Electrophysiological Evidence for Tetrodotoxin-Resistant Sodium Channels in Slowly Conducting Dural Sensory Fibers

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Strassman, A. M. and S. A. Raymond. Electrophysiological evidence for tetrodotoxin-resistant sodium channels in mechanosensitive nerve endings of slowly conducting fibers in the intracranial dura. J. Neurophysiol. 81: 413–424, 1999. A tetrodotoxin (TTX)-resistant sodium channel was recently identified that is expressed only in small diameter neurons of peripheral sensory ganglia. The peripheral axons of sensory neurons appear to lack this channel, but its presence has not been investigated in peripheral nerve endings, the site of sensory transduction in vivo. We investigated the effect of TTX on mechanoresponsiveness in nerve endings of sensory neurons that innervate the intracranial dura. Because the degree of TTX resistance of axonal branches could potentially be affected by factors other than channel subtype, the neurons were also tested for sensitivity to lidocaine, which blocks both TTX-sensitive and TTX-resistant sodium channels. Single-unit activity was recorded from dural afferent neurons in the trigeminal ganglion of urethan-anesthetized rats. Response thresholds to mechanical stimulation of the dura were determined with von Frey monofilaments while exposing the dura to progressively increasing concentrations of TTX or lidocaine. Neurons with slowly conducting axons were relatively resistant to TTX. Application of 1 μM TTX produced complete suppression of mechanoresponsiveness in all (11/11) fast A-d units (conduction velocity (c.v.) 5–18 m/s) but only 50% (5/10) of slow A-d units (1.5 <c.v.< 5 m/s) and 13% (2/15) of C units (c.v. ≈1.5 m/s). The mean TTX concentration that produced complete suppression of mechanoresponsiveness was ~270-fold higher in C units than in fast A-d units. In contrast, no significant difference was found between C and A-δ units in the concentration of lidocaine required for complete suppression of mechanoresponsiveness, indicating that the greater TTX resistance of mechanoresponsiveness in C units is not attributable to differences in safety factor unrelated to channel subtype. These data offer indirect evidence that a TTX-resistant sodium current subtype is expressed in the terminal axonal branches of many of the more slowly conducting (C and slow A-δ) dural afferents. The channel appears to be present in these fibers, but not in the faster A-δ fibers, in sufficient numbers to support the initiation and propagation of mechanically induced impulses. Comparison with previous data on the absence of TTX resistance in peripheral nerve fibers suggests that the TTX-resistant sodium channel may be a distinctive feature of the receptive rather than the conductive portion of the sensory neuron’s axonal membrane.

INTRODUCTION

Small-diameter peripheral sensory neurons exhibit a distinctive type of voltage-gated sodium current that is resistant to blockade by tetrodotoxin (TTX) (Arbuckle and Docherty 1995; Bossu and Feltz 1984; Caffrey et al. 1992; Christian and Togo 1995; Elliott and Elliott 1993; Ikeda and Schofield 1987; Ikeda et al. 1986; Kostyuk et al. 1981; McLean et al. 1988; Ogata and Tatebayashi 1992a,b; Rizzo et al. 1994; Roy and Narahashi 1992; Roy et al. 1994; Stea and Nurse 1992, 1993; Yoshimura and De Groat 1996; Yoshimura et al. 1996), which blocks voltage-gated sodium current in most other central and peripheral neurons (Yoshida 1994). This sensory-neuron-specific TTX-resistant sodium current is both pharmacologically and kinetically distinct from sodium currents that have been described in other neural and nonneural cells (Yoshida 1994). Recently a sensory neuron-specific sodium channel (SNS/PN3) was cloned that is resistant to TTX and exhibits kinetics that match those of the TTX-resistant current of small-diameter sensory neurons (Akopian et al. 1996; Sangameswaran et al. 1996). Expression of the channel is highly restricted, in that it is present only in the small-diameter neurons of peripheral sensory ganglia (dorsal root and trigeminal and nodose ganglia) but not in the large diameter sensory neurons or in other peripheral or central neurons, glia, or nonneural tissues.

Because nociceptive neurons comprise the major population of small sensory neurons, the TTX-resistant sodium current may be important in the transmission of sensory signals evoked by painful stimuli. This idea is supported by the observations that TTX resistance is specific to nociceptive neurons (Ritter and Mendell 1992) and that the TTX-resistant current is enhanced by agents such as prostaglandin E2 that produce pain or hyperalgesia in vivo (England et al. 1996; Gold et al. 1996b). Consequently, the SNS/PN3 sodium channel has attracted great interest as a possible pharmacological target for producing a selective suppression of activity in nociceptive neurons. However, electrophysiological studies have typically found that TTX resistance is present in the cell bodies but not in the peripheral axons of small-diameter sensory neurons (Villiere and McLaughlan 1996; Yoshida and Matsuda 1979), an observation that was recently corroborated by immunohistochemical localization of the SNS/PN3 channel (Novakovic et al. 1998). This restricted cellular distribution of the TTX-resistant sodium channel raises some question about its potential role in nociception, since impulse activity in the cell bodies of pseudounipolar sensory neurons is not thought to be necessary for the propagation of sensory signals to the CNS.

