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Differential Contribution of Sodium Channel Subtypes to Action Potential Generation in Unmyelinated Human C-type Nerve Fibers

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Background: **Multiple voltage-dependent sodium channels (Na_v) contribute to action potentials and excitability of primary nociceptive neurons. The aim of the current study was to characterize subtypes of Na_v that contribute to action potential generation in peripheral unmyelinated human C-type nerve fibers.**

Methods: **Registration of C-fiber compound action potentials and determination of membrane threshold was performed by a computerized threshold tracking program. Nerve fibers were stimulated with a 1-ms current pulse either alone or after a small ramp current lasting 300 ms.**

Results: **Compound C-fiber action potentials elicited by supramaximal 1-ms current pulses were rather resistant to application of tetrodotoxin (30–90 nM). However, the same concentrations of tetrodotoxin strongly reduced the peak height and elevated membrane threshold of action potentials evoked at the end of a 300-ms current ramp. A similar effect was observed during application of lidocaine and mexiletine (50 μM each).**

Conclusions: **These data indicate that more than one type of Na_v contributes to the generation of action potentials in unmyelinated human C-type nerve fibers. The peak height of an action potential produced by a short electrical impulse is dependent on the activation of tetrodotoxin-resistant ion channels. In contrast, membrane threshold and action potential peak height at the end of a slow membrane depolarization are regulated by a subtype of Na_v with high sensitivity to low concentrations of tetrodotoxin, lidocaine, and mexiletine. The electrophysiologic and pharmacologic characteristics may indicate the functional activity of the Na_v 1.7 subtype of voltage-dependent sodium channels.**

SEVERAL types of voltage-dependent sodium channels (Na_v) are expressed in the cell bodies of primary nociceptive neurons.[1–4] Two main groups of channel proteins can be separated by their sensitivity to tetrodotoxin. The channel pore of Na_v 1.7 is blocked by low nanomolar concentrations of tetrodotoxin. In contrast, Na_v 1.8 and Na_v 1.9 are resistant to this toxin.[5] Other differences between the subunits of Na_v are found in electrophysiologic characteristics such as voltage and time dependency of activation and inactivation.[4] Recently, mutations in SCN9a, the gene encoding the sodium channel Na_v 1.7, have been identified to underlie human pain syndromes such as erythromelalgia[6] and the congenital inability to experience pain.[7] Recombinant Na_v1.7 produces a fast-inactivating tetrodotoxin-sensitive current[8] and differs from other Na_v by slow closed-state inactivation.[9] However, the precise contribution of Na_v 1.7 to the excitability of peripheral human unmyelinated, including nociceptive, axons is not known. Pharmacologic studies on unmyelinated human C-type nerve fibers can be performed by using isolated nerve segments obtained by biopsy or after amputation of a limb. In such experiments, action potentials of C fibers were rather resistant to application of tetrodotoxin.[10,11] Therefore, it is puzzling how the tetrodotoxin-sensitive sodium channel Na_v 1.7 can produce abnormal excitability in peripheral human nociceptive nerve fibers.

It has been suggested that the adequate stimulus for activation of Na_v 1.7 is a small, slow membrane depolarization, *e.g.*, a "generator" potential.[9,12] In the current study, we tested this idea by means of a threshold tracking technique on unmyelinated human C-fiber axons *in vitro*. Threshold tracking reveals changes in membrane potential also under conditions that do not generate or inhibit existent action potentials. Therefore, threshold tracking represents a method for detection of changes in axonal excitability that is more sensitive than recordings of action potentials only. By using this method, we demonstrate that C-fiber action potentials produced by a 1-ms electrical pulse are resistant to tetrodotoxin. In contrast, the threshold current of action potentials after a depolarizing ramp stimulus lasting 300 ms is strongly raised in the presence of 30 nM tetrodotoxin. Likewise, action potential threshold at the end of this small, slow membrane depolarization was elevated in the presence of lidocaine and mexiletine. These two drugs have been administered effectively in the treatment of erythromelalgia.[13–15] These data suggest that at least two types of Na_v contribute to the generation of action potentials in unmyelinated human nerve fibers.

