Electrophysiological and Chemical Properties in Subclassified Acutely Dissociated Cells of Rat Trigeminal Ganglion by Current Signatures

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Xu S, Ono K, Inenaga K. Electrophysiological and chemical properties in subclassified acutely dissociated cells of rat trigeminal ganglion by current signatures. J Neurophysiol 104: 3451–3461, 2010. First published June 23, 2010; doi:10.1152/jn.00336.2010. In the present study, we subclassified acutely dissociated trigeminal ganglion (TRG) cells of rats using a current signature method in whole cell patch-clamp recordings. Using modified criteria for cell classification for the dorsal root ganglion (DRG), TRG cells were subclassified into nine cell types: 1–5, 7–9, and 13. Types 1, 3, and 7 were in the small cell groups (15–24 μm); types 4, 5, and 8–13 were in the medium cell groups (25–38 μm); and type 2 was a mixed group of both cell sizes. Types 1–3, 5, and 7 showed high-input resistance and types 1, 2, and 7 showed more depolarized resting membrane potentials. Types 1, 2, and 5–13 expressed long-duration action potentials (APs), but types 3 and 4 expressed short-duration APs. Sensitivities to capsaicin, protons, and adenosine 5′-triphosphate (ATP) in TRG cell types largely corresponded to DRG cell types. However, different from the matched DRG types, half of TRG type 1 cells were capsaicin insensitive, showing desensitizing proton-induced currents, and types 5, 7, and 9 exhibited slow-desensitizing ATP-induced currents. Types 4, 5, and 8–13 had nicotine sensitivity, but the other cell types were insensitive. These results indicate that the “current signatures” classification is a useful means to separate TRG cells into internally homogeneous subpopulations that were distinct from other cell types. Furthermore, the data suggest some specific differences in the chemical responsiveness of some cell types between the TRG and DRG.

INTRODUCTION

In the investigation of sensory physiology, cell classifications are very important for the interpretation of data from primary sensory neurons in in vitro experiments. Cells in the trigeminal and dorsal root ganglion (TRG and DRG) can be separated into many functionally diverse populations specialized to encode a variety of sensory events, such as touch, itch, thermal, and pain sensations. The last event is further divided into many kinds of nociceptive subgroups: high-threshold mechanoreceptor, polymodal, and mechano-heat and -cold nociceptors in Aδ and C afferent neurons. In the early years, in vivo recordings from DRG cells revealed that Aδ and C afferents showed short-duration action potentials (APs) with short afterhyperpolarization (AHP) and long-duration APs with long AHP, respectively (Harper and Lawson 1985a,b; Yoshida and Matsuda 1979). Based on the in vivo studies, dissociated TRG and DRG cells have been classified into two (F- and S-types) or four (types I–IV) cell groups by AP duration and whether they have a pronounced deflection (hump) on the falling phase (Cabanes et al. 2003; Liu et al. 2001; Park et al. 2009). To identify nociceptive neurons, capsaicin and tetrodotoxin (TTX) are widely combined in the investigation of cell properties to classify cells (Liu et al. 2001; Park et al. 2009; Veiga Moreira et al. 2007). However, the combination is not useful to discriminate capsaicin-insensitive and TTX-sensitive nociceptive cell groups.

To get a higher internal homogeneity in cell types of primary sensory neurons, another cell classification method using voltage-dependent current signatures has been developed in acutely dissociated DRG cells of rats. Scroggs and colleagues first described a current signature method, developed to identify and divide DRG cells into four specific cell groups, although many neurons were left without classification (Cardenas et al. 1995, 1997a,b, 1999). Subsequently, based on the former current signature method, many small- and medium-sized DRG cells were further classified into nine cell groups (types 1–9). The predictive validity of these classes was verified using a nonhierarchical cluster analysis for amplitudes of transient outward current (TOC), hyperpolarization-activated inward current (Ih), and kinetics of depolarization-activated transient outward K+ current (IK) and inward current (Petruska et al. 2000a). DRG cell classification has been more easily performed by combining the aforementioned factors with cell sizes in many studies (Cooper et al. 2004; Jiang et al. 2006; Rau et al. 2005a,b, 2006, 2007). DRG cell types, except for type 8, showed good internal homogeneities in AP and AHP features as well as chemical responsiveness to capsaicin, adenosine 5′-triphosphate (ATP), protons, and acetylcholine, and histochemical properties (Cooper et al. 2004; Jiang et al. 2006; Petruska et al. 2000a,b, 2002; Rau et al. 2005a,b, 2006, 2007). More recently, other cell groups, types 13 and 15, have been reported in small and large cell groups, respectively (Jiang et al. 2006). Cell classification by the current signature method has the substantial advantage of being able to predict AP features, chemosensitivities, and histochemical properties (Petruska et al. 2000a).

To our knowledge, there is no report demonstrating a systematic current signature classification for acutely dissociated TRG cells. Many studies have reported current signatures of Ih, I, and voltage-activated Na+ inward currents in TRG neurons (Ikeda and Matsumoto 2003; Matsumoto et al. 2010; Takeda et al. 2002, 2004a,b, 2006; Tsutsui et al. 2008; Yoshida and Matsumoto 2005; Yoshida et al. 2007). Because the TRG is generally thought to have a neuronal population comparable to that of the DRG, the current signature classification may be available to separate TRG cells into many internally homogeneous cell types that are distinct from other subclassified cell types.
Sensitivities to capsaicin and other TRPV1 (transient receptor potential cation channel, subfamily V, member 1) agonists and antagonists in TRG cells are similar to those in DRG cells (McDonald et al. 2008). On the other hand, desensitizing proton-induced current was observed in many TRG cells (40%, 44 of 110 tested cells) but in only a small population of DRG cells (12%, 10 of 85 tested cells; Krishtal and Pidoplichko 1981). Immunohistochemical studies suggest that expression of P2X<sub>2</sub> and P2X<sub>3</sub>, which are representative ATP receptors in primary sensory neurons, are considerably higher in myelinated neurons in the TRG than those in the DRG (Ambalavanar et al. 2005; Staikopoulos et al. 2007). Heteromeric P2X<sub>2/3</sub> and homomeric P2X<sub>3</sub> receptors generate slow- and fast-desensitizing currents induced by ATP, respectively (Lewis et al. 1995; Luo et al. 2006; Tsuda et al. 2000; Ueno et al. 1999). If possible to classify TRG cells using “current signatures,” some differences in proton and ATP responses may exist between TRG and DRG cell types.

