/CIS cells had responses, but 16 of 65 MS/CS neurons had responses (Fig. 6F), and the ratio of menthol-responsive neurons for the two populations was 3:1 (51/16). At 1 $\mu M$ menthol, 13 of 70 MS/CIS cells had responses but only 2 of 65 MS/CS neurons had responses (Fig. 6E), and the ratio of menthol-responsive neurons for the two populations was 6:1 (13/2). Overall averaged responses to menthol at different concentrations were also analyzed, and the
responses were larger in MS/CIS neurons than in MS/CS neurons at all three concentrations tested. At 1 μM menthol, the responses (ΔF/ΔF₀) for MS/CIS neurons were 0.12 ± 0.02 (n = 70) and for MS/CS neurons were 0.06 ± 0.01 (n = 65; P < 0.05). At 10 μM menthol, the responses (ΔF/ΔF₀) for MS/CIS neurons were 0.57 ± 0.05 (n = 70) and for MS/CS neurons were 0.21 ± 0.04 (n = 65, P < 0.05). At 100 μM menthol, the responses (ΔF/ΔF₀) for MS/CIS neurons were 1.31 ± 0.08 (n = 70) and for MS/CS neurons were 0.88 ± 0.04 (n = 65, P < 0.05).

Electrophysiological characterizations of the two menthol-sensitive neuron populations

We performed patch-clamp recordings to examine differences between the two classes of menthol-sensitive neurons. Menthol (100 μM) evoked inward currents in both MS/CIS (Fig. 7A) and MS/CS neurons (Fig. 7B). For MS/CIS neurons, menthol evoked large inward currents, but capsaicin did not have any effect (Fig. 7A). For MS/CS neurons, both menthol and capsaicin evoked inward currents (Fig. 7B). It was noted that menthol-evoked currents had different degrees of desensitization in MS/CIS neurons, but the desensitization was absent or weak in MS/CS neurons. When menthol-evoked currents in MS/CIS and MS/CS neurons were compared (Fig. 7C), it was found that menthol-evoked currents were significantly larger in MS/CIS cells (1,258.39 ± 111.04 pA, n = 8) than in MS/CS cells (504.94 ± 72.19 pA, n = 8), consistent with the results in Ca²⁺ imaging experiments (Fig. 1D). We normalized current amplitude by membrane surface areas, i.e., TRPM8 current density, and it was found that the current density of MS/CIS cells (1.13 ± 0.07 pA/μm², n = 8) was over four times higher than that of MS/CS cells (0.25 ± 0.05 pA/μm², n = 8). When voltage ramp was applied, menthol-evoked currents showed strong outward rectification in both MS/CIS (Fig. 7E; n = 3) and MS/CS neurons (Fig. 7F; n = 2). The reversal potentials of menthol-evoked currents were near 0 mV (0.9 ± 0.3 mV, n = 5), indicating that menthol activated nonselective cation channels. Thus menthol-evoked currents...
on both MS/CIS and MS/CS neurons were consistent with TRPM8 receptor activation shown in heterologous cell system expressing TRPM8 receptors (McKemy et al. 2002).

We determined several intrinsic membrane properties in MS/CIS and MS/CS neurons to see if there are differences between these two classes of neurons. The membrane properties tested include hyperpolarization-activated currents (HACs), depolarization-activated outward currents (DAOC), depolarization-activated inward currents (DAIC), action potentials, and TTX sensitivity. The three protocols for voltage-activated currents (Fig. 8, A–C) were based on previous studies for sensory neuron current signatures (Petruska et al. 2000). Hyperpolarization activated small inward currents in both MS/CIS and MS/CS neurons (Fig. 8A), and HACs were not significantly different between the two classes of neurons (Table 1). Depolarization activated large outward currents in both MS/CIS and MS/CS neurons (Fig. 8B). For both MS/CIS and MS/CS neurons, a depolarization-activated outward current always had two components: a desensitizing current component (i.e., A-current-containing component) and a steady-state current component. Neither the desensitizing current component nor the steady-state currents were found to be significantly different between MS/CIS and MS/CS neurons (Fig. 8B; Table 1). DAICs in both MS/CIS and MS/CS neurons (Fig. 8C, top) had similar amplitudes (Table 1). However, the inward currents were significantly different in decay phases.