No study has yet investigated the presence of TTX resistance in C-fiber peripheral nerve endings, the site of sensory transduction in vivo. Therefore we examined TTX resistance and its relation to fiber conduction velocity in mechanosensitive axonal branches of sensory neurons that innervate the intracranial dura. The dura receives a sensory innervation from A-δ and C
fibers originating in the trigeminal ganglion and is a convenient preparation for testing the effects of chemical agents on sensory nerve endings in vivo (Strassman et al. 1996). Because the degree of TTX resistance of axonal branches could potentially be affected by factors other than channel subtype that affect impulse conduction and generation, the selectivity of TTX for A-δ versus C-fiber neurons was compared with that of lidocaine, which blocks both TTX-sensitive and -resistant channels (Roy and Narahashi 1992).

METHODS

Surgical preparation and recording

Urethan-anesthetized male rats (400–600 g) were placed in a stereotaxic headholder. The right transverse sinus and the caudal part of the superior sagittal sinus were exposed by craniotomy. The exposed dura was bathed in a synthetic interstitial fluid (SIF) consisting of 135 mm NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM CaCl₂, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and 10 mM glucose, pH 7.2. Tungsten microelectrodes were advanced into the trigeminal ganglion by a dorsal approach through the cerebral cortex. Single-unit recordings were obtained from dural afferent neurons in the trigeminal ganglion that were identified by their constant latency response to single shock stimuli (0.5-ms pulses, 5 mA, 0.7 Hz) applied to the dura overlying the ipsilateral transverse sinus through bipolar stimulating electrodes (Strassman et al. 1996).

Identification of shortest latency site

Response thresholds and latencies to electrical stimuli were mapped at multiple sites across the surface of the exposed dura. The dural stimulation site associated with the shortest latency response was identified. The shortest latency site was typically on the transverse sinus at a distance of 3–6 mm from the midline, which corresponds to the superior sagittal sinus. In the experimental preparation, the brain and dura were intact, and recording microelectrodes were driven into the trigeminal ganglion while stimuli were applied to the dura overlying the transverse sinus.

FIG. 1. Drawing of structures within the rat cranium, with the dura partially cut away and the brain removed for purposes of illustration only. The tentorial nerve is shown exiting the trigeminal ganglion at the base of the skull and coursing caudally and dorsally up the tentorium to reach the outer dural surface of the transverse sinus. In the experimental preparation, the brain and dura were intact, and recording microelectrodes were driven into the trigeminal ganglion while stimuli were applied to the dura overlying the transverse sinus.

Identifying the site with lowest mechanical threshold

Neurons were then tested for responses to mechanical stimulation of the dura by stroking the surface of the dura with a blunt probe and indenting it with von Frey monofilaments. Only neurons for which a mechanical receptive field could be found were included in this study. For such neurons, the dura was probed with von Frey hairs of progressively decreasing intensities until the site or sites with the lowest von Frey threshold were located. The receptive field typically consisted of a single spot (<1 mm diameter) of lowest threshold, which was surrounded by an area of up to several millimeter radius from which responses could be evoked only by more intense stimuli. A few neurons exhibited two to four such spots separated by distances of ≤6 mm. After the spot(s) with the lowest mechanical threshold was identified, the distance of each of these spots from the shortest latency site (identified with electrical stimulation as described previously) was measured (±1 mm). This was used as a rough estimate of the length of the axon that coursed across the dura to the mechanosensitive ending and thus the length of axon that would be exposed to solutions applied topically to the dura (see Figs. 1 and 2). It should be noted that, as explained previously, the calculation of conduction velocity was determined from the site with the shortest response latency (which presumably corresponds to the site at which the main axon first reaches the dura) and thus does not reflect the conduction velocity of the axonal branches within the dura, which was generally much lower.

Testing of suppression of mechanical responses by lidocaine and TTX

For threshold determinations, individual von Frey hairs were applied three to six times at 5-s intervals (e.g., Figs. 3 and 4). The von Frey hairs were applied in increasing order until threshold was reached, defined as the lowest force that evoked a response in >50% of the stimulus applications. Baseline measurements of the von Frey threshold at the lowest threshold spot were made three or four times at ~10-min intervals.
Threshold measurements were then repeated in the presence of progressively increasing concentrations of lidocaine applied topically to the outer surface of the dura. Lidocaine concentration was increased until mechanical responsiveness was completely abolished, as defined by lack of response to 4-g stimulation, which was the highest intensity tested. Higher intensities were not used to avoid damage to the dura. The lidocaine concentrations used were 1.25, 2.5, 5, 10, 20, 40, and 100 mM, in SIF. Each concentration was initially applied for 10 min, and the threshold was then determined. If the threshold showed a change, the drug exposure was continued, and threshold measurements were repeated at 3- to 5-min intervals until no further changes were observed. (More frequent testing was not done to avoid stimulation-induced changes in sensitivity.) In most cases the drug effects were complete within 10 min. The next concentration in the test series was then applied, with no washout period between different concentrations. Recovery from lidocaine was determined by return to baseline von Frey threshold. After recovery from the lidocaine (typically 30 – 60 min), baseline measurements were again made, and the testing process was repeated with increasing concentrations of TTX (10^{-2} 8, 10^{-7} 5, 10^{-6} 2, and 10^{-5} M).