Materials and Methods

Preparations

The study was conducted according to the Helsinki Declaration and was approved by the local ethics committee (Munich, Germany). The patients gave written informed consent. The human nerves were obtained from four patients with biopsy of a sural nerve segment for histologic studies (neuropathy of unknown etiology) and from one patient who underwent an amputation above the knee due to peripheral vascular disease. None

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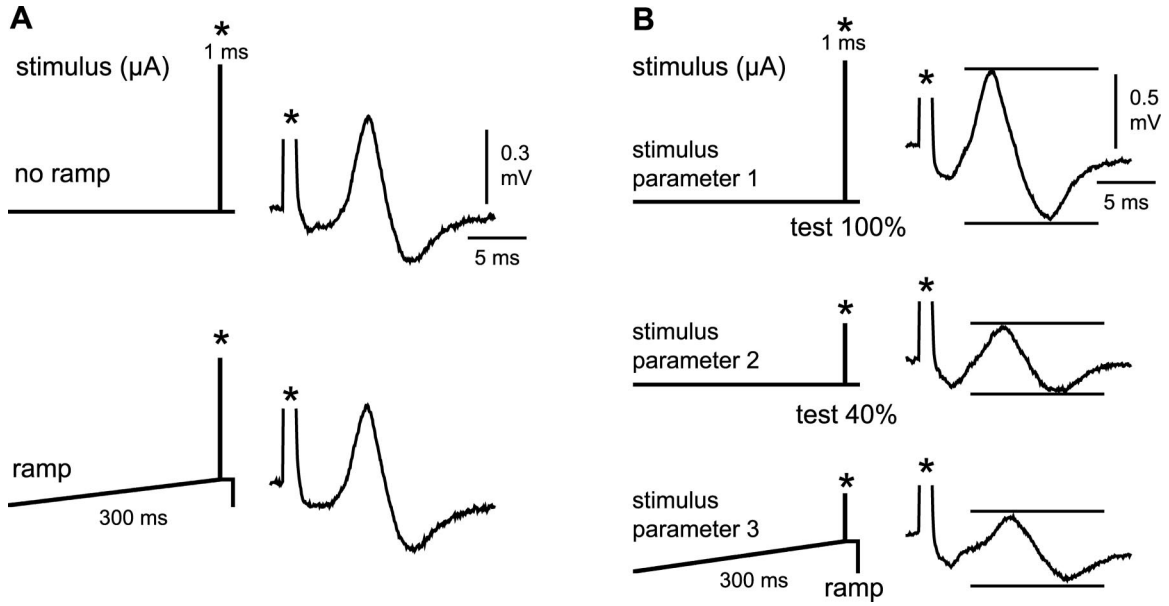

Fig. 1. Stimulation sequences and representative examples of human C-fiber compound action potentials used in the two different stimulus protocols. (*A*) (No ramp): Action potential peak height was tested by a constant supramaximal stimulus (duration 1 ms). (Ramp): A constant supramaximal stimulus (duration 1 ms) was used at the end of a slow (300-ms) current ramp (20% of current amplitude in (no ramp)). (*B*) Three stimulus conditions were tested in succession. (Stimulus parameter 1; test 100%): A constant supramaximal stimulus (duration 1 ms) evoked the maximum peak height. (Stimulus parameter 2; test 40%): A target response was set at 40% of the maximum response on stimulus parameter 1, and the test stimulus current was adjusted by computer to maintain the response close to the target. (Stimulus parameter 3; ramp): A test stimulus after a preceding ramp current of 300 ms duration and a maximal current amplitude of 50% of the test stimulus determined in stimulus parameter 2. The target response was set at 40% of the maximum response on stimulus parameter 1. Asterisks (*) indicate the short pulse in the stimulus condition and the corresponding stimulus artifact in the recordings of compound action potentials (see also Materials and Methods).

