Role of TTX-Sensitive and TTX-Resistant Sodium Channels in Aδ- and C-Fiber Conduction and Synaptic Transmission

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1Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal; 2Laboratório de Biologia Celular e Molecular, Faculdade de Medicina, Universidade do Porto, Porto, Portugal; and 3Vollum Institute, Oregon Health and Science University, Portland, Oregon

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Pinto V, Derkach VA, Safronov BV. Role of TTX-sensitive and TTX-resistant sodium channels in Aδ- and C-fiber conduction and synaptic transmission. J Neurophysiol 99: 617–628, 2008. First published December 5, 2007; doi:10.1152/jn.00944.2007. Thin afferent axons conduct nociceptive signals from the periphery to the spinal cord. Their somata express two classes of Na+ channels, TTX-sensitive (TTX-S) and TTX-resistant (TTX-R), but their relative contribution to axonal conduction and synaptic transmission is not well understood. We studied this contribution by comparing effects of nanomolar TTX concentrations on currents associated with compound action potentials in the peripheral and central branches of Aδ- and C-fiber axons as well as on the Aδ- and C-fiber-mediated excitatory postsynaptic currents (EPSCs) in spinal dorsal horn neurons of rat. At room temperature, TTX completely blocked Aδ-fibers (IC50, 5–7 nM) in dorsal roots (central branch) and spinal, sciatic, and sural nerves (peripheral branch). The C-fiber responses were blocked by 85–89% in the peripheral branch and by 65–66% in dorsal roots (IC50, 14–33 nM) with simultaneous threefold reduction in their conduction velocity. At physiological temperature, the degree of TTX block in dorsal roots increased to 93%. The Aδ- and C-fiber-mediated EPSCs in dorsal horn neurons were also sensitive to TTX. At room temperature, 30 nM blocked completely Aδ-input and 84% of the C-fiber input, which was completely suppressed at 300 nM TTX. We conclude that in mammals, the TTX-S Na+ channels dominate conduct in all thin primary afferents. It is the only type of functional Na+ channel in Aδ-fibers. In C-fibers, the TTX-S Na+ channels determine the physiological conduction velocity and control synaptic transmission. TTX-R Na+ channels could not provide propagation of full-amplitude spikes able to trigger synaptic release in the spinal cord.

INTRODUCTION

Slowly conducting fine-caliber myelinated Aδ- and unmyelinated C-fibers terminate in the superficial dorsal horn of the spinal cord (Brown 1981; Light and Perl 1977). Many of them convey nociceptive inputs and are involved in the establishment and maintenance of pathophysiological states, including neuropathic pain, caused by abnormal change in the expression of ion channels. A number of studies have shown a pivotal role of voltage-gated Na+ channels in both normal nerve conduction (Hille 2001) and development of neuropathic states (Black et al. 1996; Jennings et al. 1997; Jeftinija and Urban 1994; Steffens et al. 2001; Villiere and McLachlan 1996; Yoshida and Matsuda 1979), it is still unclear whether they are also capable of generating propagating action potentials in the central and peripheral branches of afferent fibers. Little is also known about the TTX-S and TTX-R Na+ channel distribution along the afferent axon and their involvement in conduction and synaptic transmission. A number of studies used TTX as a tool to address the question whether TTX-R Na+ channels have sufficient density to provide spike propagation in unmyelinated axons. Differences in experimental designs, peripheral nerve regions, sites of TTX application, and the blocker concentrations gave quite contradictory results. On one hand, conduction in C-fibers was found to persist in TTX for frog sciatic nerve (100 μM, Kobayashi et al. 1993; 1 μM, Buchanan et al. 1996), rat dorsal root (0.5 μM, Jefinjia 1994; Jefinjia and Urban 1994; 1 μM, Steffens et al. 2001) and biopsied human sural nerve (1–100 μM) (Grosskreutz et al. 1996; Quasthoff et al. 1995). On the other hand, C-fiber conduction was reported to be blocked by TTX in mouse peripheral nerve (~3 μM) (Yoshida and Matsuda 1979), rat dorsal root, and sciatic nerve (0.3–1 μM) (Villiere and McLachlan 1996). In a similar manner, synaptic transmission from C-fibers to the spinal dorsal horn neurons was found to be blocked by 0.5 μM TTX applied to the spinal cord slice with attached dorsal root

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(Yoshimura and Jessell 1990) but to persist in 0.5 µM TTX applied to DRG neurons (Jefftinija and Urban 1994).

Therefore we studied the contribution of TTX-S and TTX-R Na⁺ channels to spike propagation along the fine-caliber Aδ- and C-fiber axons. For this purpose, we measured effects of nanomolar concentrations of TTX on Aδ- and C-fiber responses along the peripheral and central branches of primary afferents as well as on their synaptic transmission in the spinal cord. We found that, regardless of the location along the peripheral and central branches of sensory axons, the TTX-S Na⁺ channels strongly dominate conduction in both Aδ- and C-fibers and are necessary for synaptic release.