The effect of TTX on response threshold to electrical stimulation of the dura was also determined in some neurons. Threshold was determined with single-pulse stimuli delivered at 0.5 Hz through bipolar electrodes with a constant-current stimulus isolation unit that could deliver up to 10 mA at a compliance of 100 V (Winston Electronics). Pulse width was 0.02–0.1 ms for A-delta neurons and 0.1–0.5 ms for C neurons. Pulse width was chosen so that baseline threshold was ~500 uA. Baseline was determined as the mean of three to four measurements. Threshold measurement was repeated after suppression of mechanoresponsiveness by TTX.

**Data analysis**

For each neuron, two parameters of drug sensitivity were analyzed, 1) the lowest applied drug concentration that produced partial suppression of mechanoresponsiveness, defined as an increase in von Frey threshold, and 2) the lowest applied drug concentration that produced complete suppression of mechanoresponsiveness, defined as a lack of response to 4-g stimulation. For neurons with more than one mechanosensitive spot in their dural receptive field, partial suppression was considered to have occurred if any spot exhibited an increased threshold, whereas complete suppression was not considered to have occurred until all spots showed complete loss of responsiveness.

Because drugs were applied in log increments of concentration, log values of applied concentrations were used for statistical comparisons and correlations between TTX and lidocaine concentrations and conduction velocity (log_{10} for TTX and log_{2} for lidocaine and conduction velocity). For purposes of statistical analyses, neurons that did not show suppression at the highest drug concentrations applied were assigned a value 1 log unit higher (10^{-2} M for TTX and 200 mM for lidocaine). Analysis of variance (ANOVA) with the Bonferroni/Dunn posthoc correction for multiple comparisons was used for comparisons between fast A-delta, slow A-delta, and C-fiber neurons. \chi^2 was used for comparing the incidence of TTX resistance in the three classes of neurons. Fisher’s r-to-Z calculation was used to determine the signif-
RESULTS

General observations on suppression of mechanosensitivity by TTX and lidocaine

Extracellular unit recordings were obtained from 38 mechanosensitive dural afferent neurons in the trigeminal ganglion that were identified by their response to single shock stimulation of the dura overlying the ipsilateral transverse sinus. These included 16 C units (c.v. ≤ 1.5 m/s), 10 slow A-δ units (1.5 m/s < c.v. < 5 m/s), and 12 fast A-δ units (c.v. 5–18 m/s). All neurons in this study exhibited mechanical receptive fields on the dura overlying or immediately adjacent to the ipsilateral transverse sinus or the caudalmost part of the superior sagittal sinus (e.g., Fig. 2). Baseline response thresholds to dural stimulation ranged from 0.03 to 2.9 g (mean ± SD, 0.59 ± 0.73 g; median, 0.38 g), with no significant differences between C, slow A-δ, and fast A-δ units (P > 0.1, ANOVA).

The effect of TTX (n = 36) and lidocaine (n = 32), applied topically to the dura, was tested on the neurons’ responsiveness to mechanical stimulation of the dura. Thirty of these neurons were tested with both TTX and lidocaine. TTX (0.01–100 μM) produced a decrease in mechanoresponsiveness, as shown by an increase in response threshold, in 34 of 36 neurons. The remaining two neurons, which were both C units, showed no change in threshold at the highest TTX concentration applied (100 μM). Of the 34 neurons that showed an increased threshold, 31 showed a complete loss of responsiveness, and the remaining 3 neurons (all C units) continued to show some mechanoresponsiveness at the highest TTX concentration applied. The elevation in response threshold developed in a graded manner with increasing TTX concentration in 24 neurons (e.g., Figs. 3B, 4, and 5, A–C), whereas in the remaining 10 neurons the entire increase in threshold occurred at one increment in applied concentration (e.g., Figs. 3A and 5D).

Lidocaine (1.25–100 mM) produced an increase in mechanical response threshold in all neurons tested and a complete loss of mechanoresponsiveness in 27 neurons. The increase in threshold developed in a graded manner with increasing lidocaine concentration in 24 neurons (e.g., Figs. 3B, 4, and 5, A–C), whereas in the remaining 10 neurons the entire increase in threshold occurred at one increment in applied concentration (e.g., Figs. 3A and 5D).

