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Electrophysiological Properties and Chemosensitivity of Acutely Dissociated Trigeminal Somata Innervating the Cornea

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Abstract

Adult rat sensory trigeminal ganglion neurons innervating the cornea (cTGNs) were isolated and identified following retrograde dye labeling with FM1–43. Using standard whole-cell patch clamp recording techniques, cTGNs could be subdivided by their action potential (AP) duration. Fast cTGNs had AP durations < 1 ms (40%) while slow cTGNs had AP durations > 1 ms and an inflexion on the repolarization phase of the AP. With the exception of membrane input resistance, the passive membrane properties of fast cTGNs were different from those of slow cTGNs (capacitance: 61 ± 4.5 pF vs. 42 ± 2.6 pF, resting membrane potential: -59 ± 0.7 mV vs. -53 ± 0.9 mV, for fast and slow cTGNs respectively). Active membrane properties also differed between fast and slow cTGNs. Slow cTGNs had a higher AP threshold (-25 ± 1.6 mV vs. -38 ± 0.8 mV), a larger rheobase (14 ± 1.9 pA/pF vs. 6.8 ± 1.0 pA/pF), and a smaller AP undershoot (-56 ± 1.7 mV vs. -67 ± 2.5 mV). The AP overshoot, however was similar between the two types of neurons (46 ± 3.1 mV vs. 48 ± 4 mV). Slow cTGNs were depolarized by capsaicin (1 μ M, 80%) and 60% of their APs were blocked by TTX (100 nM). Fast cTGNs were unaffected by capsaicin and 100% of their APs were blocked by TTX. Similarly, cTGNs were also heterogeneous with respect to their responses to exogenous ATP and 5-HT.

The current work shows that cTGNs have distinctive electrophysiological properties and chemosensitivity profiles. These characteristics may mirror the distinct properties of corneal sensory nerve terminals. The availability of isolated identified cTNGs constitutes a tractable model system to investigate the biophysical and pharmacological properties of corneal sensory nerve terminals.

Keywords

nociceptor; pain; primary sensory neuron; autacoids; patch clamp

The cornea is the most densely innervated structure in the body (Belmonte and Gallar, 1996). All sensory corneal nerve terminals (CNTs) are considered nociceptive because their activation by disparate stimuli exclusively elicits the perception of pain (Beuerman and

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Tanelian, 1979; Chen et al., 1995). Two types of nociceptive nerve fibers can be distinguished in the cornea, C-fibers with action potential (AP) conduction velocities < 1m/s and A δ -fibers with AP conduction velocities > 1m/s (MacIver and Tanelian, 1990). In the cornea, these fiber types show distinct differences in modality and architecture; the majority of A δ -fibers are mechanoreceptive with thin elongate (0.1–1.2 mm) nerve endings running parallel to the epithelial surface, while the C-fiber nerve endings terminate as short (< 50 μ m) vertically-directed processes clustered within the epithelium (MacIver and Tanelian, 1990). Both types of CNT fibers are responsive to a variety of neurotransmitters, autacoids, and chemicals (Belmonte and Gallar, 1996; MacIver and Tanelian, 1993). The sensory cell bodies that give rise to CNTs reside in the ophthalmic branch of the trigeminal ganglia (TG; Marfurt et al, 1989). Some of the functional differences between C- and A δ -fiber nerve terminals observed in the cornea can also be distinguished electrophysiologically at the level of the cell body. Lopez de Armentia et al. (2000) reported the electrophysiological properties of retrograde labeled TGNs innervating the cornea (cTGNs). Using ‘sharp’ intracellular micropipettes, they observed that cTGNs were not a homologous neuronal population. The cells bodies could be identified as giving rise to C- or A δ -fibers based on the fibers’ conduction velocities. In addition, both types of neurons could be identified as either polymodal or mechanosensitive. The modality of the cells correlated well with the presence or absence of an inflexion (“hump”) during the repolarization phase of an AP see also Belmonte & Gallego (1983). The occurrence of this hump was correlated with neurons that were polymodal, just as its absence corresponded to mechanosensitive neurons. Ninety-one percent of neurons with conduction velocities compatible with C-fibers had a hump during the action potential (AP) repolarization phase; conversely, only 68% of the neurons with fast conduction velocities, compatible with A δ -fibers, also showed humped APs. Mechanosensory neurons give rise exclusively to A δ -fibers, while polymodal neurons are associated with either A δ or C-fibers.