METHODS

Preparations

Laboratory Wistar rats were killed in accordance with the national guidelines (Direcc¸a˜o Geral de Veterina ´ria, Ministe ´rio da Agricultura) after the anesthesia by intraperitoneal injection of Na⁺-pentobarbital (30 mg/kg). Compound action potential current responses were recorded from different regions of the central and peripheral branches of afferent axons (see Fig. 3A) using L₅ dorsal root, L₅ mixed spinal nerve with a proximal sciatic nerve and sural nerve from 4- to 18-wk-old rats. The nerves and roots were dissected and cleaned from the connective tissue sheath in ice-cold oxygenated artificial cerebrospinal fluid (ACSF). After isolation, the roots and nerves were kept at room temperature of 22–24°C until use. Entire L₅ dorsal roots were 13–27 mm long. In the experiments studying the TTX sensitivity in the central branch of afferents, each L₅ root was divided in two equal parts, rostral dorsal root (adjacent to the spinal cord) and caudal dorsal root (near the dorsal root ganglion). To record from the proximal part of the peripheral branch of sensory axons, we used the spinal-sciatic nerve preparation that included the L₅ mixed spinal nerve and the proximal sciatic nerve. This spinal-sciatic nerve preparation allowed recordings from those sciatic nerve fibers that originated from the L₅ segment. In the experiments with TTX, the nerve was shorter (10–13 mm), to reduce the temporal dispersion of the C-fiber response and therefore to increase its amplitude. When the conduction velocity (CV) was measured, longer spinal-sciatic nerve preparation (28–48 mm) was used to increase the accuracy of the measurements. To record from the distal part of the peripheral branch of the axon, we isolated the sural nerves of 8- to 12-mm length.

Tight-seal recordings from dorsal horn neurons were done using the whole spinal cord preparation with attached segmental L₅ root (Safronov et al. 2007) from 3- to 6-wk-old rats. The vertebral column was quickly cut out and immersed in ice-cold oxygenated ACSF. The whole lumbar enlargement with attached 10- to 15-mm-long L₅ root was dissected and laterally glued by cyanoacrylate adhesive to a 1-mm-thick metal plate (Fig. 7). One sagittal cut was done using a tissue slicer (Leica VT 1000S, Germany) to create an access to gray matter. After incubation during 45 min at 33°C, the spinal cord with the metal plate was transferred into the recording chamber where the metal plate provided mechanical stability of the preparation. Dorsal horn neurons were visualized in the whole spinal cord by using the light-emitting diode (LED) illumination according to a previous description (Safronov et al. 2007). In this study, we used blue and white LEDs positioned outside the solution meniscus for oblique illumination (Fig. 7). When the preparation was positioned in the recording chamber, care was taken to avoid direct shadow imposed by the dorsal root on the cut spinal cord surface. Tight-seal whole cell recordings from spinal dorsal horn neurons were done as previously described (Melnick et al. 2004; Safronov et al. 1997).

Recordings

We used a patch-clamp amplifier to record in nerves and roots the compound action potential currents (CAPCs), which are the currents associated with a propagation of compound action potentials (CAPs). In voltage-clamp mode, the amplifier monitored the extracellular current which had to be injected, to hold at 0 mV the voltage drop on the leakage resistance of the recording suction pipette at the moment when CAP invaded it. As shown in Fig. 1A, CAPCs represented recordings from the same population of conducting fibers as conventional CAPs, and CAPCs could be directly converted to CAPs by multiplication by the leakage resistance. We have chosen to record CAPCs instead of conventional CAPs because of higher resolution limits of patch-clamp amplifiers in voltage-clamp mode (the current-clamp mode resolution was limited by the size of the voltage digitization bin). CAPCs were recorded using EPC9 or EPC10 amplifiers (HEKA, Lambrecht, Germany). In control experiments comparing CAPs and CAPCs (Fig. 1A), we used EPC10 amplifier that had a current-clamp circuitry constructed as a real voltage-follower and thus allowed recording of fast voltage changes without signal distortion (Magistretti et al. 1996,1998). Suction electrodes for stimulation and recording were fabricated from borosilicate glass tubes with 1.5 mm OD and 0.86 or 1.2 mm ID (Modulohm, Denmark) and were fire-polished to fit the size of the nerve. Leakage resistance of the recording suction pipette with inserted nerve was 17–53 kΩ for dorsal roots, 27–87 kΩ for sural nerves, and 6–28 kΩ for spinal-sciatic nerve with a recording electrode always positioned on narrower mixed spinal nerve. Recording electrodes were voltage-clamped at 0 mV when CAPCs were measured. The voltage error due to resistance in series was supposed to be negligible for extracellular recording. The effective corner frequency of the low-pass filter was 14.3 kHz (with an exception of 2 and 3 recordings filtered at 2.9 and 8.5 kHz, respectively), and the frequency of digitization was 20–200 kHz. The traces with slow C-fiber responses were filtered off-line at 1 kHz. EPSCs evoked by attached root stimulation, were recorded in the spinal dorsal horn neurons using voltage-clamp mode of EPC9 amplifier. The patch pipettes were pulled from thick-walled borosilicate glass tubes with 1.5 mm OD and 0.86 mm ID (Modulohm, Denmark) and had after fire-polishing a resistance of 3–5 MΩ. The effective corner frequency of the low-pass filter was 2.9 kHz. The frequency of digitization was 10 kHz. Offset potentials were compensated directly before formation of a seal. Liquid junction potentials were calculated and corrected for in all experiments. In neurons, the series resistance measured in the current-clamp mode was <14 MΩ and was not compensated. The mean input resistance of the neurons was 2.3 ± 0.4 GΩ (n = 10), and the mean resting potential measured with balanced amplifier input (Santos et al. 2004) was −81.0 ± 3.5 mV (n = 10).