of the five patients reported (abnormal) pain states. The nerve segments (length 12–25 mm) were immediately put into fresh artificial cerebrospinal fluid solution containing 117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-glucose, pH 7.4 (bubbled with 95% O_2–5% CO_2). Several fascicles from the nerve segments were desheathed under a microscope and then held at each end by suction electrodes in an organ bath. One suction electrode was used to elicit action potentials, while the other one was used as a recording electrode. The distance between stimulating and recording electrodes was approximately 3 mm. The organ bath (volume approximately 2 ml) was continuously perfused with artificial cerebrospinal fluid solution at a flow rate of 8 ml/min and a temperature of 32°C. Drugs were applied for several minutes by addition to the perfusion system followed by a washout period. Lidocaine and mexiletine were purchased from Sigma-Aldrich (Taufkirchen, Germany); tetrodotoxin was purchased from ICN Biomedicals (Meckenheim, Germany).

Electrophysiology

Axonal excitability was measured using the threshold tracking technique, making use of the QTRAC program.§ QTRAC is a flexible, stimulus–response data-acquisition program, originally written for studies of human nerves *in vivo*[16,17] but also suitable for electrophysiologic recordings from isolated human peripheral nerve segments *in vitro*.[18–20] In the current study, QTRAC was used to record compound action potentials from peripheral C fibers, to generate stimuli, and to display the results. Isolated fascicles were stimulated with a linear stimulus isolator (A395; WPI, Sarasota, FL) with a maximal output of 500 μA. The stimulator was controlled by a computer *via* a data-acquisition board (National Instruments NI PCI-6221; Munich, Germany). Two types of stimulation sequences (A and B) were used in the experiments (fig. 1). In both conditions, successive sweeps were delivered every 2 s.

Stimulation sequence A: (no ramp) a supramaximal 1-ms stimulus (to monitor the maximal peak response amplitude); (ramp) a supramaximal 1-ms stimulus after a preceding ramp current of 300 ms duration and with 20% of the current amplitude used in (no ramp).

Stimulation sequence B: (1) a supramaximal 1-ms stimulus (to monitor the maximal peak response amplitude); (2) a test stimulus automatically adjusted to maintain the C-fiber compound action potential at a constant amplitude (40% of the maximum); (3) a test stimulus after a preceding ramp current of 300 ms duration and with 50% of the current determined by stimulus parameter (2).

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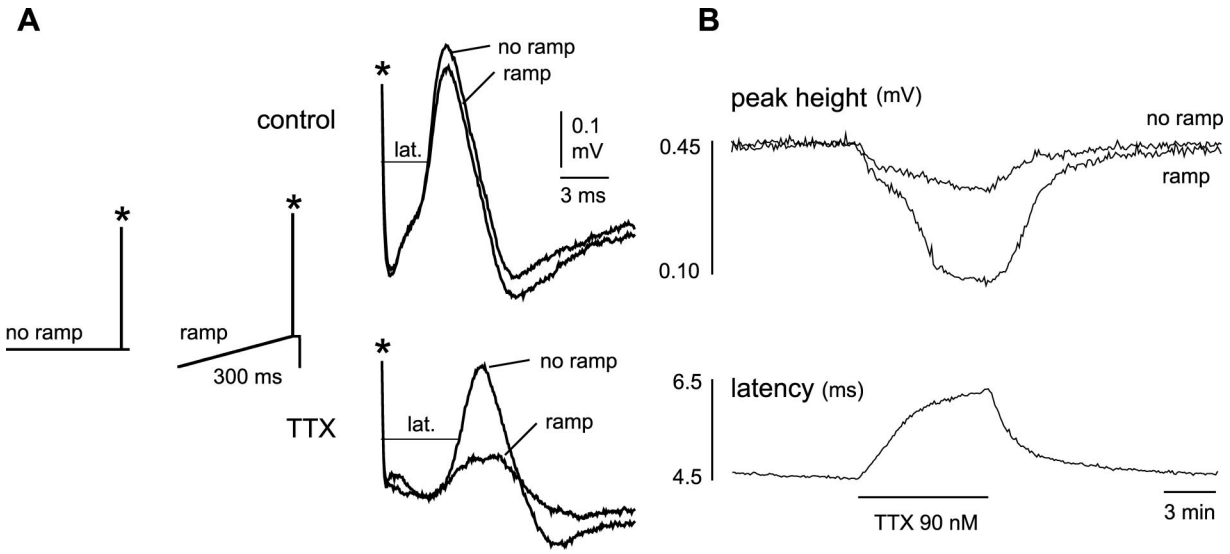