Relationship of TTX sensitivity to conduction velocity

Neurons with slowly conducting axons were relatively resistant to TTX. Application of 1 μM TTX was sufficient to
produce complete suppression of mechanoresponsiveness in all (11/11) fast A-δ units (c.v. > 5 m/s) but only 50% (5/10) of slow A-δ units (1.5 ≤ c.v. ≤ 5 m/s) and 13% (2/15) of C units (c.v. < 1.5 m/s) (Figs. 6B and 7A; P < 0.001, χ²). The mean concentration that produced complete suppression was ~25-fold higher in C units than in slow A-δ units and 270-fold higher in C units than in fast A-δ units (ANOVA, P < 0.01, and P < 0.0001, respectively; mean ± SD in log units of molarity, -4.2 ± 1.2, -5.6 ± 1.0, and -6.6 ± 0.8, for C, slow A-δ, and fast A-δ, respectively). Figure 7A illustrates the graded relationship between TTX sensitivity and conduction velocity. The concentration of TTX that produced complete suppression of mechanoresponsiveness showed a strong inverse correlation with conduction velocity (r = -0.77, P < 0.0001).

Although mechanoresponsiveness was not completely suppressed in most C units with application of 1 μM TTX, this concentration produced at least partial suppression (i.e., increased response threshold) in 67% (10/15) of C units and 80% (8/10) of slow A-δ units (and complete suppression in 100% of fast A-δ units, as noted previously) (Fig. 6A). The TTX concentration that produced partial suppression of mechanoresponsiveness tended to be higher in more slowly conducting neurons, although the correlation with conduction velocity (r = -0.49, P < 0.01) was much weaker than that found for the concentration that produced complete suppression. The concentration that produced partial suppression was significantly higher in C units than in fast A-δ units (ANOVA, P < 0.05; -6.0 ± 1.7, -6.8 ± 1.2, and -7.5 ± 0.7, respectively, for C, slow A-δ, and fast A-δ in log units of molarity).

**Relationship of lidocaine sensitivity to conduction velocity**

Slowly conducting neurons tended to be more resistant to lidocaine, but this relationship was much weaker than that...
found for TTX (Figs. 6 and 7). The concentration of lidocaine required for complete suppression of mechanoresponsiveness was not significantly correlated with conduction velocity, but there was a nonsignificant trend for this concentration to be higher in more slowly conducting neurons (Fig. 7B; \( r = -0.33, P > 0.06 \)). There was no significant difference in this concentration between A-\( \delta \) and C units (\( P > 0.06, \) ANOVA). C units as a group showed a (nonsignificant) twofold greater resistance to lidocaine than fast A-\( \delta \) neurons, with respect to the applied concentration required for complete suppression.

The concentration of lidocaine that produced partial suppression of mechanoresponsiveness was weakly correlated with conduction velocity (\( r = -0.47, P < 0.01 \)). There was no significant difference in this concentration between A-\( \delta \) and C units (\( P > 0.06, \) ANOVA).

**Time course of TTX suppression**

The relative TTX resistance of C units could in principle result from differential access of TTX, as might occur if a diffusion barrier were present around C units but not A-\( \delta \) units (see DISCUSSION). In such a case, a temporary failure to observe suppression in C units could be explained by a relative delay in access that would disappear as exposure time was increased.

However, the time course of TTX suppression did not support this possibility. As noted previously, TTX did produce an increase in threshold in 87% of C units. In all cases, these increases in threshold occurred within 10 min of exposure and appeared to have reached an endpoint within a maximum of 15 min (Fig. 5, A–D), just as they did in A-\( \delta \) units. More prolonged exposures were never observed to produce further increases in threshold.

The absence of a progressive suppression of C units over time is illustrated in Fig. 5B by a C unit that continued to show mechanoresponsiveness after prolonged exposure to \( 10^{-6} \) M TTX, a concentration sufficient to produce complete suppression in 100% of fast A-\( \delta \) units. The neuron showed partial suppression after just 10 min of exposure to \( 10^{-8} \) M TTX but then showed no further increase in threshold after >6 h of exposure to a 100-fold increase in concentration (\( 10^{-4} \) M). In view of the rapid effect of a much lower concentration (\( 10^{-6} \) M), the lack of further suppression by \( 10^{-4} \) M cannot be explained by inadequate access or exposure time.

Figure 5, C and D, illustrates two C units that showed only partial suppression after exposure to TTX concentrations of \( \geq 10^{-6} \) M for 50 and 69 min, respectively. As in all neurons, the increases in threshold could be observed within 10 min of a concentration increment, and there was no tendency for progressive threshold increases with exposures times >15 min.