Stimulation

Fibers were stimulated through suction electrodes using an isolated pulse stimulator (2100, A-M Systems). Short pulses of 50 µs were applied to activate CAPCs in Aαβ- and Aδ-fibers. The intensity of effective stimulation depended on the type of fiber and was in the range of 10–250 µA for dorsal roots, 10–450 µA for spinal-sciatic nerve, and 10–200 µA for sural nerve. These stimulations were not sufficient to activate C-fibers. For activation of CAPCs in C-fibers, 1-ms pulses were used: 20–150 µA for dorsal root, 30–600 µA for spinal-sciatic nerve, and 10–120 µA for sural nerve. In the presence of TTX, the intensity of stimulation was increased as shown in Fig. 3C. CVs were calculated from latencies of corresponding CAP waves measured from the end of 50-µs pulse for Aαβ- and Aδ-fibers and from the middle point of 1-ms pulse for C-fibers. Stimulus utilization time, i.e., the delay between the stimulus and beginning of the spike in the axon (Waddel et al. 1989), was not taken into account, assuming its negligible effect on pharmacological results. The Aαβ- and Aδ-fibers were stimulated at 0.5–1 Hz. The C-fibers were stim-
TTX-sensitive conduction in sensory Aδ- and C-fiber axons

Uncited at 0.1 Hz because stimulations of our preparation at higher frequencies of 1, 0.5, and 0.2 Hz evoked responses with latencies progressively increasing with the number of stimulus (Gee et al. 1996). Except those in Figs. 1B and 6C, CAPCs are shown as averages of ≥10 traces.

Solutions

ACSF contained (in mM) 115 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 11 glucose, 1 NaH₂PO₄, and 25 NaHCO₃ (pH 7.4 when bubbled with 95%-5% mixture of O₂-CO₂). Patch pipettes were filled with a solution containing (in mM) 3 KCl, 150 K-gluconate, 1 MgCl₂, 1 BAPTA, and 10 HEPES (pH 7.3 adjusted with KOH, final [K⁺] was 161 mM).

Evaluation of the effect of TTX

TTX (Alomone labs, Jerusalem, Israel) was applied at 1- to 1,000-nM concentrations. In control experiments, we found that at low TTX concentrations, the nerve conduction block developed during 30–40 min (not shown). Therefore to ensure complete diffusion of the blocker within the nerve, each concentration was applied to the recording chamber for ≥1 h, and all measurements were done on stabilized responses. The wash out from TTX lasted 1–2 h. The effect of TTX was estimated by measuring reduction in the total area under the “positive” and “negative” deviations of the CAPC. In the spinal cord preparation, the block developed within few minutes (see Fig. 7), indicating that the primary afferent terminals within the spinal cord were likely better exposed to the blocker. Degree of the block was estimated from the area under the EPSC traces. For each measurement, at least five consecutive EPSCs evoked at 0.1 Hz were averaged.

All numbers are given as means ± SE. The values obtained by data fitting with a nonlinear least-squares procedure are given as means ± SE. In all figures, the error bars are shown when exceeding the symbol size. The parameters were compared by paired or independent Student’s t-test. The present study is based on recordings from 72 dorsal roots, 23 spinal-sciatic nerves, 23 sural nerves, and 36 superficial dorsal horn neurons. All experiments, except those in Fig. 2 and 5, were carried out at room temperature of 22–24°C. To measure CVs and TTX effects at physiological conditions (Figs. 2 and 5), the bath temperature was increased to 35–37°C using the in-line solution heater (Warner Instruments, Hamden).

RESULTS

Recording of CAPCs

To test whether the voltage-clamp mode of the patch-clamp amplifier can be used for adequate measurements of extracellular currents in dorsal roots and nerves, we compared the fast Aαβ- and Aδ-components of both compound action potential current (CAPC) and compound action potential (CAP) recorded in L₃ dorsal root (Fig. 1A). In these experiments, EPC10 amplifier with a current-clamp circuitry designed as a real voltage-follower was used. Responses of the same root were recorded first under voltage-clamp conditions as CAPCs and then in current-clamp as CAPs. The Aαβ-fiber CAPCs had amplitudes of 20–420 nA, and the corresponding CAPs were ≤15 mV. To compare the shape of both signals, we scaled the trace of the CAPC by a factor close to the value of the leakage resistance for the recording electrode and superimposed (dotted line) on the CAP trace (Fig. 1A, right). In total five dorsal roots tested both CAPCs and CAPs showed the same kinetics. We therefore concluded that CAPCs recorded under voltage-clamp conditions can be used, instead of traditional CAPs, for adequate study of excitability in the whole nerve preparation. Thus the standard patch-clamp amplifier can be used for intracellular recordings from neurons as well as for study of population nerve action potentials. The following experiments were done in voltage-clamp mode and CAPCs were recorded.