In the present study, to address the specific features of TRG cells, we subclassified acutely dissociated TRG cells of rats by whole cell patch-clamp recordings based on the early DRG cell classification parameters (Petruska et al. 2000a, 2002) and compared with electrophysiological and chemical properties of DRG cell types. Although voltage protocols for current inductions used in our experiments were the same as those used for DRG cell classification, it was necessary to slightly modify some criteria for TRG cell classification due to TRG-specific cell size ranges and current signatures (see METHODS). In current-clamp mode, current injections for 100 ms were applied to generate an AP. The AP features, including AHP kinetics, were investigated in identified cell types. In all subclassified TRG cells, responses to capsaicin and protons at pH 5.0 were examined by measurement of other electrophysiological properties in voltage-clamp mode. Additionally, responses to nicotine and ATP were examined.

METHODS

The care and handling of the animals used in these experiments were consistent with all the National Institutes of Health recommendations for the humane use of animals. All experimental procedures were reviewed and approved by the appropriate Animal Experiment Committees of the Kyushu Dental College where the experiments were performed. The number of animals used was kept to the minimum necessary for a meaningful interpretation of the data and animal discomfort was kept to a minimum.

Preparation of TRG cells

Male Wistar rats (4 wk of age) were deeply anesthetized with pentobarbital (60 mg/kg, administered intraperitoneally). The TRGs were quickly removed bilaterally and transferred into modified Tyrode’s solution containing (in mM): NaCl 130, NaHCO<sub>3</sub> 20, KCl 3, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 2, glucose 10, and HEPES 10 (pH 7.3 adjusted with NaOH, 320 mOsm/kg), with a perfusion flow rate of 1 ml/min at room temperature. Patch pipettes were double-pulled (PP-83; Narishige, Tokyo, Japan) and filtered at 1 kHz by a low-pass filter. Series resistance was not compensated and cells with a series resistance <15 MΩ were excluded from the present study. The values of membrane potentials were corrected for the liquid junction potential (10 mV) present in the pipette. In the voltage-clamp recordings, membrane potential was held at −60 mV (V<sub>holding</sub>), and input resistance (R<sub>input</sub>) was measured by a protocol (average of eight consecutive steps to −70 mV, 10 ms duration) and evaluated as the average. Cell diameter was evaluated as an average of the longest and shortest axis in captured microphotographs of cells by a charge-coupled device camera (DS-5M-L1; Nikon, Tokyo, Japan). Only cells with diameters <38 μm were analyzed.

Cell classification protocol

Three kinds of protocols were used in the present study. The protocols were the same as those used previously for DRG cell classification (Petruska et al. 2000a, 2002). Classification protocol 1 (CP1) was used to produce TOC and I<sub>A</sub> (Fig. 1, Aa and Ab, respectively). A series of hyperpolarizing command steps (10 mV steps, 500 ms duration, 10 s interval) was applied from V<sub>holding</sub> to a final potential of −110 mV (Fig. 1Aa, inset). Peak amplitude of the TOC, which was observed in repolarization to V<sub>holding</sub> from the hyperpolarization step of −110 mV, was evaluated. The TOC was sensitive to the general I<sub>K</sub> blocker 4-aminopyridine (4-AP; Kanto Chemical, Tokyo, Japan) at 5 mM in 13 cells (Fig. 1Aa). Amplitude of I<sub>A</sub> was evaluated by subtracting an instantaneous inward current at the beginning of the hyperpolarization step of −110 mV from a current at the end of the voltage step. The I<sub>K</sub> was sensitive to the specific I<sub>K</sub> blocker ZD-7282 (Zeneca Pharmaceuticals, London, UK) at 100 μM in six cells (Fig. 1Ab). Classification protocol 2 (CP2) was used to produce depolarization-activated outward currents that involve I<sub>A</sub>. After a 500 ms conditioning pulse to −100 mV from V<sub>holding</sub>, a series of depolarizing command steps (20 mV steps, 200 ms duration, 10 s interval) was applied to a final potential of +40 mV (Fig. 1B, inset). Threshold voltage of I<sub>A</sub> (AT) was obtained from given outward current traces by CP2 (Fig. 1B). The I<sub>A</sub>-like current components were confirmed to be sensitive to 4-AP at 5 or 10 mM in 39 cells (Fig. 1B). A curve of the form A exp[−(t − k)/τ] + C was single-exponentially fit to the trace at +40 mV pulse (Fig. 1B, bottom traces) and the τ of I<sub>A</sub> (τ<sub>A</sub>) was estimated. However, the given traces at the highest voltage stimulation sometimes showed a smaller I<sub>A</sub> component than that at lower voltages or lacked I<sub>A</sub> components. In these cases, the traces at the lower voltage (+20 or 0 mV) were used for curve-fitting (Fig. 1B, top traces). Classification protocol 3 (CP3) was used to produce an inward