**Exposure length**

In studies of differential sensitivities of nerve fibers to local anesthetics, the concentration required to produce impulse blockade depends strongly on the length of the axonal segment that is exposed to the drug (Raymond et al. 1989). In this study the length of exposed axon could not be controlled, but an estimate of this length was made for each neuron by measuring the distance from the mechanosensitive site on the dura to the site at which the main axon appeared to reach the dura in its
course from the tentorium below (based on measurements of response latency to single shock stimuli, as described in the METHODS). A negative correlation was found between the length of the axonal branch that was exposed to the drug and the applied concentration required for complete suppression of mechanoreponsiveness for both TTX (Fig. 8A; \( r = -0.57, P < 0.001 \)) and lidocaine (Fig. 8B; \( r = -0.58, P < 0.001 \)).

To determine whether this effect of exposure length on drug sensitivity might contribute to the differences in drug sensitivity between C- and A-\( \delta \) fibers, the relationship between exposure length and conduction velocity was examined. There was no significant difference in exposure length between A-\( \delta \) and C fibers (ANOVA, \( P > 0.1 \)) and no significant correlation between exposure length and conduction velocity. However, there was a trend for slowly conducting fibers to have shorter exposure lengths which, although not significant \( (r = -0.34, P > 0.05) \), might still contribute to the higher TTX resistance of C fibers. Therefore partial correlations were calculated between exposure length, conduction velocity, and TTX sensitivity (applied concentration of TTX required for complete suppression of mechanosensitivity) to remove the effect of exposure length from the correlation between conduction velocity and TTX sensitivity. The correlation between conduction velocity and TTX sensitivity was only slightly reduced by this calculation \( (r = -0.74) \). Stepwise regression analysis indicated that differences associated with conduction velocity...
accounted for 59% of the variance in TTX sensitivity, and differences in exposure length accounted for an additional 11%.

Sensitivity to TTX was significantly correlated with sensitivity to lidocaine (Fig. 7C; $r = 0.59$, $P < 0.001$). Partial correlation analysis indicated that approximately one-third of this correlation was accounted for by the effect of exposure length on drug sensitivity. Overall, the statistical analyses indicate that 1) TTX and lidocaine sensitivity were influenced by a number of common factors, one of which is exposure length, and 2) sensitivity to TTX, but not to lidocaine, was also strongly dependent on conduction velocity, which was more predictive of TTX sensitivity than all other factors combined.

Effect of calcium removal

Because calcium currents could in theory generate TTX-resistant impulses (see DISCUSSION), the effect of the removal of calcium from the bathing solution on mechanosensitivity was investigated in three neurons (2 C and 1 A-δ). Removal of calcium by application of calcium-free SIF with 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N′,N″,N‴-tetraacetic acid (EGTA) had no detectable effect on mechanical response threshold, although it produced an increase in ongoing discharge (in agreement with the results of Pozo et al. 1992 in corneal afferents). In one of these neurons, a C unit, TTX at $10^{-6}$ M was applied first in normal SIF and then for 40 min in calcium-free/EGTA SIF, with no change in von Frey threshold.

Effect of TTX on electrical stimulation thresholds

It is theoretically possible that the differential TTX sensitivity was entirely due to an action of TTX on mechanotransduction and that impulse conduction was not affected by TTX in either A-δ or C neurons. If this were the case, the threshold for electrical stimulation of the dura should be relatively unaffected by TTX concentrations that suppress mechanoresponsiveness. To test this possibility, response threshold to electrical stimulation of the dura was measured before and after suppression of mechanoresponsiveness in 6 fast A-δ neurons and 5 C neurons. The TTX concentrations that produced complete suppression of mechanoresponsiveness also produced a large elevation in electrical threshold (13.3 ± 10.8 and 8.3 ± 3.9, for A-δ and C neurons, respectively, expressed as multiples of baseline threshold), consistent with an effect on conduction. The responses to these elevated currents occurred at shortened latencies, indicating that they result from current spread to the parent axons in the underlying tentorial nerve, which was not exposed to the TTX (see DISCUSSION).

Discussion

Differential sensitivity to TTX

The results demonstrate a differential sensitivity to TTX according to conduction velocity and fiber classification in mechanosensitive dural afferent neurons. An applied TTX concentration of 1 μM, which blocks TTX-sensitive sodium channels in dorsal root ganglion cells, produced complete suppression of mechanoresponsiveness in all fast A-δ fibers (≥5 m/s) but not in most C fibers. The majority of C-fibers showed a partial suppression at 1 μM, but most C-fibers as well as many of the slower A-δ fibers required higher concentrations for complete suppression of mechanoresponsiveness. The concentration of TTX required for complete suppression of mechanoresponsiveness was ~270-fold higher in C fibers than in fast A-δ fibers.