Classification of Aαβ-, Aδ-, and C-fiber CAPCs

Stimulation of nerves with short (50 μs) current pulses evoked CAPCs in myelinated Aαβ- and Aδ-fibers, without activating slow C-fibers (Fig. 1B, top traces, n = 10). We considered the inflection point on the fast CAPC as the end of the Aαβ-component and the beginning of the Aδ-component. The end of the Aδ-component was individually determined for each response by analyzing its decay at higher magnification.

FIG. 1. Voltage-clamp recording of the compound action potential currents (CAPCs). A: a dorsal root was stimulated by a short (50 μs) current pulse and the Aαβ/Aδ-fiber CAPC (left) and CAP (right) were recorded in voltage- and current-clamp modes, respectively. The CAPC trace was scaled by a factor close to the value of the leakage resistance (Rₐ) of the recording suction electrode and the resulting trace (…) is shown superimposed on the CAP trace. B: classification of the Aαβ-, Aδ-, and C-fiber CAPC components. Top: Aαβ- and Aδ-components activated by a 50-μs stimulation of the dorsal root (conduction distance, 11 mm). Bottom: stimulation of the same root with 1-ms current pulses activated additionally C-fibers. Ranges of Aαβ-, Aδ-, and C-fiber CAPCs are indicated above the traces.

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(not shown). In many cases, Aαβ-fibers started to activate at lower stimulation intensities than Aδ-fibers. Stimulation of the nerve with long (1 ms) current pulses activated both the fast Aαβ/Aδ- and the slow C-fibers (Fig. 1B, bottom traces). The C-fiber CAPC had a typical biphasic shape; an inward current was followed by an outward current. The C-fiber response was measured from the beginning of the inward current to the end of the outward current. All events observed between the Aδ- and C-responses were classified as Aδ/C-fiber currents (Fig. 1B).

The CVs were measured in long (conduction distance, 24–44 mm) fragments of spinal-sciatic nerve at room temperature of 22°C (n = 5) and physiological temperature of 36–37°C (n = 5), and the results are shown in Fig. 2 and Table 1. The highest and lowest CVs were calculated for each component by dividing its shortest and longest latency times by the conduction distance. At room temperature, CV was 9.4–26.7 m/s (mean slowest CV – mean fastest CV) for Aαβ-fibers, 1.1–9.4 m/s for Aδ-fibers and 0.23–0.7 m/s for C-fibers. The CV values increased at 36–37°C to 17.4–44.2 m/s for Aαβ-fibers, 1.8–17.4 m/s for Aδ-fibers and 0.34–1.3 m/s for C-fibers. Taking into account that the CV changes along the afferent axons (Waddell et al. 1989), we also did measurements for dorsal roots (Table 2, n = 5; 11–13 mm conduction distance, 22–24°C). In comparison with the spinal-sciatic nerve, the root conducted more slowly giving the CV values of 4.2 to >16 m/s for Aαβ-fibers, 0.56–4.2 m/s for Aδ-fibers, and 0.16–0.43 m/s for C-fibers.

CAPC block by TTX

The effect of TTX on CAPCs was studied in four regions along the central and peripheral branches of primary afferent axon (Fig. 3A): the rostral dorsal root, the caudal dorsal root, the mixed spinal nerve with the proximal sciatic nerve and the sural nerve. TTX at 3–1,000 nM blocked the C-fiber CAPCs, simultaneously increasing their latency and temporal dispersion as well as the stimulation intensity needed to evoke the corresponding maximum response (Fig. 3B). To account for the temporal dispersion of the C-fiber CAPC, we analyzed changes in its integrated area as a measure of block (Gokin et al. 2001; Huang et al. 1997). At each TTX concentration, the stimulation with increasing intensity was done and the corresponding maximum response was determined during the offline analysis (Fig. 3C, all corresponding maximum responses are shown normalized to 1). The C-fibers in all four regions of primary afferents were sensitive to TTX, which at 1,000 nM suppressed 0.85 ± 0.02 (n = 5) of the CAPCs in the sural nerve, 0.89 ± 0.02 (n = 5) in the spinal-sciatic nerve, 0.65 ± 0.03 (n = 5) in the caudal dorsal root, and 0.66 ± 0.05 (n = 5) in the rostral dorsal root (Fig. 3D). The IC50 values obtained by fitting the data points for all regions ranged between 14 and 33 nM. The CAPC block in both regions of the peripheral branch occurred in a narrower concentration range. The remaining TTX-resistant component of the C-fiber CAPC was significantly larger in the central branch of the afferents (mean 0.34 and 0.35 for the rostral and caudal dorsal roots, respectively) compared with the peripheral branch (0.11 and 0.15 for the spinal-sciatic nerve and the sural nerve, respectively). Comparison using the independent Student’s t-test gave the following values: P < 0.001 for the caudal dorsal root versus the spinal-sciatic nerve, P < 0.001 for the caudal dorsal root versus the sural nerve, P < 0.003 for the rostral dorsal root versus the spinal-sciatic nerve, and P < 0.01 for the rostral dorsal root versus the sural nerve.