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currents using this protocol (second classification protocol (CP2). Because the voltage-activated outward K currents (were called fast (or slow (bottom traces classification protocol (CP3). The B current. After a 500 ms conditioning pulse to (declining phase in the first suprathreshold trace (double-headed arrow in (s interval) was applied from (of inward current (n) was obtained from given outward current traces (arrows). The τ of Iₙ (τₙ) was derived from a single-exponential curve-fitting in the Iₙ-like component (double-headed arrow in B). C: voltage-activated currents using the third classification protocol (CP3). The τ of inward current (τᵢᵢ) using this protocol (inset of Ca) was derived from a single-exponential curve-fitting in the declining phase of the first suprathreshold trace (double-headed arrow in Ca). If the τᵢᵢ was under or over 0.55 ms (dashed line in Ch), the inward currents were called fast (bottom traces) or slow (top traces), respectively. Many slow inward currents (n = 9 of 11) were tetrodotoxin-resistant (1 μM; TTXr) and all fast inward currents (n = 10) were tetrodotoxin-sensitive (TTXs) (Cb).

current. After a 500 ms conditioning pulse to −80 mV from Vᵢᵢ, a series of depolarizing command steps (10 mV steps, 2 ms duration, 10 s interval) was applied from Vᵢᵢ to a final potential of +10 mV (Fig. 1Ca, inset). A curve of the form mentioned earlier was fit to the single-exponential-like component in the declining phase of the first suprathreshold trace (Fig. 1Ca) and the τ of inward current (τᵢᵢ) was estimated. The CP3-induced inward currents were divided into two different types, slow and fast. The former type was higher-threshold (from −20 to 0 mV) and slowly activated currents, with a long declining phase after CP3 stimulation (Fig. 1Ca, top traces). The latter type were lower-threshold (generally −40 mV) and fast-activated currents followed by outward currents during CP3 stimulation (Fig. 1Ca, bottom traces). Many slow inward currents in 10 of 12 cells were resistant to TTX (Wako Pure Chemical Industries, Osaka, Japan) at 1 μM (Fig. 1, Ca, top traces, and Cb), but all fast inward currents in 10 cells were sensitive to TTX (Fig. 1, Ca, bottom traces, and Cb).

Criteria for TRG cell classification

Table 1 shows the modified criteria for acutely dissociated TRG cell classification in the present study. In preliminary classification trials, we used a variety of ranges for cell size but realized that the ranges of cell size (small: 15–24 μm and medium: 25–38 μm) used in another TRG study (Park et al. 2006) were suitable for classification in the present study because the cell-size grouping led to the highest homogeneities of AP features and chemical properties in each TRG cell type. The ranges of cell size were different from those in the original DRG cell classification (Petruska et al. 2000a). After many tests and verifications, the cutoff value for TOC and Iₜₐ of TTX-resistant (TTXr) and TTX-sensitive (TTXs) was used. The value was applied to discriminate TTX-sensitive and TTX-resistant inward currents (Fig. 1Cb). In return for not using the criterion of large Iₜₐ, the CP3 signature (slow or fast) was used to discriminate TRG type 4 from other cell types in the present study.

AP generation

In the current-clamp mode, membrane potentials were manually set to −60 ± 2 mV after observing the resting membrane potential (Eᵢᵢ). A series of current steps (0.2 nA steps to a final current of 1 nA, 10 ms duration, 10 s interval; Fig. 2A, inset) was applied to produce an AP. The period from the peak to 95% of repolarization in the first generated AP by a current injection of 1 nA was evaluated as AP duration (Fig. 2Bb). Because AP generation was frequently repetitive during the injection of 1 nA, AHP durations were evaluated in voltage traces induced by the threshold current injection in all cells. The τ of AHP (τₐₜₐ) was determined by single-exponentially fit (as before) to the AHP phase (Fig. 2Bb).

<table>
<thead>
<tr>
<th>Type</th>
<th>TOC</th>
<th>Iₜₐ</th>
<th>Cell Size</th>
<th>InC</th>
<th>AT</th>
</tr>
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<tr>
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<td>Small</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
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<td>No</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
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<td>Yes</td>
<td>Small</td>
<td>Fast</td>
<td>NR</td>
</tr>
<tr>
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<td>Yes</td>
<td>Medium</td>
<td>Fast</td>
<td>NR</td>
</tr>
<tr>
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<td>No</td>
<td>Yes</td>
<td>Medium</td>
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</tr>
<tr>
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<td>Yes</td>
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<td>Slow</td>
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<tr>
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<td>Small</td>
<td>Slow</td>
<td>NR</td>
</tr>
<tr>
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<td>Medium</td>
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</tr>
<tr>
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<td>No</td>
<td>No</td>
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<td>10</td>
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Criteria for nine trigeminal ganglion (TRG) cell types. The criteria of current signatures were modified from dorsal root ganglin (DRG) cell classification (Petruska et al. 2000, 2002). “Yes” and “No” indicate >30 pA and <30 pA, respectively, in the peak amplitudes of transient outward current (TOC) and hyperpolarization-activated inward current (Iₜₐ) by application of CP1. “Small” and “Medium” indicate 15–24 and 25–38 μm, respectively, in cell diameter. “Fast” and “Slow” indicate <0.55 ms (lower threshold) and >0.55 ms (higher threshold), respectively, in τᵢᵢ of suprathreshold inward currents by application of CP3. In NR, the test was “not required”. Note that there were no subclassified type 6 cells in acutely dissociated TRG cells in the present study.
Drug applications

In voltage-clamp mode, drugs were acutely applied to cells for 4–6 s using the Y-tube method (Nakagawa et al. 1990). To avoid chemical effects on voltage-activated currents and neuronal excitability (Chen et al. 2010; Liu et al. 2001, 2004), drug applications were performed after investigations of electrophysiological parameters. Capsaicin (Nacalai Tesque, Kyoto, Japan) was dissolved to 1 mM in DMSO. Nicotine (Research Biochemicals International, Natick, MA) and ATP (Sigma–Aldrich) were dissolved to 100 and 10 mM, respectively, in distilled water. These stock solutions were finally diluted to 0.1% with the perfusion solution. DMSO itself at 0.1% was confirmed to have no effect on base currents in 10 cells (data not shown). When applying an acidic solution with pH 5.0, MES (Sigma–Aldrich) was substituted for HEPES. The applications were performed at 30- to 60-s intervals in the following order: nicotine, ATP, capsaicin, and protons. To avoid cross-desensitization of ATP reactivity after capsaicin application (Piper and Docherty 2000), ATP was applied prior to the capsaicin application. If the recorded cells showed drug-induced current >1 pA/pF, the cells were deemed to be sensitive to the drugs.