A similar differential sensitivity to TTX has also been found in dorsal root ganglion, for impulses evoked by intracellular current injection. Most of the small ganglion cells are resistant to suppression by TTX concentrations (0.1–3 μM) that block activity in the larger cells (Caffrey et al. 1992; Yoshida and Matsuda 1979; Yoshimura et al. 1996). In dorsal root ganglion, the basis for this differential TTX sensitivity was determined in whole cell patch clamp studies of dissociated neurons, which showed that TTX-resistant sodium channels are present in most of the small ganglion cells, whereas larger cells possess only TTX-sensitive channels (Caffrey et al. 1992; McLean et al. 1988; Rizzo et al. 1994; Roy and Narahashi 1992; Yoshimura et al. 1996). Some small cells possess both TTX-sensitive and -resistant channels. The TTX-sensitive current is entirely blocked by TTX concentrations of <0.1 μM, whereas the TTX-resistant current is unaffected at 0.1 μM and requires concentrations of ≥60–100 μM for 50% suppression (Akopian et al. 1996; Elliott and Elliott 1993; Ogata and Tatebayashi 1993; Roy and Narahashi 1992; Sangameswaran et al. 1996).

Direct measurement of single-cell membrane currents has not been accomplished in small-diameter nerve fibers, so inferences about the presence of TTX-resistant channels in such fibers have had to rely on recordings of impulse activity. However, in nerve fibers the sensitivity of impulse activity to sodium channel blockers is affected by a number of factors in addition to sodium channel subtype, such as the locus and density of sodium channels and other channels, fiber geometry, and cable properties (Raymond and Gissen 1987). For example, myelinated fibers might be more susceptible to blockade as a result of the restricted distribution of sodium channels to nodal regions of the axonal membrane, with long internodes of inexcitable membrane. If, as a result of such factors, C fibers had greater conduction safety than A-δ fibers (Gissen et al. 1982) or stronger mechanical transduction, they could in principle exhibit a greater resistance to TTX without having TTX-resistant sodium channels. We assessed the importance of these other factors by determining the differential sensitivity of A-δ and C fibers to lidocaine, which acts on both TTX-sensitive and -resistant channels (Roy and Narahashi 1992). If the differential TTX sensitivity resulted from general differences in conduction safety or impulse generation, a comparable differential sensitivity should also be found with lidocaine. This was not the case. The difference in lidocaine sensitivity between A-δ and C fibers was <1% as large as the difference in TTX sensitivity. From this it would appear that factors other than channel subtype can account for only a small part of the differential TTX sensitivity.

Two additional factors should be noted that could potentially affect this comparison between TTX and lidocaine. First, concomitant blocking of potassium channels, which occurs with lidocaine and not with TTX, would partially offset the effect of sodium channel blockade. However, the predicted effect would be relatively small (Raymond 1992) and would tend to produce a greater differential block of A-δ over C fibers, given that potassium currents appear to be larger in C than in A-δ fibers.
Such an effect would be in the opposite direction needed to explain our results, which showed a far greater differential block of A-δ fibers by TTX than by lidocaine.

Second, differential sensitivity to TTX could in principle result from differential access, as might occur if a diffusion barrier to TTX were present around C units but not A-δ units. Any such barrier would have to selectively impede diffusion of TTX but not the relatively more lipophilic lidocaine, since there was little differential sensitivity to lidocaine. No such lipid barrier is known in any tissue, including the dura (Andres et al. 1987). Rather, it is A-δ fibers, not C fibers, that are known to have a lipid barrier (myelin). Furthermore, both TTX and lidocaine are thought to diffuse in the aqueous interstitial fluid rather than in lipid, and both have access to myelinated and unmyelinated fibers. Aside from these theoretical considerations, the time course of the TTX effects is incompatible with a selective failure of access of TTX to C fibers. To explain the data by differential access, there would have to be a much longer time course for any effect of TTX on C fibers as compared with A-δ fibers. This was not the case. TTX, even at concentrations of 10 μM, increased the threshold within 10 min of drug exposure in C fibers as well as A-δ fibers. These data are incompatible with the idea that TTX has significantly less access to C fibers than to A-δ fibers. Conceivably, C fibers might have one population of sodium channels that is highly accessible and another population along the same axons that is inaccessible, but such a novel hypothesis of spatially segregated channels that differ vastly in accessibility is generally invoked only for myelinated axons, not unmyelinated ones. In contrast, the alternative explanation of separate channel populations that differ in TTX affinity is consistent with the known properties of sodium channel subtypes in small-diameter sensory neurons.

The results of this study thus offer strong indirect evidence for the presence in dural C fibers of an ion channel that is resistant to TTX but not to lidocaine. In addition to a TTX-resistant channel, the majority of C-fibers also appear to possess TTX-sensitive sodium channels, since they showed partial suppression of mechanoresponsiveness with 1 μM TTX. However, the TTX-resistant channels apparently are present in sufficient numbers to support the initiation and propagation of mechanically induced impulses in most C fibers, as well as in many slow A-δ fibers, but not in fast A-δ fibers.