The electrophysiological and chemosensitivity properties of acutely isolated cTGNs of the rat have not been reported. Our goals in this study are to characterize rat corneal sensory nerve cell bodies and compare their properties with corresponding sensory nerve terminals in the cornea (Gover et al., 2003), and with mouse corneal sensory nerve cell bodies (Lopez de Armentia et al., 2000). Using patch-clamp technique we examined the electrophysiological properties of isolated, dye-labeled, primary sensory afferent cell bodies. This approach allows us to make direct comparisons of physiological and pathophysiological profiles between peripheral nerve terminals and the cell bodies that give rise to those nerve terminals. If the profiles of these two cellular compartments are similar to one another, larger more accessible cell body can be used as a surrogate preparation for the study of the putative currents and receptors of the nerve terminal. In addition, numerous properties of target-identified primary sensory cell bodies can be compared with non-labeled neurons to determine whether identified cells possess distinct characteristics.

The purpose of the current work was three-fold. First, we wanted to utilize a retrograde fluorescent labeling technique to identify cTGNs that would minimize damage to the corneal nerve terminals. The fluorogold method to label cTGNs used by Lopez de Armentia and colleagues (Lopez de Armentia et al., 2000) required *n*-heptanol wounding of the corneal epithelium in order to allow dye access to CNTs. We developed a technique that retrogradely labels cTGNs without the need to damage the cornea surface. A small styryl dye, FM1–43 that can enter neurons through non-selective cation channels such as TRPV1 (Meyers et al., 2003), was utilized. Second, we wanted to apply patch-clamp recording techniques to determine whether isolated cTGNs possessed electrophysiological properties distinct from unlabeled, non-corneal TGNs. Finally, we wanted to discern whether cTGNs had chemosensitive response profiles similar to those of CNTs.

Our result showed that, although cTGNs and unlabeled TGNs share the same passive and active properties, they have a distinct chemosensitivity profile.

EXPERIMENTAL PROCEDURE

Retrograde Labeling Method

Male Sprague Dawley rats (120–200g) were anesthetized with 60 mg kg⁻¹ ketamine and 10 mg kg⁻¹ xylazine (i.p.). A small piece of surgifoam (2 mm in dia., Johnsons & Johnsons, New Brunswick, NJ, USA) saturated with 5 mM FM1–43 (Biotium Inc, Hayward, CA, USA) diluted in DMSO, was carefully placed in the center of the cornea to retrogradely label TG sensory cell bodies whose axons innervate the cornea. After 20–40 min, the surgifoam was removed and the eyes were repeatedly washed with warm (30°C) phosphate buffered saline (PBS). After a period of 4–5 days, rats were killed by CO₂ inhalation as approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore.

Trigeminal neuron dissociation

The TG were removed and maintained in a cold (4–5°C) Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution (HBSS; Gibco BRL, Rockville, MD, USA) until dissociation. TGs were dissociated enzymatically with HBSS containing collagenase type IA (1mg ml⁻¹, Sigma, St Louis, MO, USA) and dispase II (1 mg ml⁻¹, Boehringer Mannheim, Mannheim, Germany). After 2 hr of incubation (37°C), the ganglia were subjected to mechanical dissociation with fired-polished Pasteur pipettes. After three washes in L15 medium (Gibco BRL, Rockville, MD, USA) containing heat inactivated 10 % fetal bovine serum (FBS; Gibco BRL, Rockville, MD, USA), cell suspensions were transferred to circular 25 mm glass coverslips (Fisher, Newark, DE, USA) coated with poly-D-lysine (0.1 mg ml⁻¹; Sigma, St Louis, MO, USA) and incubated at 37°C for 2 hr. Dishes containing coverslips were then transferred to a room temperature incubator and stored for up to 3 days. About 1–4 neurons per coverslip were labeled with FM1–43.

Whole-cell patch-clamp recording

Whole cell patch-clamp techniques were used in conjunction with an Axopatch 200B amplifier, Digidata 1322A interface, and pCLAMP-9 software (Axon Instruments, Union City, CA, USA). Patch pipettes (1–4 MΩ) were filled with an intracellular solution (mM): 135 KCl, 10 NaCl, 2 MgCl₂, 1 CaCl₂, 10 N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (HEPES), 11 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 2 Mg-ATP, and 1 Li-GTP; pH 7.3 adjusted with KOH, 324 mosmol l⁻¹. Pipette voltage offset was neutralized prior to the formation of a GΩ seal. Membrane input resistance (*R*_{in}), series resistance (*R*_s), and capacitance (*C*_m) were determined from current transients elicited by 5 mV hyperpolarizing voltage steps from a holding potential of –60 mV using the membrane test application of pCLAMP-9. Capacitance compensation and 70% *R*_s compensation were used. In current-clamp mode, resting membrane potential was maintained at –60 mV. The identification of the AP hump was subjective. Threshold (TH, the most hyperpolarized potential at which the cell is able to fire an AP) was determined by injecting increments of depolarizing current (Δ of 50 pA) for 10 ms until the cell started to elicit AP. Coverslips were superfused (2–4 ml min⁻¹) continuously during recording with room temperature (22–24 °C) Locke solution (mM): 10 dextrose; 136 NaCl; 5.6 KCl; 1.2 MgCl₂; 2.2 CaCl₂; 1.2 NaH₂PO₄; 14.3 NaHCO₃ equilibrated with 95 % O₂-5 % CO₂; pH adjusted between 7.3 and 7.5. In experiments where we used nominal extracellular zero calcium we substituted MgCl₂ for CaCl₂. Retrogradely labeled TG neurons were identified by epifluorescence microscopy. Cells were excited by the output of a 100 W mercury arc lamp that passed through a 480 nm bandpass filter (30 nm bandwidth). Fluorescence