For comparison, we also re-evaluated the data from the Fig. 3D and constructed the concentration-effect curves where the C-fiber CAPCs were measured as a negative peak current amplitude (Fig. 3E). Under these conditions, the apparent TTX block was stronger. The reduction of the peak CAPCs was seen at lower concentrations and the TTX-resistant CAPC components measured at 1,000 nM were smaller.

Control experiments were done to ensure that the C-fiber CAPCs remaining in the presence of 1,000 nM TTX were Na+-dependent. A substitution of 2 mM Ca2+ with a mixture of inorganic Ca2+ channel blockers (2 mM Co2+, 0.1 mM Cd2+, and 2 mM Mg2+) did not reduce the remaining CAPCs (n = 5; 2 rostral dorsal roots, 1 caudal dorsal root, and 2 sciatic nerves, not shown). In absence of TTX, the CAPCs completely disappeared when 115 mM NaCl in ACSF was substituted with 115 mM choline-Cl (n = 4, 2 rostral dorsal roots, 1 caudal dorsal root, 1 entire dorsal root, not shown). Thus it could be concluded that the C-fiber CAPCs were Na+-dependent.

The Aαβ- and Aδ-fiber CAPCs in all studied regions of the afferent axons were blocked by TTX in a narrow concentration range of 1–30 nM (Fig. 4, A and B). For this reason, the magnitudes of the Aαβ- and Aδ-fiber CAPCs were plotted as a function of TTX concentration for pooled data (n = 8) from two caudal dorsal roots, two rostral dorsal roots, two spinal-sciatic nerves, and two sural nerves (Fig. 4B). The half-maximum block was obtained at IC50 of 5–7 nM TTX.

These experiments have shown that TTX at concentrations insufficient to affect TTX-R Na+ channels (insensitive to 75–100 μM) (Elliott and Elliott 1993; Ogata and Tatebayashi 1993) has a strong effect on conduction in both Aδ- and C-fibers. It could be concluded that the TTX-S Na+ channels blocked by 1–1,000 nM TTX play dominant role in conduction of both myelinated and unmyelinated sensory axons.
**TABLE 1. Conduction velocities in the peripheral nerve**

<table>
<thead>
<tr>
<th>Spinal-Spinal</th>
<th>Fastest</th>
<th>Slowest</th>
<th>Fastest</th>
<th>Slowest</th>
<th>Fastest</th>
<th>Slowest</th>
</tr>
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<tbody>
<tr>
<td>22 °C Mean</td>
<td>26.7 ± 3.1</td>
<td>9.4 ± 1.2</td>
<td>9.4 ± 1.2</td>
<td>1.1 ± 0.4</td>
<td>0.7 ± 0.04</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>36–37 °C Mean</td>
<td>34.7–19.7</td>
<td>11.9–6.4</td>
<td>11.9–6.4</td>
<td>2.6–0.6</td>
<td>0.8–0.6</td>
<td>0.3–0.17</td>
</tr>
<tr>
<td>Range</td>
<td>44.2 ± 5.2</td>
<td>17.4 ± 2.1</td>
<td>17.4 ± 2.1</td>
<td>1.8 ± 0.6</td>
<td>1.3 ± 0.05</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td>Range</td>
<td>60.3–31.9</td>
<td>21.2 ± 11.2</td>
<td>21.2 ± 11.2</td>
<td>3.9–0.8</td>
<td>1.5–1.2</td>
<td>0.55–0.21</td>
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Conduction velocities (CVs) were measured in long (conduction distance, 24–44 mm) fragments of spinal-sciatic nerve at 22 and 36–37 °C (n = 5). The highest and lowest CVs were calculated for Aαβ-, Aδ-, and C-components of the compound action potential current (CAPC) by dividing their shortest and longest latency times by the conduction distance. Values are means ± SE.

**C-fiber CAPC block by TTX increases with temperature**

It has been recently shown for the rat cutaneous C-fiber terminals that the relative contribution of TTX-R Na⁺ channels to the spike generation was smaller at physiological temperature than at low (10°C) temperature (Zimmermann et al. 2007). Therefore we checked whether the magnitude of the TTX-R component of C-fiber CAPC depends on temperature. These experiments were performed on dorsal roots (n = 6; 8- to 11-mm conduction distance) because of the largest portion of TTX-R response observed in this region (see Fig. 3D). Following this, the perfusing solution was heated to 35–37°C, and then the blocker was washed out. All changes in the latency to the peak of negative wave, the time of the peak was normalized to the first control measurement at 22°C. It is clear from Fig. 5, A and B, that the remaining TTX-R portion of the C-fiber response was much smaller at physiological temperature than at room temperature.