Statistics

After the current and voltage traces were converted into ASCII files, calculations and measurements were carried out using a custom macro-program and “solbar” function for curve fitting (least-squares method) in Microsoft Excel (Microsoft, Tokyo, Japan). Peak amplitudes of capsaicin-, ATP-, and proton-induced currents were normalized for cell size (pA/pF). The numerical data are given as means ± SE. For statistical analyses of electrophysiological and chemical parameters between each pair in all cell types, the Dunn’s multiple-comparison test was used as a post hoc test followed by the Kruskal–Wallis test. When cell numbers in the cell type were less than three, the groups were excluded from the analysis. Because a parameter for nondesensitizing ATP-induced currents was limited to only two groups by the exclusion, the Mann–Whitney test was applied to compare two independent groups. All statistical analyses were performed in Prism (GraphPad Software, San Diego, CA). Values of P < 0.05 were considered to be significant.

RESULTS

Cell classification for TRG cells

According to the modified criteria for acutely dissociated TRG cell classification (Table 1), all small and medium TRG cells (n = 170) could be subclassified into nine cell types: 1–5, 7–9, and 13. There were no subclassified type 6 cells in TRG cells in the present experimental condition. Representative current signatures in subclassified TRG cell types are illustrated in Fig. 3.

Current signatures and cell sizes in nine TRG cell types

Type 1 (n = 40) was classified with little TOC (1.2 ± 0.6 pA) and I_{H} (9.0 ± 1.6 pA) in small cells (18.6 ± 0.4 μm,
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ranging from 13.0 to 23.8 μm; 13.3 ± 0.8 pF; Table 1, Fig. 3A). Most type 1 cells (n = 27) showed slowly inactivated IA-like components (τ1A: 13.2 ± 1.5 ms) in CP2-induced outward current traces, in which ATs indicated variable values from −40 to +20 mV. Nine cells showed IA-like components (τ1A: 19.4 ± 3.8 ms) at low voltage stimulations, but they did not show this component at the highest voltage stimulation of +40 mV (Fig. 3A), probably due to being masked by delayed-rectified outward current components. The other four cells did not show any IA-like components through all outward current traces (data not shown). All CP3-induced inward currents in the type 1 group were the slow type (τinc: 0.72 ± 0.02 ms, Fig. 3A).

The type 2 group (n = 53) was classified with TOC (159.8 ± 18.3 μA) and little Ith (2.1 ± 0.7 pA) (Table 1, Fig. 3B). Type 2 cells were distributed over both small and medium cells (23.6 ± 0.5 μm, ranging from 17.1 to 31.0 μm; 22.0 ± 0.9 pF). CP2-induced outward currents had either slowly inactivated IA-like (τ1A: 10.1 ± 0.8 ms, n = 30) or very slowly inactivated IA-like components (τ1A: 68.8 ± 7.5 ms, n = 22, Fig. 3B), except for one cell that showed no IA-like component. The values of ATs varied from cell to cell and ranged from −40 to +20 mV. All inward currents were of the slow type (τinc: 0.69 ± 0.02 ms, Fig. 3B).

The type 3 group (n = 8) was classified with Ith (309.6 ± 82.0 pA) and fast inward current (τinc: 0.43 ± 0.02 ms) without TOC in small cells (20.3 ± 0.9 μm, ranging from 16.0 to 23.5 μm; 21.8 ± 2.2 pF; Table 1, Fig. 3C). All type 3 cells showed AT at −40 mV and rapidly inactivated IA-like outward currents (τ1A: 3.3 ± 0.2 ms; Fig. 3C).

The type 4 group (n = 16) was classified with Ith (353.3 ± 44.7 pA) and fast inward current (τinc: 0.39 ± 0.01 ms) without TOC in medium cells (33.7 ± 0.5 μm, ranging from 31.0 to 37.4 μm; 45.1 ± 1.9 pF; Table 1, Fig. 3D). All type 4 cells showed AT at −40 mV and rapidly inactivated IA-like outward currents (τ1A: 3.1 ± 0.1 ms; Fig. 3D). These current signatures and cell size in the type 4 group were very similar to those of the type 3 group, but there was a difference in cell size (cell ranges: 31–37 μm in the type 4 and 16–24 μm in the type 3).

The type 5 group (n = 6) was classified with Ith (49.0 ± 7.2 pA), slow inward current (τinc: 0.81 ± 0.09 ms), and AT at 0 mV without TOC (0.3 ± 0.3 pA) in medium cells (34.2 ± 0.7 μm, ranging from 32.1 to 36.4 μm; 50.8 ± 3.9 pF; Table 1, Fig. 3E). The current signatures and cell size in the type 5 group were similar to those of the type 8 group, but there was a difference in the value of AT with 0 mV for type 5 cells and −20 mV for type 8 cells (Table 1, Fig. 3, E and G). Type 5 cells showed slowly inactivated IA-like outward currents (τ1A: 14.1 ± 1.1 ms; Fig. 3E).