emission was passed through a 590 nm longpass filter before detection. The effects of bath applied ATP (100 μ M), serotonin (5-HT, 10 μ M), histamine (10 μ M), and muscimol (100 μ M) were studied using the voltage-clamp mode of the patch clamp technique. In order to monitor membrane conductance, ramp voltage commands were applied repetitively (from -70 to -50 mV, 0.2 mV ms^{-1} , 1 Hz). The neurons were considered responsive if the agonist evoked a current > 10 pA. The criteria for cell inclusion in the study were as follows: $R_s \leq 5$ M Ω , $R_m > 100$ M Ω , and stable recording with 70% R_s compensation throughout the experiment.

Statistical Analyses

All data is expressed as mean $\pm$ SEM and were analyzed by unpaired Student t-test. Statistical analyses were performed using Sigma Plot 2000 (Jandel Scientific Software, Chicago, IL, USA).

RESULTS

Retrograde Labeling of Trigeminal Ganglion Neurons

After 20 to 40 min of FM1–43 application to the center portion of the cornea, many corneal nerve terminals (CNTs) were labeled (Fig. 1A and B). The pattern of CNT labeling was similar to that observed using dextran-conjugated Oregon Green 488 BAPTA 1 (Gover et al., 2003), or to that revealed with immunostaining for substance P or CGRP (Miller et al., 1981; Jones and Marfurt, 1991). Four days after FM1–43 application, about one percent of the dissociated trigeminal ganglion neurons (TGNs) displayed FM1–43 fluorescence (Fig. 1C and 1D). This corresponded to about one to four labeled TGNs per coverslip or about 40 labeled dissociated TGNs for each FM1–43 stained cornea. The number of TGNs innervating the cornea (cTGN) range between 50 and 450 neurons depending upon the species (Muller et al., 2003); in the rat Marfurt et al. (1989) estimated about 143 TGNs project axons to the cornea. Thus, the approximately 30% labeling of the cTGNs observed in the present work is not unreasonable given an unknown loss of TGNs associated with the dissociation procedure and restricting dye application to the center of the cornea. We restricted dye application to the center of the cornea in order to minimize dye spread to limbal sensory nerve terminals. Extending the time period between dye application and cell dissociation from four to ten days did not measurably increase the number of labeled TGNs.

Electrophysiological Classification of Corneal Trigeminal Ganglion Neurons

Patch-clamp recording of labeled TGNs showed that cTGNs were not a homogenous population with respect to their electrophysiological properties. Two types of cTGNs could be distinguished by their action potential (AP) properties: 61% of the labeled TGNs (60/98, Table 1) had APs with an inflexion (hump) on the repolarization phase and relatively long durations, averaging about 3 ms at 0 mV (Fig. 2A, Table 2). These cTGNs were designated slow type neurons (S-neurons). The other 39% (38/98, Table 1) of the cTGNs, had shorter duration APs, averaging 0.6 ms and there was no hump on the falling phase of the AP (Fig. 2B). These neurons were designated fast type neurons (F-neurons). All the labeled TGNs studied could be classified as either F-neurons or S-neurons.

The two types of cTGNs had different passive and active membrane properties (Tables 1 and 2). The F-neurons were larger as measured by membrane capacitance (61 pF vs. 42 pF for F-neurons and S-neurons, respectively), had a significantly more polarized resting membrane potential (-59 mV vs. -54 mV for F-neurons and S-neurons, respectively), a more hyperpolarized undershoot (-67 mV vs. -56 mV for F-neurons and S-neurons, respectively), a lower AP threshold (-39 mV vs. -25 mV for F-neurons and S-neurons,

respectively), and required less current to reach spike threshold (7 pA/pF vs. 15 pA/pF for F-neurons and S-neurons, respectively).

The unlabeled TGNs could also be classified into F- and S-neurons. Their active and passive membrane properties were not significantly different from the properties of cTGNs (Tables 1 and 2).

TTX Sensitivity

To test the cTGN AP sensitivity to TTX, the AP TH was determined. TTX (100nM) was then perfused and the cells were examined for their ability to sustain an AP. APs recorded in corneal F-type neurons were abolished by bath application of TTX (4/4; 100 nM; Fig. 3B and Table 3). By contrast, 60% of APs recorded in corneal S-neurons were unaffected by TTX (6/10; Fig. 3D and Table 3), indicating TTX-resistant sodium currents (TTXr) likely participate in the generation of APs in some of the corneal S-neurons. Similar results were obtained with unlabeled TGNs, where all F-neurons APs were blocked by 100 nM TTX (4/4) and 57% of APs in the S-neurons were not affected by TTX (8/14).