Fig. 2. Differences in the sensitivity to tetrodotoxin (TTX) of action potentials in unmyelinated human nerve fibers induced by stimulation with or without a ramp current. (*A*) Stimulus waveforms and representative superimposed examples of compound action potentials. Two stimulus conditions (no ramp, ramp; see fig. 1A; stimulus interval 2 s) were tested in the control solution and during application of tetrodotoxin. (*B*) Continuous recording of peak height evoked by the two stimulus conditions (no ramp, ramp) before, during, and after application of tetrodotoxin (90 nM). Also illustrated is the effect of tetrodotoxin on the latency (lat.) to 50% of the action potential produced by the 1-ms current pulse (no ramp). Note that action potentials at the end of the current ramp are more sensitive to the blocking effect of tetrodotoxin. Asterisks (*) indicate the short pulse in the stimulus condition and the corresponding stimulus artifact in the recordings of compound action potentials.

Statistical Analysis

All data are given as mean ± SEM and represent changes from baseline (in percentages). The statistical uncertainty of our findings was quantified using the 95% confidence intervals (CIs).

Results

Electrophysiologic studies were performed on isolated fascicles from short segments of human sural and tibial nerves. Electrical stimulation with current pulses of 0.1 or 1 ms in duration produced A- and C-fiber compound action potentials.[20] The C-fiber component had a peak amplitude of 0.6 ± 0.1 mV (mean ± SEM; n = 16; 95% CI, 0.5–0.8). Nerve fascicles were stimulated by a series of different current pulses (fig. 1). Within a few minutes after the onset of the stimulation protocol, stable compound C-fiber action potential amplitudes were elicited by the different stimulus parameters, and test substances were added to the bathing solution.

Tetrodotoxin Reduces Action Potential Peak Height at the End of a Current Ramp

In a series of experiments, we tested the effects of tetrodotoxin on the peak height of action potentials recorded either by supramaximal stimulation with a 1-ms current pulse alone or by such a current pulse at the end of a slow current ramp (see fig. 1A for stimulus protocol). A representative example of the observations is illustrated in figure 2. There was no clear difference in the peak height of the two types of action potentials in the normal bathing solution. However, in the presence of tetrodotoxin (90 nM), a strong reduction of the action potential peak height elicited at the end of the current ramp was observed (−73.6 ± 1.8%, mean ± SEM; n = 6; 95% CI, −69.1 to −78.1). In contrast, the peak height of the action potential produced by the 1-ms current pulse only (no ramp) was less affected (−22.8 ± 1.6%, mean ± SEM; n = 6; 95% CI, −18.7 to −26.9). We also observed that tetrodotoxin produced an increase in the latency to half of the maximal C-fiber compound action potential peak height determined by the stimulus without a ramp (fig. 2B, bottom panel).