The type 7 group (n = 16) was classified with Ith (60.6 ± 10.9 pA) and slow inward current (τinc: 0.73 ± 0.04 ms) without TOC (1.6 ± 1.3 pA) in small cells (18.5 ± 0.6 μm, ranging from 15.0 to 22.0 μm; 15.9 ± 1.1 pF; Table 1, Fig. 3F). Most of the type 7 cells (n = 11) showed slowly inactivated IA-like current (τ1A: 10.1 ± 1.4 ms; Fig. 3F), whereas ATs varied from cell to cell and ranged from −40 to 0 mV. Similar to some type 1 cells, three of the type 7 cells showed slowly inactivated IA-like components (τ1A: 10.7 ± 3.8 ms) at low-voltage stimulations, but they did not show these components at the highest voltage stimulation of +40 mV.

The type 8 group (n = 16) was classified with Ith (191.2 ± 25.7 pA), slow inward current (τinc: 0.68 ± 0.02 ms), and AT at −20 mV without TOC in medium cells (33.3 ± 0.9 μm, ranging from 26.8 to 37.5 μm; 47.1 ± 3.2 pF; Table 1, Fig. 3G). Type 8 cells showed relatively rapidly inactivated IA-like components (τ1A: 4.4 ± 0.3 ms; Fig. 3G).

The type 9 group (n = 5) was classified with little TOC (7.0 ± 5.1 pA) and no Ith in medium cells (31.1 ± 0.9 μm, ranging from 28.1 to 33.2 μm; 49.6 ± 7.4 pF; Table 1, Fig. 3H). As shown in Fig. 3H, CP1 signature of type 9 cells looked leaky, but the type 9 group clearly lacked the Ith component. Type 9 cells showed slowly inactivated IA-like outward current (τ1A: 6.9 ± 0.8 ms; Fig. 3H) and ATs were −20 mV (n = 3) or 0 mV (n = 2). All inward currents were of the slow type (τinc: 0.99 ± 0.08 ms; Fig. 3H). The criteria for type 9 current signatures were the same as those of type 1, except for a difference in cell size (cell ranges: 28–33 μm in the type 9 and 13–24 μm in the type 1).

The type 13 group (n = 10) was classified with both TOC (132.4 ± 44.6 pA) and Ith (85.6 ± 15.6 pA; Table 1, Fig. 3I). Type 13 cells were distributed only in medium cells (31.6 ± 1.0 μm, ranging from 26.8 to 38.0 μm; 51.5 ± 4.4 pF) showing slowly inactivated IA-like outward current (τ1A: 9.4 ± 1.2 ms; Fig. 3I). ATs varied from cell to cell and ranged from −40 to 0 mV. All inward currents were of the slow type (τinc: 0.76 ± 0.06 ms; Fig. 3I).

Electrophysiological features in TRG cell types

It has been suggested that values of Rin and Em can predict myelination status in vivo (López de Armentia et al. 2000). There were significant differences in Rin and Em values among cell types (P < 0.01 for both; Table 2). The Rin values in types 1, 3, and 7 were significantly higher than those in the type 4 group (P < 0.05 for type 1; P < 0.01 for types 3 and 7), the type 8 group (P < 0.05 for all), and the type 9 group (P < 0.05 for type 1; P < 0.01 for types 3 and 7) (Table 2). Em in the type 1 group was significantly higher than that in the type 8 and type 13 groups (P < 0.05 and 0.01, respectively; Table 2).

AP features (duration and AHP) can also predict nociceptor status and other afferent characteristics (Harper and Lawson 1985a,b; Yoshida and Matsuda 1979). There was a significant difference in AP duration among cell types (P < 0.01). AP duration in the type 1 group indicated the longest value among nine groups of TRG cell types (Fig. 4, Table 2) and was significantly longer than types 2, 3, 4, and 8 (P < 0.01 for all). AP durations in types 2, 7, and 13 were significantly longer than those in the type 3 group (P < 0.05 for type 3; P < 0.01 for types 2 and 7) and the type 4 group (P < 0.01 for all; Fig. 4, Table 2). AP duration in the type 5 group was significantly longer than that in the type 4 group (P < 0.05; Fig. 4, D and E; Table 2). There was significant difference in τAHP (P < 0.01) among cell types. Among TRG cell types, the mean τAHP in the type 1 group indicated the longest value (Fig. 4A, Table 2) and was significantly longer than that in types 2, 4, 8 (P < 0.01 for all), 9, and 13 (P < 0.05 for both). τAHP in the type 7 group was significantly longer than that in the type 4 group (P < 0.01; Fig. 4, D and F, Table 2).
Responses to capsaicin and protons

Responses to capsaicin at 1 μM and protons at pH 5.0 were examined in all TRG cells (n = 170). Capsaicin-sensitive cells accounted for 69% (n = 117) of TRG cells and capsaicin-induced currents indicated a variety of kinetics (Fig. 5), consistent with other studies (Liu and Simon 1996, 2000; Liu et al. 1997). On the other hand, similar to DRG studies, pH 5.0 induced inward currents with various kinetics in all TRG cells (Petruska et al. 2000, 2002; see Liu and Simon 2000).

All cells in the type 5, 9, and 13 groups, 91% in the type 2 group (n = 48 of 53), and 81% in the type 7 and type 8 groups (each n = 13 of 16) were capsaicin sensitive (Fig. 5, Table 3). All cells in the type 4 group and most cells in the type 3 group were capsaicin insensitive (Fig. 5, C and D, Table 3), showing a high homogeneity in each TRG cell type. However, in the type 1 group, capsaicin-sensitive cells accounted for 53% (n = 21 of 40, Table 3), showing a low homogeneity. Peaks of capsaicin-induced currents in Table 3 basically represent the offset of the superfusion. There was no significant difference in the peak amplitudes among cell types.