Because control CAPCs slightly increased after each heating-cooling cycle (see Fig. 5B), we measured the TTX block by normalizing the responses in 1,000 nM to their nearest (preceding/following) controls (Fig. 5C). At 22°C, the remaining TTX-R responses of 0.29 ± 0.03 (n = 6; TTX 22°C normalized to control* 22°C) and 0.36 ± 0.04 (n = 6, TTX 22°C normalized to recovery 22°C) were in agreement with our data from Fig. 3D. At 35–37°C, the remaining TTX-R component was 0.07 ± 0.02 (n = 6, TTX 35–37°C normalized to control 35–37°C). This increase in the block at physiological temperature was significant (P < 0.002, paired Student’s t-test).

These experiments indicated that the contribution of TTX-R Na⁺ channels to spike propagation in dorsal root C-fibers at physiological temperature is much smaller than at room temperature.

**Slowing of C-fiber conduction by TTX**

To study the slowing of the C-fiber conduction with a block of TTX-S Na⁺ channels by nanomolar TTX, we measured the changes in the latency to the peak of negative wave in different blocker concentrations at room temperature (Fig. 6A, shown for a caudal dorsal root). The exact time moment of the peak of the negative C-wave was determined by locally fitting the data points (±2–30 ms from the peak) with a Gaussian function (see Fig. 6C). Latencies obtained for different TTX concentrations and normalized to those for control are shown in Fig. 6B. The first significant conduction slowing was seen at 3 nM (1.10 ± 0.02, n = 6, P < 0.003, paired Student’s t-test). The effect increased with TTX concentration reaching its saturation at 300 nM, where the conduction was slowed by a factor of 2.8 ± 0.1 (n = 6). Fitting the concentration dependence of the conduction slowing with an isotherm gave the half-maximum effect concentration of 26 ± 2 nM (Fig. 6B).

In the following experiments, we tested whether the TTX-dependent increase in the C-fiber CAPC latency is also accompanied by an increase in the latency time variation. Both these parameters are important for defining criteria of identification of monosynaptic EPSCs in spinal dorsal horn neurons in the presence of TTX. We did measurements for 30 nM TTX, a concentration at which the C-fiber CAPC latency was in-
increased twice (2.0 ± 0.1, n = 6, Fig. 6B). In these experiments, individual (not averaged) CAPC traces recorded in control and in 30 nM TTX were fitted with Gaussian function to obtain the current peak times (indicated on the left) was increased with TTX concentration in agreement with curves shown in C to obtain the maximum response. The Aαβ- and Aδ-waves were truncated. C: the C-fiber CAPC magnitude (integrated area) as a function of the stimulation intensity in control and in 30 and 1,000 nM TTX plotted for the root from B. The maximum CAPC in each curve was normalized to 1. The CAPCs evoked by the saturating stimulations were selected for comparison at different TTX concentration (B) and for construction of the concentration-effect curves in D. D: concentration dependence of C-fiber CAPC block by TTX in the rostral dorsal root (n = 5), the caudal dorsal root (n = 5), the spinal-sciatic nerve (n = 5), and the sural nerve (n = 5). The data corresponding to the central afferent branch are shown by filled symbols and those to the peripheral branch by open symbols. The CAPCs were measured by integrating the area under the trace. The data points were fitted using equation: 

\[ I_{\text{Max}}/(1+Le^{-IC_{50}/[\text{TTX}]}) \]

where \( I_{\text{Max}} \) was a maximum inhibition measured at 1,000 nM TTX, \( IC_{50} \) was a blocker concentration of a half-maximum inhibition and \( n \) was the Hill coefficient. The values of \( I_{\text{Max}}, IC_{50}, \) and \( n \) were 0.66, 14 ± 2 nM, and 0.95 ± 0.17 for the rostral dorsal root, 0.65, 33 ± 7 nM, and 1.43 ± 0.49 for the caudal dorsal root, 0.89, 33 ± 1 nM, and 2.4 ± 0.2 for the spinal-sciatic nerve, and 0.85, 30 ± 1 nM, and 3.3 ± 0.7 for the sural nerve, respectively.

Our observations that nanomolar TTX blocked the major part of the C-fiber CAPC in dorsal roots and peripheral nerve and reduced by several times the axonal CV raised a question about the functional role of the TTX-R Na⁺ channels in the C-fiber conduction and synaptic transmission. To answer this question we studied how the application of 30 nM TTX changes the efficiency of synaptic transmission from C-fiber terminals to spinal dorsal horn neurons.
Effect of TTX on synaptic transmission

EPSCs activated by stimulation of attached L5 dorsal root were recorded at room temperature in superficial dorsal horn neurons most of which were located in substantia gelatinosa. The input was classified as Aδ- or C-type on the basis of calculated CV (with a 1-ms allowance for synaptic transmission) and duration of stimulation used for its activation. Of a total of 36 dorsal horn neurons responding monosynaptically to primary afferent stimulation, 4 had only Aδ-input, 15 only C-input, and 17 neurons had both Aδ- and C-fiber inputs. Aδ-fiber-mediated EPSCs were elicited by a short (50 μs) pulse stimulation of dorsal roots and the corresponding CV for the fiber ranged from 0.74 to 3.56 m/s. The activation of C-input always needed a 1-ms stimulation of the root and the calculated CV ranged from 0.19 to 0.65 m/s. In control, the EPSCs were considered as monosynaptic if no failure was seen in 10 consecutive stimulations (0.1 Hz) and a variation of the EPSC latency did not exceed 1 ms. In the case of the multi-component C-fiber-mediated EPSCs, each component was analyzed using these criteria. Polysynaptic responses were not considered.