Tetrodotoxin Raises Threshold Current after Ramp Stimulation

In further experiments, we determined the maximal compound C-fiber action potential peak height, and the currents necessary to maintain the C-fiber action potential at 40% of the maximum by using either a 1-ms current pulse alone or by such a current pulse at the end of a slow current ramp (see fig. 1B for stimulus protocol). A representative experiment that demonstrates the effects of tetrodotoxin on these stimulus conditions is illustrated in figure 3. There was a clear difference in the sensitivity to tetrodotoxin (30 nM) of the maximal peak amplitude, elicited by a 1-ms supramaximal current pulse (fig. 3A, middle panel/test 100%; fig. 3B, left panel) and on the membrane threshold determined at the end of a 300 ms ramp current (fig. 3A, top panel/ramp). Quantitatively, only a slight reduction in the maximal peak amplitude was observed (−5.4 ± 3.5%, mean ±

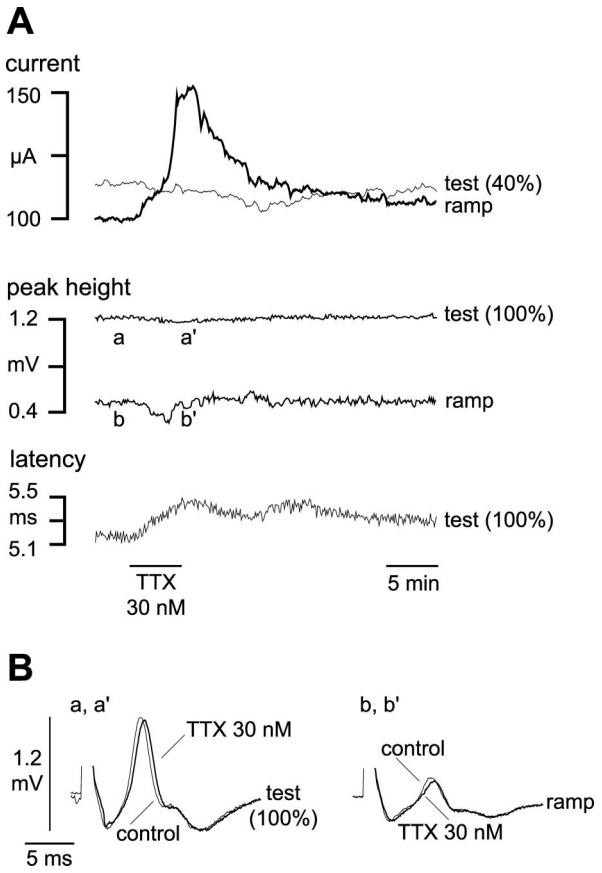

Fig. 3. Effects of tetrodotoxin (TTX) on peak height and membrane threshold of isolated human C fibers. (*A*) Continuous recordings of currents, peak amplitudes, and latency of C-fiber compound action potentials determined with the tracking stimulation parameters described in figure 1B. Note that tetrodotoxin (30 nM) had a minimal effect on the maximal peak height (test 100%) but strongly elevated the current amplitude necessary to maintain a constant peak amplitude after a preceding ramp stimulus (ramp). (*B*) Representative examples of the maximal (test 100%) C-fiber compound action potential and of C-fiber potentials recorded after a ramp stimulus (ramp) before and in the presence of 30 nM tetrodotoxin at time points indicated by the *letters*.

SEM; n = 10; 95% CI, −2.5 to −13.4). In contrast, the current necessary to produce an action potential with 40% of the maximal amplitude after a depolarizing ramp stimulation (fig. 3A, top panel/ramp) was elevated by 54.4 ± 11.8% (mean ± SEM; n = 10; 95% CI, 27.7–81.1). We also tested the effects of tetrodotoxin on a 1-ms current pulse necessary to maintain the C-fiber compound action potential peak amplitude at 40% of the maximum (fig. 3A, top panel/test 40%). This parameter, which is sensitive to changes in the axonal membrane potential,[19,20] remained almost unchanged (< 5%).