As in DRG, many cell types exhibited both desensitizing and/or nondesensitizing proton-induced currents (Petruska et al. 2000a, 2002). Desensitizing proton-induced currents were observed in types 3 and 5–13 cells and 78% (n = 31 of 40) of type 1 cells (Fig. 5, Table 3). There was a significant difference in the peak amplitudes of desensitizing proton-induced currents among cell types (P < 0.01). Peak amplitude of the desensitizing proton-induced currents in the type 1 group (Fig. 5A) was significantly larger than that in types 5 (P < 0.05; Fig. 5E), 8, and 13 (P < 0.01 for both; Fig. 5, G and I, Table 3). That in the type 3 group (Fig. 5C) was significantly larger than that in the type 8 group (P < 0.01; Fig. 5G). The desensitizing proton-induced currents were often followed by nondesensitizing components (see Fig. 5, A, B, and E–I). The amplitudes of the nondesensitizing components were dependent on whether the cell showed capsaicin sensitivity. In 89% (n = 59 of 66) of capsaicin-sensitive cells, the desensitizing proton-induced currents were followed by relatively large nondesensitizing components (Fig. 5, A, B, and E–I). The remaining 11% of cells (n = 7) with capsaicin sensitivity showed only desensitizing currents. Meanwhile, in all capsaicin-insensitive cells, desensitizing proton-induced currents were followed by very small nondesensitizing components, if present (Fig. 5, A and C). On the other hand, 89, 88, and 23% of types 2, 4, and 1 cells, respectively, showed only nondesensitizing proton-induced current components (Fig. 5, A, B, and D, Table 3). Peak amplitude of the nondesensitizing proton-induced currents in the type 4 group (Fig. 5D) was significantly smaller than that in the type 1 (P < 0.05; Fig. 5A) and type 2 groups (P < 0.01; Fig. 5B, Table 5).

Responses to nicotine and ATP

Responses to nicotine at 100 μM and ATP at 10 μM were examined in 115 TRG cells. Nicotine-sensitive cells accounted for 43% (n = 50) of TRG cells, consistent with 45% in a previous TRG study (Liu and Simon 1996). On the other hand, ATP-sensitive cells accounted for 88% (n = 101) of TRG cells and ATP-induced currents showed three patterns of current...
kinetics: fast-desensitizing current (37%, \( n = 37 \); Fig. 5, B and G), slow-desensitizing current (51%, \( n = 52 \); Fig. 5, A and D–I), and the intermediate current (or also known as “the mixed current”; 12%, \( n = 12 \); Fig. 5, G and I). The populations of the three patterns of ATP-induced currents were similar to those of a previous TRG study (Luo et al. 2006).

All cells in the types 5, 9, and 13 groups and >80% in the type 4 and type 8 groups (\( n = 12 \) of 14 and 13 of 16, respectively) were nicotine sensitive, but all cells in the types 1 and 3 groups, 89% in the type 2 group (\( n = 32 \) of 36), and 78% in the type 7 group (\( n = 7 \) of 9) were nicotine insensitive (Fig. 5, Table 4). It is interesting to note that nicotine-sensitive cells were seen in medium cell groups and nicotine-insensitive cells in small cell groups. There was no significant difference in the peak amplitudes among cell types.

Fast-desensitizing ATP-induced currents were observed in 89% of type 2 cells (\( n = 32 \) of 36) and 25% of type 8 cells (\( n = 4 \) of 16) (Fig. 5, B and G, Table 4). Peak amplitude of the fast-desensitizing ATP-induced current in the type 2 group was significantly larger than that in the type 8 group (\( P < 0.05 \) in Mann–Whitney test, Table 4). Slow-desensitizing ATP-induced currents were observed in all cells in the type 4 and 9 groups and roughly 80% in the type 5 and 7 groups [\( n = 5 \) of 6 (83%) and 7 of 9 (78%), respectively] (Fig. 5, A and D–I, Table 4). There was no significant difference in the peak amplitudes among cell types. Intermediate currents were ob-

### Table 3. Responses to capsaicin and proton in subclassified cells (\( n = 170 \))

<table>
<thead>
<tr>
<th>Type</th>
<th>( n )</th>
<th>Capsaicin</th>
<th>Proton, Desen.</th>
<th>Proton, Nondesen.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( % (n) )</td>
<td>pA/pF</td>
<td>( % (n) )</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>53 (21)</td>
<td>79 ± 16</td>
<td>78 (31)</td>
</tr>
<tr>
<td>2</td>
<td>53</td>
<td>91 (48)</td>
<td>65 ± 6</td>
<td>11 (6)</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>13 (1)</td>
<td>20</td>
<td>100 (8)</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>0 (0)</td>
<td>—</td>
<td>13 (2)</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>100 (6)</td>
<td>33 ± 5</td>
<td>100 (6)</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>81 (13)</td>
<td>79 ± 19</td>
<td>100 (16)</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>81 (13)</td>
<td>31 ± 10</td>
<td>100 (16)</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>100 (5)</td>
<td>53 ± 10</td>
<td>100 (5)</td>
</tr>
<tr>
<td>13</td>
<td>10</td>
<td>100 (10)</td>
<td>49 ± 17</td>
<td>100 (10)</td>
</tr>
</tbody>
</table>

Values are means ± SE. “Nondes.” and “Desen.” indicate nondesensitizing and densensitizing currents, respectively. *Significantly larger than types 5, 8, and 13. **Significantly larger than type 8. **Significantly larger than type 4. Probabilities are presented in the text.
served in small populations of all cell types, except for the type 3, 4, and 9 groups (Fig. 5, B and G, Table 4). On the other hand, ATP-insensitive cells were observed in all type 3 cells and about 30% of type 1 and type 8 cells (n = 5 of 18 and n = 4 of 14, respectively).