These results do not directly demonstrate the identity of the TTX-resistant channel in the dural afferents, but the voltage-gated TTX-resistant sodium channel found in small diameter dorsal root ganglion cells seems the most likely candidate among known ion channels. It is conceivable that a C-fiber-specific voltage-gated calcium channel could also contribute to the differential TTX resistance of C fibers because voltage-gated calcium channels are resistant to TTX (Dichter and Fischbach 1977; Heyer and Macdonald 1982; Matsuda et al. 1978; Ransom and Holz 1977; Yoshida et al. 1978) and are suppressed by lidocaine (Oyama et al. 1988), although not as strongly as sodium channels. However, it is unlikely that calcium channels would be capable of supporting impulse propagation on their own, as axonally propagating calcium spikes have been observed only rarely, primarily in invertebrates, and usually only in the presence of potassium channel blockers (Horn 1978; Stockbridge and Ross 1986; Yoshida and Matsuda 1980). In addition, our observations in a few neurons that the removal of calcium did not affect TTX susceptibility or mechanoresponsiveness, as well as similar observations on mechanosensitivity in small-diameter corneal afferents (Pozo et al. 1992), make it unlikely that calcium currents have a major role in the TTX-resistant responses we observed.

**Cellular localization of TTX resistance**

In the preparation used in this study, the axons of the dural afferent neurons reach the dura through the tentorial nerve, which courses caudally from the trigeminal ganglion along the base of the skull and then dorsally up the tentorium to reach the dura covering the dorsal surface of the brain (Andres et al. 1987; unpublished observations). On reaching the dura, the axons give rise to fine branches that travel horizontally across the dura for variable distances of up to several millimeters to supply mechanosensitive endings (see Figs. 1 and 2). Solutions applied topically to the outer surface of the dura would be expected to access the dural branches but not the main axons in the tentorial nerve below. TTX resistance in this preparation thus requires that mechanical transduction, impulse initiation, and impulse propagation from the mechanosensitive ending to the point where the dural branch joins the main axon are all TTX resistant. These findings thus demonstrate TTX resistance in the nerve endings and the dural axonal branches supplying them but provide no evidence on the TTX sensitivity of the main (parent) axons in the tentorial nerve.

A number of previous electrophysiological studies found that mammalian peripheral nerve fibers in nerve trunks are not resistant to TTX. In recordings of the compound action potential in rabbit sciatic and vagus nerves, it was found that the C-fiber component was slightly less sensitive to TTX than the A-δ fiber component, but this difference was no greater than that found in the sensitivity to lidocaine, and all fibers were blocked at TTX concentrations (1–3 μM) far below that required to suppress the TTX-resistant sodium current present in the cell bodies of sensory neurons (Colquhoun and Ritchie 1972; Gaumann et al. 1992; Gissen et al. 1980). Intracellular recording studies in preparations of rodent dorsal root ganglion with peripheral nerve attached have found that TTX blocked axonally conducted impulses in all neurons studied, including C- and A-δ fiber neurons whose cell bodies were TTX resistant (Ritter and Mendell 1992; Villiure and McLachlan 1996; Yoshida and Matsuda 1979). Conduction through the central axonal processes in the dorsal roots was also blocked by TTX. Similarly, Noda et al. (1997) found that TTX blocked propagation of impulses evoked by application of bradykinin to the distal processes of small-diameter cultured dorsal root ganglion cells. On the other hand, recordings of the compound action potential in peripheral nerve biopsy material from patients with peripheral neuropathies found that the C-fiber wave was partially resistant to TTX (Quasthoff et al. 1995), although it is not known whether the property of TTX resistance was affected by the nerve pathology (Novakovic et al. 1998). In addition, one study reported that the synaptic response of rat spinal cord neurons to stimulation of C fibers in peripheral nerve was not blocked by application of 0.5 μM TTX to the nerve (Jeftinija 1994). However, the length of peripheral nerve exposed to TTX in this study, although not reported, was clearly much...
shorter than that used in the studies cited previously and may have been inadequate for producing impulse blockade. Alternatively, there may exist a subpopulation of TTX-resistant C fibers in peripheral nerve that were not sampled in the single-cell recording studies. Overall, the weight of available electrophysiological evidence indicates that the peripheral axonal branches of most mammalian C fibers do not possess TTX-resistant channels in sufficient numbers to support impulse propagation. This conclusion is further supported by the recent observation that PN3, the sensory neuron-specific TTX-resistant sodium channel, can be localized immunohistochemically in small-diameter dorsal root ganglion cells but cannot be detected in peripheral nerve unless the nerve has been damaged, in which case the channel accumulates at the site of injury (Novakovic et al. 1998).