Lidocaine and Mexiletine Raise Membrane Threshold after Ramp Stimulation

In another series of experiments, we tested the effects of lidocaine and mexiletine on the maximal C-fiber peak amplitude produced by a 1-ms current pulse and on the membrane threshold at the end of a depolarizing ramp current. A representative experiment is illustrated in figure 4. Both local anesthetics differentially affected these two parameters. Peak height (fig. 4A, middle panel/test 100%; fig. 4B, top panel) was slightly reduced by −7.0 ± 1.3% (mean ± SEM; n = 9; 95% CI, −3.9 to −10.0) in the presence of lidocaine (50 μM) and by −9.8 ± 1.4% (mean ± SEM; n = 5; 95% CI, −6.0 to −13.5) during application of mexiletine (50 μM). In contrast, the threshold current at the end of a depolarizing current ramp stimulation was increased by 40.3 ± 5.3% (mean ± SEM; n = 9; 95% CI, 28.0–52.6) during application of 50 μM lidocaine (fig. 4A, top panel/ramp). Likewise, a rise in threshold by 44.2 ± 11.9% (mean ± SEM; n = 5; 95% CI, 11.1–77.3) was observed in the presence of mexiletine (50 μM). We also tested the effects of lidocaine and mexiletine on a 1-ms current pulse necessary to maintain the C-fiber compound action potential peak amplitude at 40% of the maximum (fig. 4A, top panel/test 40%). This parameter remained almost unchanged (< 5%).

Discussion

Multiple voltage-dependent sodium channels (Na_v) have been described in primary afferent, including nociceptive, neurons.[1–4,21] Suitable preparations for detailed electrophysiologic studies of Na_v in primary nociceptive neurons are dorsal root ganglion cells. Voltage-dependent sodium currents in such neurons can be observed with intracellular recording techniques. The aim of the current study was to characterize Na_v in the peripheral axons of unmyelinated human nerve fibers. These fibers are of very small diameter and are not accessible with intracellular recording techniques. A useful technique for functional studies on human C fibers is microneurography. This method has helped to gain much insight into the regulation of membrane excitability of nociceptive fibers in normal and pathophysiologic conditions.[22] Alternatively, information about the presence of voltage- or ligand-gated channels in the axonal membrane of human C-type nerve fibers can be obtained by a combination of threshold tracking techniques with pharmacologic tools using isolated segments of peripheral human nerve segments.[10,20,23]

The nerve segments used in the current study were obtained from patients, and we do not have "control" preparations from healthy volunteers. However, data from patients with chronic ischemia and with neuropathy of unknown etiology apparently do not differ from each other. This indicates that the disease condition does not seem to affect our findings. However, a definite conclusion would require experiments on nerve segments from healthy human subjects. Compound C-fiber action potentials are composed of action potentials from afferent and efferent fibers. However, the contribution of efferent sympathetic axons to the total number of