**DISCUSSION**

The present study is the first to demonstrate TRG cell classification using the current signature method. Acutely dissociated TRG cells could be subclassified into nine cell types—types 1–5, 7–9, and 13—using modified criteria for voltage-dependent currents and cell sizes based on the early DRG cell classification system (Petruska et al. 2000a, 2002). Cell sizes and electrophysiological and chemical properties in TRG cell types are summarized in Table 5. Characteristics in TRG cell types were mostly similar to those in DRG cell types (Petruska et al. 2000a, 2002; Jiang et al. 2006), although there were some minor differences in some cell types between the TRG and DRG (Table 5). The high similarities of TRG and DRG cell types allow us to quote already given physiological and immunohistochemical knowledge of the DRG cell types and to compare various characteristics between TRG and DRG cells.

**Current signatures in TRG cells**

Some current signature features for classification differed between TRG and DRG cell types to the extent that some misclassification could occur. In the TRG type 3 group, the mean amplitude of $I_H$ (309.6 pA) was larger than that in the DRG type 3 group (35.7 pA; Petruska et al. 2000a). If a criterion of large $I_H$ (>300 pA) for discrimination of DRG type 4 was used in the present TRG study, many type 3 cells would be misclassified into the TRG type 4 group due to the considerably larger $I_H$ in TRG type 3 cells. Therefore, the criterion of large $I_H$ (>300 pA) was not suited to TRG cell classification under our experimental conditions. Other criteria for the TRG type 4 group (fast inward current and medium cell size) were used to discriminate the cell type in the present study. Consequently, the classification of TRG type 4 cells by the modified criteria showed chemical properties similar to those in the DRG type 4 group (Table 5). Furthermore, AT in the TRG type 4 group differed from the DRG type 4 group (−40 vs. −20 mV in DRG; Petruska et al. 2000a). Similarly, whereas AT in the DRG type 9 group was −20 mV, two cells of five TRG type 9 cells showed 0 mV at AT. Because of no compensation of series resistance in the present study, some of the AT data might be distorted.

**Cell sizes in TRG cells**

TRG cell size ranges for small and medium cell groups (15–24 and 25–38 μm of cell diameters) used in the present study were the same as those in a previous TRG study (Park et al. 2006). In the original DRG cell classification (Petruska et al. 2000a), the range of cell size was set to <22 μm for very small, 24–32 μm for small, and 32–48 μm for medium cell groups; DRG cells in type 3 and type 7 groups were very small, small, 24–32 μm, and medium, respectively. Some small populations of TRG cell types (Petruska et al. 2000a, 2002) were not included in the present study.
type 1 and type 2 groups were small, and the other type groups were medium cells. Although cell sizes of TRG types 3–7 were similar to the DRG cells of the same type, cell sizes of other TRG types were smaller than those of the DRG types.

**AP features in TRG cells**

AP and AHP durations in TRG cell types, except for AHP in the type 4 group, showed properties similar to those in matched DRG cell types and there was an intrinsic relationship between AHP and AP, showing that the longer AHP, the longer the AP (Table 5). When AP and AHP features were compared between TRG and DRG cell types, the difference of current injection protocols between the present TRG and earlier DRG studies should be noted. The long current injection used in the present study has frequently been used to generate AP and AHP in many other TRG in vitro studies (López de Armentia et al. 2000; Park et al. 2009; Takeda et al. 2004a,b, 2005, 2006; Tsutsui et al. 2008; Veiga Moreira et al. 2007; Yoshida and Matsumoto 2005; Yoshida et al. 2007). The durations of AP and AHP are thought to be prolonged and shortened, respectively, by the injected current compared with those of physiologically induced AP and AHP in vivo. On the other hand, a very short current injection (1 ms) was used in the DRG cell classification studies (Petruska et al. 2000a, 2002). The AP and AHP properties produced by the protocol were similar to physiologically induced AP characteristics in vivo (Djouhri et al. 1998; Villiére and McLachlan 1996). A difference of AHP in the type 4 group between the TRG and DRG (Table 5) may be due to the different current injection protocols.

**Capsaicin- and proton-induced currents in TRG cells**

Capsaicin sensitivities in TRG cell types were almost the same as those of DRG cell types (Petruska et al. 2000a, 2002), except for the many capsaicin-insensitive cells in the TRG type 1 group (Table 5). In a previous study, there were no differences in the sensitivity to TRPV1 agonists, including capsaicin, between TRG and DRG cells (McDonald et al. 2008). Capsaicin is frequently used as a selective stimulant of polymodal nociceptor and activates TRPV1. Since many nonnociceptive itch-related DRG cells have been reported to express TRPV1 (Sun and Chen 2007; Sun et al. 2009), it is considered that capsaicin-sensitive TRG types may be included in the nonnociceptive itch-related group.

Most of the TRG type 1 cells and a small population of type 2 cells showed a desensitizing proton-induced current component (Table 5), which is different from DRG types 1 and 2 that show uniformly nondesensitizing proton-induced current without a desensitizing component (Petruska et al. 2000a, 2002). In an early electrophysiological study (Krishtal and Pidoplichko 1981), the desensitizing proton-induced current was observed in 40% of TRG cells but only in 12% of DRG cells. This supports the existence of the desensitizing proton-induced current in TRG type 1 and type 2 cells in the present study, which is different from the matched DRG cell types. Amiloride-sensitive acid-sensing ion channels (ASICs) are the most important candidates for inducing desensitizing proton-induced currents in primary sensory neurons (Jiang et al. 2006). Although there is no report documenting comparative ASIC protein expressions in TRG and DRG cells, our data indicate that ASICs may distribute more widely to small cells in the TRG than those in the DRG. Because ASICs are thought to be mechanoreceptors (García-Añoveros et al. 2001), the type 5–13 groups and the subgroups of type 1 and type 2 cells that showed desensitizing proton-induced currents with capsaicin sensitivities may be mechano-heat nociceptors.