Effect of Zero Extracellular Ca^{2+} on the AP Hump

To investigate the ionic mechanism underlining the inflexion (hump) during the repolarization phase of corneal S-neurons, we measured the hump in normal Locke's solution and then in Locke's solution with nominally zero Ca^{2+} , where MgCl_2 was substituted for CaCl_2 (Fig. 4). In 57% (11/20) of corneal S-neurons, the hump was completely abolished in the absence of extracellular Ca^{2+} (Fig. 4D). In corneal F-neurons, nominally zero extracellular Ca^{2+} reduced the AP overshoot (data not shown).

We tested whether a relationship existed between AP duration, the Ca^{2+} dependence of the repolarization hump and the TTX sensitivity of the AP in corneal S-neurons. The data shown in Fig. 5 indicate that most corneal S-neurons with short AP durations (< 2 ms) were TTX-sensitive (TTXs; 3/4) and their humps were unaffected by nominally zero extracellular Ca^{2+} (4/5). In contrast, most corneal S-neurons with AP durations > 2 ms were resistant to 100 nM TTX (TTXr; 5/6) and their humps were abolished by nominally zero extracellular Ca^{2+} (7/8). Thus, there seems to be two populations of corneal S-neurons based upon their AP duration, their sensitivity to TTX, and the sensitivity of the hump to low extracellular Ca^{2+} . The majority (14/21) of corneal S-neurons had long duration APs (> 2ms), Ca^{2+} sensitive, humps and TTXr APs, suggesting that Ca^{2+} influx participates in the development of the hump and that TTXr sodium currents could be responsible for AP generation in the same neuronal population. On the other hand, corneal S-neurons with faster APs (< 2 ms) were mostly TTXs and their humps were unaffected by changes in extracellular Ca^{2+} . In these cells, it is likely that the hump on the repolarizing phase of the AP was due to currents other than TTXr sodium or Ca^{2+} currents.

Capsaicin Sensitivity

We tested capsaicin sensitivity of corneal S-neurons and F-neurons by bath application of 1 μM capsaicin while monitoring membrane potential. The membrane potential of the F-neurons was unaffected by capsaicin application (0/3; Fig. 6B and Table 3), but the majority (84%, 16/19) of the S-neurons showed a robust membrane depolarization in response to capsaicin (Fig. 6D and Table 3). The distribution of capsaicin responses was similar in unlabeled TGNs. None of the unlabeled F-neurons were affected by capsaicin (0/4), while most of unlabeled S-neurons were depolarized by capsaicin application (82%, 9/11; Table 3).

Chemosensitivity

We determined whether cTGNs were sensitive to a panel of agonists. Accordingly, we tested the responses of cTGNs and unlabeled TGNs to bath applied ATP (100 μ M), serotonin (5-HT, 10 μ M), histamine (10 μ M), or muscimol (100 μ M). TGNs were voltage-clamped and repetitively (1 Hz) depolarized with a ramp protocol (from -70 to -50 mV, with a slope of 0.2 mV ms^{-1}) from a holding potential of -60 mV. The neurons were considered responsive if test substance evoked a current > 10 pA. No corneal S-neurons (0/12) responded to bath applied ATP. By contrast, 33% (5/15) of unlabeled S-neurons generated inward currents in response to ATP (Table 4). Most corneal and unlabeled F-neurons were activated by ATP (75%; 8/10 and 80%; 3/4), respectively (Table 4).

A higher percentage of unlabeled TGNs responded to serotonin (47%; 7/15 and 75%; 3/4 for S-neurons and F-neurons, respectively) when compared with corneal TGNs (27%; 3/11 and 10%; 1/10 for S-neurons and F-neurons, respectively). All TGNs were unresponsive to histamine with the exception of one unlabeled S-neuron (1/25). On the other hand, the vast majority of both corneal and unlabeled TGNs responded to muscimol (Table 4). All the corneal TGNs responded to muscimol (4/4 S-neurons and 3/3 F-neurons), and 87% (13/15) of unlabeled S-neurons and 75% (3/4) of unlabeled F-neurons responded.

DISCUSSION

There were three major goals of this work: to develop a retrograde fluorescent labeling technique to identify corneal TGNs (cTGNs) that would minimize damage to the corneal nerve terminals; to determine whether cTGNs innervating the cornea of the rat expressed a unique set of electrophysiological properties that may be distinct from other TGNs; and finally, to assess whether cTGNs possessed chemosensitivity properties similar to those possessed by corneal nerve terminals (CNTs).