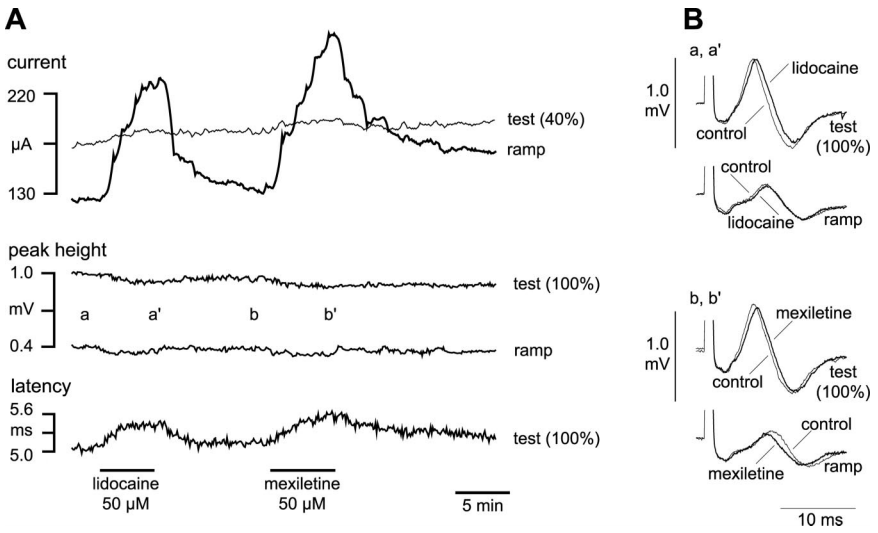

Fig. 4. Effects of lidocaine and mexiletine on peak height and membrane threshold of isolated human C fibers. (*A*) Continuous recordings of currents, peak amplitudes, and latency of C-fiber compound action potentials determined with the tracking stimulation parameters described in figure 1B. Note that the local anesthetics had a small effect on the maximal peak height (test 100%) but strongly elevated the current amplitude necessary to maintain constant peak amplitude after a preceding ramp stimulus (ramp). (*B*) Representative examples of the maximal (test 100%) C-fiber compound action potential and of C-fiber potentials recorded after a ramp stimulus (ramp) before and in the presence of lidocaine and mexiletine at time points indicated by the *letters*.

fibers is small (7–19%).[24] Also, tetrodotoxin-resistant Na_v 1.8 have not been described for postganglionic sympathetic neurons or fibers,[25] and it is unlikely that the key observation, *i.e.*, different sensitivity to tetrodotoxin of action potentials evoked with or without a preceding ramp stimulus, could be related to electrogenesis of sympathetic efferent fibers only.

In previous electrophysiologic studies of action potentials in isolated human nerve segments, the compound C-fiber action potential produced by a 1-ms current pulse was found to be rather insensitive to tetrodotoxin even in concentrations of several micromolars.[10,11] This observation in human unmyelinated C-fiber axons is similar to the presence of tetrodotoxin-resistant action potentials in C-type nerve fibers of other mammalian species.[26–29] It can be explained by the functional activity of the sodium channel subtype Na_v 1.8[30,31] because only graded action potentials can be elicited in about 80% of knockout Na_v 1.8 −/− mouse small C-type dorsal root ganglion neurons.[32] The current study describes a ramp stimulation protocol that reveals that also tetrodotoxin-sensitive sodium channels are functionally expressed in unmyelinated C-type peripheral human nerve fibers. It is possible that the molecular identity of this subtype of voltage-dependent sodium channel is Na_v 1.7 because other investigated sodium channels, such as the Na_v 1.4 and Na_v 1.6 sodium channels, show faster kinetics of closed-state inactivation.[9,12,33] However, we cannot exclude that further subtypes of Na_v expressed in dorsal root ganglion cells[1,4] contribute to membrane excitability after a ramp stimulus. The molecular identity could be deduced with more certainty by the use of subtype-specific blockers of tetrodotoxin-sensitive sodium channels. However, to our knowledge, such blockers are not publicly available at present.

It has been demonstrated that the adequate stimulus for activation of Na_v 1.7 is a small, slow membrane depolarization, *e.g.*, a "generator" potential because Na_v 1.7 is very slow in the kinetics of closed-state inactivation.[9,12,33] "Generator" potentials are produced by the transduction of (noxious) stimuli in the peripheral nerve endings. Furthermore, preferential immunoreactivity of peripheral nerve terminals to an antibody against Na_v 1.7 has been described.[34] These facts may indicate that the functional expression of Na_v 1.7 is restricted to nerve endings. However, in our studies over the past years on isolated human nerve segments, many voltage- and ligand-gated ion channels considered to be specific for terminals of unmyelinated axons were found to be present in the axonal membrane, too.[19,20] Therefore, we have used the axonal membrane of unmyelinated nerve fibers as a model for peripheral nerve terminals and have imitated a "generator" potential in the axonal membrane by a slow current ramp. Excitability of peripheral nerve fibers can be studied with high sensitivity using a computer-controlled threshold tracking technique (QTRAC).§ We have used this program to generate a current ramp with duration of 300 ms and small current amplitude.[9,12] Both membrane threshold and action potential peak height determined at the end of this current ramp were strongly altered in the presence of tetrodotoxin (figs. 2 and 3).