TRPV1 generates nondesensitizing proton-induced current in TRG cells (Liu and Simon 2000). Because the nondesensitizing proton-induced current in DRG type 2 cells is largely suppressed by the TRPV1 blocker capsazepine (Jiang et al. 2006), the large nondesensitizing proton-induced currents in many TRG type 2 cells may be mainly generated by TRPV1. Nevertheless, seven TRG cells with capsaicin sensitivity showed only desensitizing currents. It is possible that prior application of capsaicin produced a tachyphalaxis to proton activation in some cells. Besides TRPV1, TWIK-related acid-sensing K⁺ channels produce nondesensitizing currents that contribute to proton reactivity. They are highly expressed in DRG type 4 cells (Cooper et al. 2004; Rau et al. 2006).

**ATP-induced currents in TRG cells**

Although the overall pattern of ATP reactivity was similar, kinetic patterns of ATP reactivity differed between TRG and DRG. TRG type 5, 7, and 9 cells showed slow-desensitizing ATP-induced currents (Table 5), which is different from DRG type 5 and type 7 cells that show fast-desensitizing ATP-induced currents, and DRG type 9 cells that show the intermediate currents (Petruska et al. 2000a, 2002). An early TRG study using electrophysiological and immunohistochemical techniques reported that the slow-desensitizing ATP-induced current reflect cells expressing P2X₁, P2X₂, and P2X₃, the fast-desensitizing current reflect cells expressing P2X₁ and P2X₃ and the intermediate currents reflect cells expressing P2X₁, P2X₃, and P2X₄ (Luo et al. 2006). Together with our results and an earlier study, TRG type 5, 7, and 9 cells may express P2X₂ with other P2X subunits, which are different from the same DRG cell types. An immunohistochemical TRG study suggests that heteromeric P2X₂/3 receptors are frequently expressed in myelinated neurons of the TRG compared with the DRG (Staikopoulos et al. 2007). Because DRG type 5 and type 9 cells are thought of being myelinated neurons (Petruska et al. 2002; Rau et al. 2005a, 2007), the slow-desensitizing ATP-induced currents in TRG types 5 and 9 are probably reflective of specific differences of heteromeric P2X₂/3 receptor expression between TRG and DRG.

**Nicotine-induced currents in TRG cells**

Nicotine sensitivity covaried with the cell sizes in TRG cell types. Small cells in types 1, 3, and 7 groups and small/medium cells of the type 2 group were insensitive to nicotine, but medium cells in types 4, 5, and 8–13 groups were sensitive to nicotine (Table 5). The relationship between nicotine sensitivity and cell size was closely matched to an earlier TRG study (Liu and Simon 1996). Many DRG cell types have sensitivity to acetylcholine and the receptor subtypes are pharmacologically identified as α3β4(α5) and/or α7 (Rau et al. 2005a). The fact that many TRG cell types showed nicotine sensitivity in the present study is consistent with the many DRG cell types that show acetylcholine sensitivities. However, there were
some differences in types 1 and 2 groups in nicotine/acetylcholine sensitivity between the TRG and DRG cells (Table 5, nicotine-insensitive vs. acetylcholine-sensitive in DRG; Rau et al. 2005a). The differences may be due to the specific discrepancy of nicotinic acetylcholine receptor (nAChR) expression between the TRG and DRG or methodological differences of nicotine and acetylcholine application. Recently, it has been reported that nicotine at micromolar concentrations activates not only nAChRs but also TRPA1 in TRG cells (Talavera et al. 2009). Therefore nicotine-induced currents in the present study may partly reflect TRPA1 activation. However, the distribution of TRPA1 in current signature identified cells is not known.

**Subphenotypes in the TRG type 1 and type 8 groups**

Although the DRG type 1 group has been originally reported to be a uniform cell group (Petruska et al. 2000a), the TRG type 1 group showed a mixed class in the present study. The TRG type 1 group may be largely divided into capsaicin-sensitive and -insensitive subgroups. The former capsaicin-sensitive type 1 subgroup may be further divided into two subgroups that show different proton-induced currents, a nondesensitizing and a desensitizing proton-induced current. The capsaicin-sensitive and nondesensitizing proton-induced current subgroup was similar to cells in the DRG type 1 group (Petruska et al. 2000a). TRG type 8 cells showed various kinetics in ATP-induced currents, similar to DRG type 8 cells (Petruska et al. 2002). Therefore the TRG type 8 group may also be a mixed class divided by ATP-induced current kinetics.

**Myelination status of TRG cell types**

The current signature classified cells are likely to represent both Aδ and C fiber populations. An electrophysiological study of TRG neurons has reported significant differences in R∞ and E∞ between Aδ and C neurons, identified by conduction velocities, and found higher values of R∞ and E∞ in C neurons than those values in Aδ neurons (López de Armentia et al. 2000). The present study demonstrated that there were significant differences in R∞ and E∞ among TRG cell types. If the relationship in R∞ and E∞ mentioned earlier is applied to the present results, types 4, 8, 9, and 13 may be Aδ neurons and types 1, 2, and 7 may be C neurons; it is difficult to apply this analysis to the type 3 and type 5 groups. However, the classification of TRG cell types in Aδ and C neurons could be clarified by further experiments using isolectin B4 (unmyelinated marker) staining and immunohistochemistry for neurofilament (myelinated marker) previously used for DRG cell types (Petruska et al. 2000a,b, 2002).

In conclusion, the “current signatures” classification is available for acutely dissociated TRG cells for the separation into internally homogeneous subpopulations. Also, the present study clearly demonstrated that most of properties in TRG cells are similar to those in DRG cells, although there were some differences. Some of these differences may be due to different developmental courses from different embryonic origins between the TRG and DRG; many trigeminal afferents are derived from the ectodermal placode, whereas the sciatic nerve is derived from the segmental neural crest (D’Amico-Martel and Norden 1983). The present findings will be useful for the comparison of cellular mechanisms of the TRG and DRG in sensory research.

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**Disclosures**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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