FM1-43 labeling of cTGNs

To identify cTGNs, we utilized a retrograde labeling technique that minimized damage to the cornea. Previous electrophysiological studies of cTGNs relied upon a retrograde labeling procedure that injured the corneal epithelium in order to label sensory nerve terminals (Lopez de Armentia et al., 2000). In the current work, we applied the styryl pyridinium dye FM1-43 directly to the surface of the cornea. FM1-43 enters peripheral sensory nerve terminals through mechanotransducing, TRPV1, and P2X₂ channels (Meyers et al., 2003). These nonselective cation channels are known to exist in most corneal nerve terminals (Belmonte and Gallar, 1996; Gover et al., 2003; Vulchanova et al., 1998) and probably underlie the basis for staining corneal nerve terminals and cTGNs by FM1-43. Corneal sensory nerve terminals respond to capsaicin (Gover et al., 2003), a TRPV1 agonist, indicating that the nerve ending where the FM1-43 was applied has capsaicin receptors even if some of the cell bodies do not. Comparison of passive and active membrane properties between FM1-43 labeled cTGNs and unlabeled TGNs (Tables 1 and 2) revealed no evidence that FM1-43 altered any of the electrophysiological properties of labeled neurons. Thus, we believe the current retrograde labeling method produced minimal changes in the cTGNs.

Electrophysiological properties of cTGNs

The rat cTGNs were a heterogeneous population of neurons with respect to their passive and active electrical membrane properties. More than half (60%) of the cTGNs had long duration action potentials (APs, > 1 msec) with an inflexion on the repolarization phase of their APs. These neurons were designated slow neurons (S-neurons). The cTGNs that had short duration APs (< 1 msec) with no obvious inflexion on the repolarization phase of their

AP were designated fast neurons (F-neurons). F-neurons possessed a more hyperpolarized resting membrane potential and greater capacitance than S-neurons. They also had a lower AP threshold, lower rheobase, and their APs were always abolished by 100 nM TTX. By contrast, about half of the S-neurons (40%) were resistant to TTX (TTXr) indicating that corneal S-neurons are not a homogeneous cell population. They could be electrophysiologically segregated by a differential proportion of voltage-gated sodium channels. Neurons with AP abolished by 100nM TTX (TTXs) probably have a higher density of Na_v 1.6 and 1.7, sodium channel isoforms sensitive to TTX present in the peripheral neurons; while neurons with AP unaffected by TTX most likely would have a higher density of Na_v 1.8 and 1.9, sodium channels resistant to TTX (Goldin, 2001; Schild et al., 2005).

In another study, cTGNs recorded in the intact TG of the mouse could also be segregated into F-and S-type neurons based upon the duration of their AP. Eighty-six percent of mouse cTGNs were classified as S-type neurons, and of these 60% were unaffected by TTX (Lopez de Armentia et al., 2000). The percentage of S-neurons and their sensitivity to TTX in the rat cTGNs was smaller than in the mouse cTGNs perhaps reflecting differences in species, recording conditions, and/or effects of dissociation. The presence of an inflection on the repolarizing phase of an AP and the TTX resistance of an AP are characteristics often associated with primary sensory neurons that are classified as polymodal nociceptors (Djouhri et al., 2003; Fang et al., 2005). It is noteworthy that the majority of CNTs have been classified as polymodal nociceptors (Belmonte and Gallar, 1996).

In S-neurons with longer APs (>2 ms, 11/20 neurons) the hump was abolished by the removal of external Ca²⁺ and their APs were TTXr. We, therefore, conclude that in S-neurons with long duration APs, the hump is generated by Ca²⁺ influx through voltage dependent Ca²⁺ channels (VDCCs). Although the exact mechanism by which Ca²⁺ generated the AP hump is not known, two mechanisms could occur. Calcium influx through VDCC could generate enough current to produce the hump, or the rise in intracellular calcium via VDCC could activate a signal pathway capable of modulating ion channels, chloride channels for instance. Modification of the intracellular buffer capability could help differentiate between these two mechanisms. A fast calcium buffer such as BAPTA would block the hump if Ca²⁺ is activating a signal pathway; however, if the calcium influx is directly responsible for the hump a change in the intracellular buffer capability would not change the hump. Because TTXr sodium channels have a unique set of biophysics properties when compared with TTXs, such as slow activation and slow inactivation, the presence of TTXr sodium channels in these neurons may support a longer depolarization. A longer depolarization would permit the recruitment of a high percentage of VDCCs in the APs and as a consequence a Ca²⁺ conductance could directly shape the AP. TGNs possess a plethora of voltage-dependent Ca²⁺ channels including T-, L-, N-, P-/Q- and R-type (Ikeda and Matsumoto, 2003). In this context, it is noteworthy that cTGNs with long duration APs possess both L- and N-type VDCC currents (Gover et al., *in press*).