There are two possible explanations for the high sensitivity to tetrodotoxin of action potentials elicited after a ramp stimulus. First, tetrodotoxin blocks a ramp current which enhances membrane depolarization during a small current ramp.[9,12] Such an effect can contribute to the rise in "threshold" current necessary to maintain the compound action potential at a constant amplitude (fig. 3). Second, it has been described that, compared with tetrodotoxin-sensitive channels, slow inactivation of tetrodotoxin-resistant Na_v (*e.g.*, Na_v 1.8) is more complete at voltages reached with physiologic stimuli.[35] Therefore, it is likely that the sodium current during the action potential at the end of the ramp stimulus is carried by tetrodotoxin-sensitive channels (possibly Na_v1.7). This

view can explain the strong reduction of the action potential peak height by tetrodotoxin as illustrated in figure 2. Another finding in the current study is that tetrodotoxin produced an increase in the latency to the action potential, although the peak height was not changed much (figs. 2 and 3). This effect is also explainable by the assumption that, similar to the cell bodies in dorsal root ganglia,[36] multiple sodium currents contribute to action potentials in unmyelinated, including nociceptive, nerve fibers. In nociceptive sensory neurons, the tetrodotoxin-sensitive sodium conductance activates quickly and returns to zero at the peak of the action potential. In contrast, tetrodotoxin-resistant channels account for the main sodium conductance at the peak of the action potential.[36]

We also describe effects of lidocaine and mexiletine, which are used in the treatment of various types of neuropathic pain,[37,38] including erythromelalgia.[13–15] Lidocaine in concentrations of 50 μM reduced the axonal excitability at the end of a slow voltage ramp (fig. 4). This concentration is above the effective plasma concentrations of approximately 7.4–18.5 μM reached in the therapy of erythromelalgia[15] and above 6.2–33.9 μM used for patients with neuropathic pain after nerve injury[39] but far below the concentration of lidocaine necessary to induce a nerve conduction block ($>$ 1 mM).[40] The actual concentration of lidocaine at the axonal membrane in the human nerve segments may be below 50 μM because of diffusion barriers. It is also possible that voltage ramps with longer duration could increase the efficacy of lidocaine. The low concentration of lidocaine necessary to reduce axonal excitability at the end of the slow voltage ramp is in accordance with the observation that analgesic concentrations of lidocaine suppress spontaneous injury discharge in corneal C fibers but do not block action potential conduction.[41] This finding is plausible under the assumption that injury of the nerve terminal produces slow depolarizing "generator" potentials. It has been demonstrated that the steady state inactivation and the development of slow inactivation of Na_v 1.7 channels have a higher sensitivity to lidocaine as compared with blockade of the open channel.[42] Mexiletine is a structural analog of lidocaine, and the explanation of its effect in the current study seems to be identical to lidocaine.

In summary, our data indicate that the use of a slow current ramp, as suggested from observations using voltage clamp techniques,[12] is a useful parameter in electrophysiologic studies with the aim to reveal the function of Na_v 1.7 in the axonal membrane of unmyelinated, including nociceptive, peripheral nerve fibers. The findings also support the point of view[16,43] that the analysis of action potentials and membrane threshold of peripheral nerve fibers will benefit from the use of multiple measures of axonal excitability. Finally, the presence of several subtypes of Na_v in human nociceptive nerve fibers suggests that the knowledge about their role in different pain states and the availability of specific sodium channel blockers may result in a mechanism-based pain therapy.

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