In all tested neurons, monosynaptic Aδ-inputs were completely and reversibly blocked by 30 nM TTX (Fig. 7A; n = 36).
TTX at 30 nM was also applied to 21 neurons with C-fiber input. In seven of them, the C-fiber-mediated EPSCs were completely blocked (Fig. 7A). In all these cases, the C-fiber-mediated EPSCs first showed increased latencies and then completely disappeared within 5–10 min of TTX perfusion. In 14 of 21 neurons with C-fiber input, the EPSCs were incompletely blocked (Fig. 7B). In recordings done during TTX perfusion, these C-fiber-mediated EPSCs showed progressively increasing latencies and latency variations, decreasing magnitudes and increased number of failures. An increase in the stimulation strength did not reduce the number of failures. After the stabilization of the block, the variation of the EPSC latency was within the ±4% range of the mean, in agreement with predictions of our CAPC experiments. The mean increase in the EPSC latency was 1.7 ± 0.1 (n = 14, range: 1.3–2.6). To estimate the degree of the synaptic transmission block, we used the ratio of the mean EPSC areas in at least five consecutive episodes in TTX and control. In 14 neurons with incomplete EPSC block, 30 nM TTX suppressed C-fiber synaptic transmission by 76.4 ± 6.6%. In total 21 neurons tested for 30 nM TTX (77 and 81% of control). In all 14 cases, 300 nM TTX completely blocked the EPSCs within 2–5 min of perfusion (Fig. 7C). Thus the Aδ- and C-fiber inputs to the spinal dorsal horn neurons were blocked with suppression of TTX-S Na+ channels by nanomolar TTX.

**DISCUSSION**

Our experiments have shown that nanomolar concentrations of TTX blocked to a large degree conduction in C-fiber afferents and completely abolished Aδ-fiber conduction as well as synaptic transmission from both Aδ- and C-fibers to the spinal cord. This indicated that spike propagation and synaptic release in both types of thin afferent axons are predominantly based on TTX-S Na+ channels. In unmyelinated C-fibers, TTX-S Na+ channels determine physiological CVs and trigger transmitter release to the second-order sensory neurons in the spinal dorsal horn. This dominance of TTX-S Na+ channels is not changed along the peripheral and central branches of primary afferents. Under physiological conditions, TTX-R Na+ channels seem to be expressed at densities insufficient to provide synaptic transmission to the spinal cord.

We used a voltage-clamp mode of a patch-clamp amplifier to record CAPCs that showed the time course similar to conventional CAPs seen under current-clamp conditions. The voltage-clamp amplifier provided high resolution of CAPCs with amplitudes in nanoampere range, and our classification of Aδ- and C-fibers on the basis of their CVs was in agreement with previous studies done at both physiological and room temperatures (Gokin et al. 2001; Grudt and Perl 2002; Harper and Lawson 1985; Villiere and McLachlan 1996; Wadel et al. 1989).

Both Aαβ- and Aδ-fibers were completely suppressed by 30 nM TTX with IC50 of 5–7 nM. TTX-S component of the C-fiber CAPC was blocked in a concentration range between 1 and 100 nM with IC50 of 14–33 nM. These IC50 values are typical for TTX-S Na+ channels in a number of preparations (Ogata and Tatebayashi 1993; Safronov et al. 1997; Takahashi 1990). Slightly higher sensitivity obtained in our experiments for myelinated nerves might reflect structural differences in fiber organization. It is possible that TTX, as a membrane impermeant blocker, has better access to Na+ channels in nodes of Ranvier of myelinated axons than to those in unmyelinated axons grouped in Remak bundles and surrounded by Schwann cell membrane (Murinson and Griffin 2004).

The C-fiber CAPCs were blocked in 1,000 nM TTX by 65–89% at room temperature and by 93% at 35–37°C, implying that the major part of the response was carried through TTX-S Na+ channels. This observation raised a question about the physiological role of TTX-R Na+ channels in unmyelinated C-fiber axons. Presence of a small TTX-R component of CAPC could be explained either by the existence of a small population of C-fibers with pure TTX-R conduction or, alternatively, by the presence of some TTX-R Na+ channels in C-fibers with predominantly TTX-S conductance. Our experiments with a latency increase due to Na+ channel block by TTX have strongly supported the second hypothesis. If C-fibers with pure TTX-R spikes were present in the dorsal root,
one would expect that detectable portion of the C-fiber CAPC would not increase its latency in 30–300 nM TTX. In contrast, in all our experiments, the entire C-fiber CAPC was progressively slowed by two to three times in a concentration-dependent manner, indicating that there were no fibers unaffected by low TTX concentrations. Interestingly, the half-maximum effect concentrations for the CAPC suppression and the latency increase were close to each other (14–33 nM for the CAPC block vs. 26 nM for the latency increase), once more indicating that both processes are likely to depend on the block of the same type of channel. Thus one can conclude that TTX-S Na\(^+/\)H\(^+\) channels are critically important for spike propagation and determination of the physiological CV in C-fibers. After block of TTX-S channels, TTX-R Na\(^+/\)H\(^+\) channels can probably provide only slowly propagating spikes in C-fibers.