In corneal S-neurons with AP durations between 1 and 2 ms, the hump was unaffected by removing Ca²⁺ from the Locke solution. Further, their APs were TTXs, indicating that the hump in those neurons was neither due to Ca²⁺ current nor to a TTXr current. It is likely that a different expression of voltage-gated potassium channels resident in TGNs (Seifert et al., 1999) could underlie the hump in fast S-neurons. Indeed, in small diameter TGNs the inhibition of delayed rectifier potassium current causes the formation of a pronounced hump in the repolarization phase of the AP (Yoshida and Matsumoto, 2005). Therefore, if a subset of neurons expressed less of this specific class of potassium channel, it is possible that a hump in the AP is due to a potassium current, instead of VDCC or TTXr sodium channel. In

summary, the ionic mechanism responsible for the inflection on the repolarization phase of the AP is not uniform among cTGN S-neurons.

Chemosensitivity of cTGNs

The sensitivity to ATP, serotonin, histamine, muscimol, and capsaicin was determined in cTGNs and compared to unlabelled TGNs. After examining the sensitivity to agonists, capsaicin was applied. There were no obvious correlation between capsaicin sensitivity and the sensitivity of other agonist tested. ATP elicited an inward current in ~ 80% of F-neurons of both corneal and unlabelled TGNs. By contrast, ATP failed to produce a detectable current in corneal S-neurons. ATP elicited an inward current in only 33% of unlabelled S-neurons. It has been postulated that sensory afferent nerve terminals, upon stimulation, release ATP onto surrounding corneal epithelial cells (Kimura et al., 1999). However, functional purinergic receptors on CNTs have not yet been reported (Dowd et al., 1997). The current work demonstrates that a subset of cTGNs, F-neurons, have functional purinergic receptors. Because of their short duration APs, large size and TTX sensitivity these F-neurons might correspond to mechanosensitive afferents. We used a single concentration of ATP, 100 μ M, to investigate the sensitivity of TGNs to ATP. This concentration is considered a supramaximal concentration for activating P2X type purinergic receptors (Dunn et al., 2001). Our observation that some TGNs respond to ATP while none of the S-type cTGNs were responsive indicates that S-type cTGNs have few, if any, P2X receptors.

The vast majority of TGNs responded to bath application of muscimol, a GABA_A agonist, with a robust inward current. Functional GABA_A receptors have not yet been characterized on CNTs. In contrast, functional GABA_A receptors are present on peripheral sensory nerve terminals, central nerve terminals, and cell bodies of primary somatosensory neurons (Carlton et al., 1999; Ashworth-Preece et al., 1997; for review see MacDermott et al., 1999). At some peripheral sensory nerve terminals low concentrations of GABA, acting via GABA_A receptors, depolarizes the nerve terminal and reduce AP amplitude. The altered AP may evoke altered amounts of algogenic substances from the peripheral nerve terminals (Carlton et al., 1999).

In our survey of TGNs, only one unlabeled S-neuron (1/25) responded to histamine; none of the cTGNs responded. Our results suggest that TGNs in the rat have few functional histamine receptors. Because histamine responses have been reported in some guinea pig TGNs, this may reflect species variation (Hutcheon et al., 1993). After inflammation, however, functional histamine receptors may be up-regulated. Capsaicin-sensitive nasal trigeminal sensory nerve terminals are normally unresponsive to histamine but become very responsive to histamine following an acidic challenge to the nasal mucosa (Sekizawa et al., 1998).

Serotonin receptors exist in TGNs (Longmore et al., 1997; Smith et al., 2002) and their nerve terminals, including corneal nerve endings (Potrebic et al., 2003). Some of the cTGNs responded to serotonin (27% of S-neurons and 10% of the F-neurons). These values were lower than those for unlabeled TGNs (47% and 75% for S- and F-neurons, respectively). Due to the rapid nature of the inward currents evoked by serotonin in cTGNs, we assume that these responses were mediated by 5HT₃ ionotropic receptors. It is not conclusively known whether 5HT₃ receptors exist in CNTs, but CNTs stain positive for 5HT_{1D} metabotropic serotonin receptors (Potrebic et al., 2003).

Comparison between cTGNs and corneal nerve terminals (CNTs)

Nociceptive nerve terminals in the cornea are unresponsive to a P2X purinoceptor agonist (Dowd et al., 1997) or to histamine (MacIver and Tanelian, 1993). Our observation that ATP

and histamine do not evoke measurable responses in any the S-neurons is in keeping with these reports. ATP, but not histamine, however, did active some corneal F-neurons. cTGNs also mirror CNT chemosensitivity with respect to vanilloid agonists. Almost all the S-neurons (84%) were depolarized by capsaicin; none of the F-neurons tested were responsive. In CNTs, the vast majority of terminals are excited by capsaicin (Belmonte et al., 1991; Gover et al., 2003). We cannot yet judge whether responses to GABA or 5-HT in cTGNs also occur in CNTs because it is not yet known whether CNTs are excited by these signal molecules. Nonetheless, where comparisons can be made, there seems to be a correlation between the chemosensitivity of cTGNs and CNTs. Thus, it would appear that labeled cTGNs maybe an attractive model system for in-depth cellular, biophysical, and pharmacological studies of the actions of various endogenous mediators on CNTs. In the same vein, the cTGNs might also be a tractable model system to screen and test reagents that may have therapeutic actions on CNTs.