This study is the first to document TTX resistance in peripheral endings of C-fiber neurons. These results, considered together with the evidence cited previously on the TTX sensitivity of peripheral nerve fibers, suggest that the TTX-resistant sodium channel may be selectively distributed in the peripheral terminals and preterminal axonal branches of C-fibers, as well as in the cell body, but not in the main axon. A selective subcellular distribution (somal vs. axonal) has been found previously for different subtypes of the TTX-sensitive sodium channel (Westenbroek et al. 1989). The current results are consistent with the idea that the cell body of small-diameter dorsal root ganglion cells may be used as a model for the study of the electrophysiological properties of the C-fiber peripheral nerve terminal (Gold et al. 1996a). It is possible that TTX-resistant channels may be a distinctive feature of the receptive rather than the conductive portion of the axonal membrane and that their density in the preterminal axonal branch decreases at progressively greater distances from the terminal. This would be consistent with the finding that neurons with longer axonal branches within the dura tend to be more susceptible to suppression by TTX.

Interpretation of applied drug concentrations

In an in vivo preparation such as that used in this study, the actual drug concentration at the site of action on the exposed nerve fibers will inevitably be lower than the concentration that was applied, as a result of factors such as connective tissue diffusion barriers, dilution by tissue fluids, and transport in the circulation. Such differences in absolute drug concentration do not affect the determination of relative drug susceptibilities. For lidocaine, this difference between the applied concentration and the effective concentration at the site of action in our preparation may be estimated on average at roughly one order of magnitude, based on comparison of our mean applied blocking concentration (18 mM) with steady-state blocking concentrations in mammalian peripheral nerve in vitro (Huang et al. 1997; Raymond et al. 1989). Individual nerve fibers might differ considerably in their relative accessibility to topically applied solutions, for example, as a result of differences in the depth of the mechanosensitive nerve ending within the dura. Such differences in accessibility might contribute to the large interneuron variability in lidocaine sensitivity and particularly might account for the few neurons in which lidocaine failed to produce complete suppression of mechanosensitivity. However, such interneuron variation in drug accessibility apparently was not strongly related to fiber conduction velocity, because no significant relationship was found between conduction velocity and lidocaine sensitivity.

Subdivision of A-δ neurons

A-δ neurons were subdivided into fast (>5 m/s) and slow groups in our initial study of meningeal afferents to describe the variation in the incidence of mechanosensitivity across the population (Strassman et al. 1996). This subdivision was retained because of its utility in describing the presence of TTX resistance (i.e., TTX resistance was only found in neurons slower than 5 m/s). It should be noted that the conclusions of this study are also supported by correlation analyses based on conduction velocity without separation into neuronal classes. Other studies of primary afferents have also treated the more slowly conducting A-δ neurons as a separate group, either explicitly or implicitly by omission. Many studies of A-δ nociceptors in skin and other tissues did not examine fibers having conduction velocities below 5 m/s (e.g., Burgess and Perl 1967; Hoheisel et al. 1989; Light and Perl 1979; Lynn and Carpenter 1982; Meyer et al. 1991; Perl 1968; Rethelyi et al. 1982), whereas others described the slower A-δ fibers as a separate class (Hoheisel and Mense 1987; Liang and Terashima 1993; Liang et al. 1995; Lynn et al. 1995). One intracellular labeling study presented light microscopic evidence that fibers in the lower end of the A-δ range were unmyelinated on both the peripheral and central sides of their axonal bifurcation in the dorsal root ganglion (Hoheisel and Mense 1987). Thus this as well as several previous studies found that in some cases slow A-δ fibers can be more similar to C fibers than to the faster A-δ fibers with respect to certain anatomic and physiological properties, including myelination. Therefore it may be warranted in some cases to regard them as a separate, intermediate group when investigating potential differences between C and A-δ fibers.

Implications for selective block of peripheral nociception

These findings support the possibility that, if a blocking agent can be developed for the sensory neuron-specific TTX-resistant sodium channel, it would selectively inhibit impulses in slowly conducting sensory fibers in the periphery and thus might function as a peripherally acting analgesic. Such an agent might not produce complete block because TTX-sensitive sodium channels are also present in these fibers (as discussed previously), but it would be expected to inhibit activity evoked through all transductive mechanisms, in contrast to agents acting at other potential C fiber–specific targets such as the capsaicin receptor. Such an agent might thus provide for the first time the long-sought differential blockade of small-diameter nociceptive fibers, which is not produced by any of the existing blocking agents such as lidocaine (Raymond and Gissen 1987).

Conclusion

The mechanosensitive endings and distal axonal branches of most dural C fibers are resistant to TTX, whereas those of fast
A-δ fibers are not. The TTX resistance of C fibers relative to fast A-δ fibers is too large to be explained by a difference in safety factor, as demonstrated by the relatively small difference in sensitivity to lidocaine. The explanation for the differential TTX sensitivity that appears to be most consistent with currently known properties of sensory neurons is that TTX-resistant sodium channels are present in the distal axonal branches of most dural C-fibers, as well as many of the slower A-δ fibers, but not fast A-δ fibers in sufficient numbers to support the initiation and conduction of mechanically induced impulses.

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Similar results were recently reported for corneal afferents (Brock et al. J. Physiol. (Lond.) 512: 211–217, 1998).

REFERENCES