**FIG. 7.** Density of menthol-evoked currents in MS/CIS and MS/CS neurons. A: large inward current evoked by menthol (100 μM) in a neuron. Capsaicin (0.5 μM) did not have effect on the cell. Cell is a MS/CIS neuron. B: small inward current evoked by menthol (100 μM) in another neuron. Capsaicin (0.5 μM) evoked a large inward current in the same cell. Cell is a MS/CS neuron. C: pooled results of amplitude of currents evoked by menthol (open bars, n = 8) and capsain (solid bars, n = 8). D: density of menthol-evoked currents in MS/CIS and MS/CS neurons. E: I-V curve of menthol-evoked current in a MS/CIS neuron. Similar results were obtained in another 2 cells. F: I-V curve of menthol-evoked current in a MS/CS neuron. Similar results were obtained in another cell. Note that scales for currents are different between E and F. Time intervals between drug applications were 20 min in all experiments.
between the two neuron populations, and apparent decay time constants for MS/CS (\(\tau = 2.31 \pm 0.86 \text{ ms}, n = 8\)) were about three times longer than those of MS/CIS neurons (\(\tau = 0.75 \pm 0.13 \text{ ms}, n = 13\)). Furthermore, DAICs in MS/CIS cells could be inhibited 90% in the presence of 500 nM TTX (\(n = 13\); Fig. 8C, bottom; Table 1). Action potential duration was shorter in MS/CIS than in MS/CS neurons (Fig. 8D; Table 1; \(P < 0.05\)). AHP80 was also shorter in MS/CIS cells than in MS/CS cells (Fig. 8D; Table 1; \(P < 0.05\)). When injecting depolarization currents to the cells in the presence of TTX, only passive membrane potential changes were seen in MS/CIS cells, but some active membrane potential changes could be observed in MS/CS neurons (Fig. 8E).

**DISCUSSION**

In this study, we used acutely dissociated DRG neurons without culture to show that TRPM8-expressing neurons can be classified into two functionally distinct populations. One population features nonnociceptive neuron properties including capsaicin-insensitive, ATP-insensitive, rapid acid response, and predominated with TTX-sensitive sodium channels. The other population poses nociceptive neuron properties such as capsaicin-sensitive, ATP-sensitive, prolonged acid response, and having large portion of TTX-resistant sodium channels in

**TABLE 1. Comparison of electrophysiological properties of MS/CIS and MS/CS neurons**

<table>
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<tr>
<th></th>
<th>HAC, nA</th>
<th>Desensitizing Component</th>
<th>Steady-State Current</th>
<th>DAIC, nA</th>
<th>Without TTX</th>
<th>With TTX</th>
<th>AP Duration, ms</th>
<th>HP80, ms</th>
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<tr>
<td>MS/CIS ((n = 13))</td>
<td>0.29 ± 0.04</td>
<td>2.6 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>2.6 ± 0.3</td>
<td>0.2 ± 0.07*</td>
<td>6 ± 0.4*</td>
<td>52 ± 4*</td>
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<tr>
<td>MS/CS ((n = 8))</td>
<td>0.28 ± 0.05</td>
<td>3.1 ± 0.6</td>
<td>2.6 ± 0.5</td>
<td>2.7 ± 0.7</td>
<td>1.6 ± 0.6</td>
<td>8 ± 0.8</td>
<td>66 ± 6</td>
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Values are means ± SE. HAC, hyperpolarization-activated currents—values were current amplitudes of HAC at a hyperpolarization step from −60 to −110 mV. DAOC, desensitizing component—a desensitizing current component of depolarization activate outward currents; values were current amplitudes at a depolarization step from −100 to +40 mV; DAOC, steady-state current—values were current amplitudes at a steady-state current component—values were current amplitudes at a depolarization step from −80 to 10 mV; AP, action potential; AHP80, 80% recovery to baseline; MS/CIS, menthol-sensitive, capsaicin-insensitive neuron; MS/CS, menthol-sensitive, capsaicin-sensitive neuron. *\(P < 0.05\), MS/CIS vs. MS/CSs.
addition to TTX-sensitive sodium channels. Our results not only confirm the early observations for the presence of both MS/CIS and MS/CS neurons under culture conditions (McKemy et al. 2002; Reid et al. 2002; Viana et al. 2002), but also provide new information for chemical sensitivity and intrinsic properties of the two populations of menthol-sensitive neurons. Our results suggest that MS/CIS neurons are nonnociceptive neurons and MS/CS neurons are nociceptive neurons. We also showed that there are significant differences in the responsiveness to menthol between the MS/CIS neuron population and the MS/CS neuron population. The responsiveness to cold is also significantly different between the two neuronal populations.

We showed that there is a population selection of MS/CIS over MS/CS neurons with low stimulation intensity (low concentration of menthol). The population selection (Fig. 6) is suggested by the changes in the ratio of the responsive MS/CIS versus the responsive MS/CS neurons when chemical stimulation was at different intensities (i.e., different menthol concentrations). For example, at high menthol concentration of 100 \(\mu M\), the ratio was about 1:1. In contrast, the ratio became 3:1 at 10 \(\mu M\) menthol and 6:1 at 1 \(\mu M\) menthol. Thus low intensity stimulation preferentially selects the MS/CIS neuron population over the MS/CS neuron population. On the other hand, high stimulation intensity recruits both MS/CIS and MS/CS neuron populations with poor selectivity. This population selection is consistent with the phenomenon that topical menthol application produces cooling sensation at low concentrations of menthol and burning pain sensation at high concentrations of menthol (Cliff and Green 1994; Eccles 1994; Green 1992; Wasner et al. 2004). Burning pain sensation after topical application of high concentrations of menthol has recently been suggested to be caused by peripheral activation of nociceptors in human subjects (Wasner et al. 2004). We chose to use menthol as a stimulant for TRPM8 receptors in most of our experiments. This is because TRPM8 is the only menthol receptor identified thus far. Menthol evoked outward rectified currents with reversal potentials near 0 mV for both MS/CIS and MS/CS neurons. This indicates that menthol effects on both MS/CIS and MS/CS were mediated by TRPM8 receptors.