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ABBREVIATION

5-HT	Serotonin
5-HT1D	Serotonin receptor 1 D
5-HT3	Serotonin receptor 3
AP	Action potential
ATP	Adenosine triphosphate
BAPTA	(1,2-bis(o-aminophenoxy)ethane- <i>N,N,N',N'</i> -tetraacetic acid)
CGRP	Calcitonin-gene related peptide
Cm	Capacitance
CNTs	Corneal nerve terminal
cTGN	Corneal trigeminal ganglion neuron
DMSO	Dimethyl Sulfoxide
EGTA	Ethylene glycol-bis(β -aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid
GABA_A	Gamma-aminobutyric acid receptor type A
HBSS	Hanks balance solution
HEPES	10 <i>N</i> -[2-hydroxyethyl]piperazine- <i>N'</i> -[2-ethanesulphonic acid]
P2X	Purinergic receptor type 2X
R_{in}	Input resistance
R_s	Series resistance
TGN	Trigeminal ganglion neuron
TH	Threshold
TRPV1	Transient receptor potential vanilloid 1
TTX	Tetrodotoxin

TTXr	Tetrodotoxin resistant
TTXs	Tetrodotoxin sensitive
VDCC	Voltage dependent Ca ²⁺ channel

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Figure 1. FM1-43 labeling of rat corneal sensory neurons. **A.** Fluorescence image 40 min after applying a sponge saturated with FM1-43 to the center of the cornea. **B.** Same cornea observed with bright field and fluorescence. **C.** Corneal trigeminal sensory neuron retrogradely labeled with FM1-43 applied to the center of the cornea. **D.** Bright field and fluorescence image; about 1% of the total isolated trigeminal ganglia neurons showed FM1-43 labeling.

Figure 2.

Two types of trigeminal corneal sensory neurons based upon their action potential durations. Action potentials (APs) were evoked by 5 ms depolarizing incremental current pulses, starting with 20 pA pulses. **A.** Fast type neurons have relatively short duration APs and a spike threshold near -38 mV. **B.** Slow type neurons have longer AP durations and a hump on the repolarization phase of the spike. AP threshold was near -25 mV. The insert shows the current protocol used. Unlabeled trigeminal ganglia neurons could also be classified into fast and slow type. Calibration is shown in panel A. Arrows indicate 0 mV.

Figure 3.

Fast and slow type of corneal sensory trigeminal ganglion neurons have different TTX sensitivity. Action potentials (APs) were evoked by applying 500 ms depolarizing current pulses. **A.** Control fast type neuron (F-neuron). **B.** Same neuron recorded in the presence of 100 nM TTX for 1 min. The AP recorded in all F-neurons, labeled and unlabeled, were blocked by TTX. **C.** Control slow type neuron (S-neuron). **D.** Same cell recorded in the presence of 100 nM TTX. The APs in 60% of the S-neurons were unaffected by TTX application. Similar results were obtained with unlabeled S-neurons. Arrows indicate 0 mV.

Figure 4.

Effect of reducing extracellular calcium on the hump on the repolarization phase of the action potential in S-neurons. **A.** Control action potential (AP) recorded from a F-neuron. **B.** Same neuron as in A recorded in an extracellular solution with nominally zero Ca^{2+} . Lowering the extracellular Ca^{2+} did not appreciably affect the AP waveform in this and other F-neurons. **C.** Control AP recorded from a S-neuron. **D.** Same neuron as in C recorded in a nominally zero Ca^{2+} extracellular solution. The hump on the repolarization phase of the AP was abolished. dV/dt of the APs is shown below the corresponding APs in A–D. Calibrations in shown in A apply to B–D.

Figure 5.

Relationship between action potential duration, sensitivity of action potential hump to zero extracellular calcium, and AP sensitivity to TTX in S-type cTGNs. **A.** Relationship between action potential (AP) duration and zero calcium effect. The humps in neurons with AP duration longer than 2 ms (recorded at 0 mV) were abolished by nominally zero extracellular Ca^{2+} , with one exception. The humps in neurons with AP durations between 1 and 2 ms were unaffected by reducing extracellular Ca^{2+} , with the exception of one neuron. **B.** Relationship between AP duration and TTX sensitivity. Neurons with AP durations longer than 2 ms were unaffected by 100 nM TTX, with the exception of one neuron.