Our experiments have also shown that the relative contribution of TTX-R Na\(^+/\) channels to the C-fiber response changes along the axon in such a way that the central branch of the afferent axon had significantly higher percentage of TTX-R component than its peripheral branch. Within the branches, however, the difference in the relative TTX-R channel expression was found to be not significant. This may imply differential mechanisms of regulation of TTX-S/TTX-R Na\(^+/\) channel expression in the central and peripheral axon branches of primary sensory neuron. However, in spite of this variation in the expression of functional TTX-R Na\(^+/\) channels along the extension of the axon, its conduction was always dominated by TTX-S Na\(^+/\) channels.

In the spinal cord preparation, 30 nM TTX had stronger effect on C-fiber-mediated EPSCs (84% block) than on the C-fiber CAPCs in isolated dorsal roots (45% block for rostral and 30% block for caudal, calculated from the fitting curves in Fig. 3D). In the presence of TTX, the EPSCs always showed a typical increase in their latency corresponding to the slowing of conduction observed in the isolated roots. Most axonal spikes reaching the spinal cord became unable to trigger synaptic release to dorsal horn neurons. In 300 nM TTX, the synaptic conduction was completely blocked. This full block of neurotransmitter release could not be attributed to blockade of C-fiber conduction, as 300 nM TTX only reduced responses in dorsal roots to 37–38% of control (fitting curves in Fig. 3D).

Thus TTX-R action potentials could still arrive at the terminal, but transmitter release did not occur. One can assume, therefore, that TTX-R Na\(^+/\) channels alone cannot provide spikes of amplitude sufficient to activate presynaptic high-threshold voltage-gated Ca\(^{2+}\) channels critical for transmitter release from the C-fiber axon terminals (Bao et al. 1998; Heinke et al. 2004). Our data also indicate that 30 nM TTX cannot be used as a tool for separating A\(\delta\)- and C-fiber-mediated EPSCs. Separation of these components in 50 nM TTX observed by Yoshimura and Jessell (1990) might be due to incomplete perfusion of the slice with the blocker.

Our data agree with studies showing that under physiological conditions, C-fiber conduction is blocked by TTX (Villiere and McLachlan 1996; Yoshida and Matsuda 1979). Partial resistance of C-fiber-mediated transmission to the spinal cord

**Fig. 7.** TTX block of A\(\delta\)- and C-fiber-mediated excitatory postsynaptic currents (EPSCs). Tight-seal recordings were done in the whole spinal cord preparation where the dorsal horn neurons were visualized using oblique LED illumination (Safronov et al. 2007). Shown neuron was located 10 \(\mu\)m under the slice surface. A: recordings from a neuron with both A\(\delta\)- and C-fiber-mediated EPSCs completely blocked by 30 nM TTX. The uppermost trace (also in B and C) shows the superposition of 5 consecutive recordings in control. The attached dorsal root was stimulated with a 1-ms current pulse to activate both A\(\delta\)- and C-components. B: recordings from a neuron with C-fiber-mediated EPSCs incompletely blocked by 30 nM TTX. The last trace in TTX shows the superposition of 5 consecutive recordings. C: complete block of A\(\delta\)- and C-fiber-mediated EPSCs by 300 nM TTX.
observed in experiments where TTX was locally applied to primary afferents (Jeflinjia 1994; Jeflinjia and Urban 1994; Steffens et al. 2001), may be also explained in context of our results, by assuming that in some C-fibers TTX-R Na⁺ channels have sufficient density to provide slowly propagating low-amplitude spikes able to cross the region of TTX application. In this case, the TTX-S Na⁺ channels in the dorsal root part rostral to the region of TTX application could restore original spike amplitude needed to trigger synaptic release. In contrast to rat axons, in amphibian sciatic nerve the TTX-R Na⁺ channels appear to play more important role in C-fiber conduction (Buchanan et al. 1996; Kobayashi et al. 1993).

Large TTX-R C-fiber responses were also seen in human sural conduction (Buchanan et al. 1996; Kobayashi et al. 1993). In conclusion, our study has shown that TTX-S type of Na⁺ channels dominates conduction in fine-caliber primary afferents. It was the only type of Na⁺ channels underlying Aδ-fiber responses. In C-fiber axons, the TTX-S Na⁺ channels determine the physiological CV and control synaptic transmission. Although TTX-R Na⁺ channels were revealed at least in a part of C-fibers, they could not provide propagation of full-amplitude spikes able to trigger synaptic release in the spinal cord. In this context, the gain of the function of axonal TTX-R Na⁺ channels by their modulation, expression upregulation or redistribution (England et al. 1996; Gold et al. 1996; Khasar et al. 1998; Lai et al. 2002; Novakovic et al. 1998; Waxman et al. 1999; Zhang et al. 1997) may be a key factor in developing diverse pathological states.

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