Studies have indicated that cold-sensitive neurons can be classified into menthol-sensitive and menthol-insensitive neuron classes (Babes et al. 2004; Thut et al. 2003; Viana et al. 2002). Menthol-sensitive neurons have a lower threshold to cold stimulation than menthol-insensitive cold-sensitive neurons, and TRPM8 mRNA is preferentially expressed within low-threshold cold-sensitive neurons (Babes et al. 2004; Nealen et al. 2003; Thut et al. 2003). Thus other sensory molecules may be involved in encoding for high-threshold noxious cold. For example, previous studies have suggested that TRPA1 may serve as a high-threshold noxious cold sensory molecule because this receptor is expressed on sensory neurons with classical nociceptive markers (Story et al. 2003). However, different from experiments using heterologous cells that expressed mouse TRPA1, cells expressing rat TRPA1 were not shown to be activated by cold temperatures (Jordt et al. 2004; Nagata et al. 2005). The controversy remains to be solved in a future study, and it remains unknown what membrane proteins may be involved in high-threshold noxious cold (McKemy 2005).

Our experiments were performed at an ambient temperature of 26°C. Previous studies using heterologous expression systems have shown that the TRPM8 activation threshold is \(-25°C\) at resting membrane potentials (Voets et al. 2004). However, a recent study (de la Pena et al. 2005) has found that >75% of cold-sensitive trigeminal neurons are activated by temperatures >25°C, raising the possibility that gating of TRPM8 may be under intrinsic modulation. This also raises the possibility that we might have underestimated TRPM8-mediated responses because of the potential TRPM8 desensitization at the basal temperature of 26°C in our study. Therefore cold and menthol responsiveness may become more profoundly different between the two subpopulations of DRG neurons when experiments are performed at body temperature (~37°C). However, it is also possible that the differences actually become less between the two subpopulations of DRG neurons at body temperature.

We showed that MS/CIS neurons are much more specific than MS/CS neurons in their chemical sensitivity. Of the three other chemical stimulants, including capsaicin, ATP, and acid, MS/CIS neurons only responded to acid transiently. Transient acid response has been observed in other nonnociceptive neurons. These results, together with those using cultured sensory neurons (Babes et al. 2004; McKemy et al. 2002; Nealen et al. 2003; Reid et al. 2002; Thut et al. 2003), suggested that MS/CIS neurons may be those low-threshold cold-specific afferent fibers identified in previous in vivo studies (Hollon et al. 1975; Hensel and Iggo 1971; Spray 1986). The responsiveness of menthol has very large variations in the MS/CIS neuron population, presumably because of differences in the expression level of TRPM8 receptors. The expression level of TRPM8 receptors on these neurons may be one important factor in determining their thresholds for innocuous cold stimulation.

We showed that 2.7% of acutely dissociated DRG neurons were MS/CS neurons, which account for 44% of the total menthol-sensitive neurons. Our results suggest that the previous observation of 50% MS/CS neurons in cultured rat sensory neurons (McKemy et al. 2002; Reid et al. 2002; Viana et al. 2002) was not just caused by the change of receptor expression under culture conditions. Interestingly, TRPM8 mRNA was not found to be coexpressed with TRPV1-immunoreactive DRG neurons freshly dissociated from adult mice (Peier et al. 2002). One possibility for the failure of anatomically detecting the coexpression might be because of the lower expression level of both TRPM8 and TRPV1 receptors on MS/CS neurons as indicated in our study. Alternatively, TRPM8 and TRPV1 are indeed not coexpressed normally but become coexpressed very rapidly (within 4 h) after acute dissociation. However, this latter possibility is less likely because no other sensory molecules have been shown to have such a rapid upregulation in a bath solution that is not for cell culture. We have shown that MS/CS neurons respond to pain-producing stimulants including capsaicin and ATP and have other nociceptive neuron features such as expression of TTX-resistant sodium channels. This suggests that MS/CS neurons may be polymodal nociceptive neurons. In previous in vivo studies, noxious cold-sensitve afferent fibers were found to often also respond to other noxious stimuli such as intense mechanical and/or heat stimuli (Bessou and Perl 1969; Shea and Perl 1985; Simone and Kajander 1996). It would be interesting to know whether
MS/CS neurons are the same or different noxious cold–sensitive afferent fibers identified in those in vivo studies.

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REFERENCES


McKemy DD. How cold is it? TRPM8 and TRPA1 in the molecular logic of cold sensation. Mol Pain 1: 16, 2005.