Neurons with AP durations between 1 and 2 ms were sensitive to TTX; their APs were abolished by the perfusion of 100 nM TTX, with the exception of one neuron.

Figure 6.

Sensitivity of trigeminal corneal sensory neurons to capsaicin. After determining whether labeled neurons were fast or slow type, they were superfused with 1 μ M capsaicin. **A.** Action potential (AP) recorded in an F-neuron. **B.** Same neuron as in A, perfused with capsaicin; membrane potential held at -60 mV. The cell did not respond to a 40 sec application of capsaicin. Similar results were obtained with unlabeled F-neurons. **C.** AP recorded in a S-neuron. **D.** Same cell as in C perfused with capsaicin; resting membrane potential was -60 mV prior to capsaicin application. Capsaicin depolarized the S-neuron to spike threshold. Similar results were obtained with unlabeled S-neurons. APs evoked by 10 ms depolarizing current pulses. Lower horizontal line in B and D shows time of capsaicin application.

Table 1**PASSIVE PROPERTIES OF ISOLATED TRIGEMINAL GANGLION NEURONS**

	Type	<i>Cm</i> (pF)	<i>Rm</i> (MΩ)	<i>Em</i> (mV)
Cornea Sensory Neurons	Slow (n=60)	422.6	256 ± 21.8	-53 ± 0.9
	Fast (n=38)	61 ± 4.5 *	196 ± 20.4	-59 ± 0.7 *
Unlabeled Trigeminal Neuron	Slow (n=14)	49 ± 9.5	208 ± 27.7	-48 ± 3.1
	Fast (n=4)	54 ± 1.0 *	134 ± 14.9	-59 ± 0.6 *

Cm, cell membrane capacitance; *Rm*, cell membrane input resistance; *Em*, resting membrane potential. Values are expressed as mean ± s.e.m.

* significant difference (p<0.05) between slow-type and fast-type neurons. Slow and fast characterization is based upon action potential duration (see text). There was no significant difference between labeled and unlabeled slow- or fast-type neurons.

Table 2**ACTIVE PROPERTIES OF ISOLATED TRIGEMINAL GANGLION NEURONS**

	Type	Duration (ms)	Overshoot (mV)	Threshold (mV)	Rheobase (pA/pF)	AP Blocked by TTX
Cornea Sensory Neurons	Slow (n=21)	2.7 ± 0.23	46.2 ± 3.07	-25.2 ± 1.63	14.9 ± 1.94	60% (6/10)
	Fast (n=9)	0.6 ± 0.05 *	48 ± 4.05	-38.6 ± 0.84 *	6.8 ± 1.02 *	100% (4/4)
Unlabeled Trigeminal Neuron	Slow (n=14)	2.5 ± 0.3	43 ± 4.0	-30 ± 2.4	11 ± 1.8	57% (8/14)
	Fast (n=4)	0.6 ± 0.2 *	46 ± 1.6	-45 ± 1.7 *	7 ± 2.3 *	100% (4/4)

Action potential duration (AP) measured at 0 mV; Overshoot, most positive membrane potential during a single evoked AP; rheobase, minimum current necessary to evoke an AP normalized to cell size (pF); AP blocked by the perfusion of 100 nM TTX. Values are expressed as mean ± s.e.m.

* significant difference ($p < 0.05$) between slow-type and fast-type neurons. There was no significant difference between labeled and unlabeled slow- or fast-type neurons.

Table 3
CHEMOSENSITIVITY OF ISOLATED TRIGEMINAL GANGLION NEURONS

	Type	ATP (100 μ M)	5-HT (10 μ M)	Histamine (10 μ M)	Muscimol (100 μ M)	Capsaicin (1 μ M)
Cornea Sensory Neurons	Slow	0% [*] (0/12)	27% [*] (3/11)	0% (0/5)	100% (4/4)	84% (16/19)
	Fast	80% (8/10)	10% [*] (1/10)	0% (0/7)	100% (3/3)	0% (0/3)
Unlabeled Trigeminal Neuron	Slow	33% (5/15)	47% (7/15)	10% (1/10)	87% (13/15)	82% (9/11)
	Fast	75% (3/4)	75% (3/4)	0% (0/3)	75% (3/4)	0% (0/4)

Percentage and number of neurons showing an inward current in response to agonist application. Capsaicin responsive cells were depolarized by 1 μ M capsaicin.

^{*} denotes significant difference between the corneal sensory neurons and the unlabeled trigeminal ganglion neurons. The slow-type corneal sensory neurons did not respond to ATP while 33% of the slow-type unlabeled TG neurons responded. A higher percentage unlabeled TG neurons, slow- and fast-type, responded to 5-HT, than corneal sensory neurons. There were no differences in responsiveness to histamine (10 μ M) or muscimol (10 μ M) between labeled and unlabeled neurons or between fast-type and slow-type.