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Delayed Depolarization and Slow Sodium Currents in Cutaneous Afferents

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SUMMARY AND CONCLUSIONS

1. Intraaxonal recordings were obtained in vitro from the sural nerve (SN), the muscle branch of the anterior tibial nerve (ATN), or the deafferented ATN (dATN) in 5- to 7-wk-old rats. Whole-nerve sucrose gap recordings were obtained from the SN and the ATN. This allowed study of cutaneous (SN), mixed motor and muscle afferent (ATN), and isolated muscle afferent (dATN) axons.
2. Application of the potassium channel blocking agent 4-aminopyridine (4-AP) to ATN or dATN resulted in a slight prolongation of the action potential. In contrast, a distinct delayed depolarization followed the axonal action potential in cutaneous afferents (SN) exposed to 4-AP. The delayed depolarization could be induced by a single whole-nerve stimulus or by injection of constant-current depolarizing pulses into individual axons. The delayed depolarization often gave rise to bursts of action potentials and was followed by a prominent afterhyperpolarization (AHP).
3. In paired-pulse experiments on single SN axons, the recovery time (half-amplitude of the action potential) was 3.06 ± 1.82 (SE) ms ($n = 12$). After exposure to 4-AP the recovery time of the delayed depolarization was considerably longer (half-recovery time: 99.0 ± 28.3 ms; $n = 15$) than that of the action potential (18.8 ± 9.1 ms; $n = 16$).
4. Application of tetraethylammonium (TEA) to cutaneous or muscle afferents alone had little effect on single action potential waveform. However, TEA reduced the amplitude of the AHP elicited by a single stimulus in cutaneous afferent axons after exposure to 4-AP and resulted in repetitive spike discharge.
5. The delayed depolarization and spike burst activity induced by 4-AP in SN was present in Ca^{2+} -free solutions containing 1 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid and was not blocked by Cd^{2+} (1.0 mM).
6. We obtained whole-cell patch-clamp recordings to study Na^+ currents from either randomly selected dorsal root ganglion neurons or cutaneous afferent neurons identified by retrograde labeling with Fluoro-Gold. The majority of the randomly selected neurons had a singular kinetically fast Na^+ current. In contrast, no identified cutaneous afferent neurons had a singular fast Na^+ current. Rather, they had a combination of kinetically separable fast and slow currents or a singular relatively slow Na^+ current.
7. These results demonstrate a difference in the sensitivity of myelinated cutaneous and muscle afferent axons to blockade of a 4-AP-sensitive K^+ channel. Cutaneous afferent axons give

rise to a prominent depolarizing potential after the action potential, which is not present in the muscle afferent or motor axons. We propose that cutaneous afferent axons have kinetically slow Na⁺ channels not present in muscle afferent and efferent fibers, whose activation underlies the delayed depolarization and multiple spike discharge. The results indicate a difference in the Na⁺ channel organization of myelinated cutaneous versus muscle afferent axons and their cell bodies.

INTRODUCTION

Mammalian myelinated axons express a diversity of K⁺ channel types that can be distinguished on the basis of kinetic and pharmacological properties (Baker et al. 1987; Kocsis et al. 1986, 1987). These include kinetically fast [4-aminopyridine (4-AP)-sensitive] and slow [tetraethylammonium (TEA)-sensitive] K⁺ currents (Roper and Schwartz 1989) and an inward rectifier (Baker et al. 1987; Birch et al. 1991; Eng et al. 1990). It has been suggested that the organization of these channels on myelinated motor and sensory axons may differ because these two groups of axons exhibit different sensitivities to K⁺ channel blockade (Bowe et al. 1985; Kocsis et al. 1986). Although blockade of the 4-AP-sensitive fast K⁺ current in motor axons results in a modest broadening of the action potential, a similar pharmacological blockade of sensory axons produces a distinct depolarization on the falling phase of the action potential, termed the delayed depolarization (Bowe et al. 1985; Kocsis et al. 1983). The delayed depolarization, which can exceed 20 mV and last for tens of milliseconds, often gives rise to bursts of spikes. The absence of this prominent depolarizing potential in motor axons suggests a fundamental difference in the ionic channel organization of sensory versus motor myelinated axons. Although several lines of evidence suggest that the delayed depolarization of sensory axons is the result of a Na⁺ channel that is kinetically distinct from the channel that gives rise to the action potential (Kocsis et al. 1983, 1987; Pongrácz et al. 1991), the electrophysiological origin of the delayed depolarization remains unresolved.

More recently, Bowe et al. (1992) demonstrated in regenerated nerves that the delayed depolarization is not present in all regenerated sensory myelinated axons and that it may be specific for cutaneous afferents. In the present study we examined the electrophysiological properties of normal myelinated cutaneous and muscle afferent axons using intraaxonal and sucrose gap recordings to determine the electrophysiological basis for these differences in cutaneous and muscle afferent axons. Our observations on these axons implicate a difference in Na⁺ channel organization; therefore whole-cell patch-clamp techniques were used to assess the Na⁺ channel properties of the dorsal root ganglion (DRG) cell bodies of origin for cutaneous and noncutaneous sensory axons.

Our results indicate that the delayed depolarization occurs in rapidly conducting cutaneous afferent axons and is virtually absent in muscle afferent and motor axons. Moreover, whole-cell voltage-clamp experiments on the cell bodies of cutaneous afferent fibers reveal a disproportionately large distribution of neurons that express slow Na⁺ currents or both fast and slow Na⁺ currents. In contrast, muscle afferent neurons displayed primarily a singular fast Na⁺ current. The presence of kinetically fast and slow Na⁺ currents on the cell bodies of the cutaneous afferent neurons supports the hypothesis that the delayed depolarization results from the activation of a slow Na⁺ current. The specificity of the delayed depolarization to cutaneous afferents and its association with burst firing further suggest a role of a slow Na⁺ current in cutaneous signal transduction.

METHODS

Female Wistar rats, ranging in age from 5 to 7 wk, were anesthetized with pentobarbital sodium (50 mg/kg) and exsanguinated by carotid section. Relatively young rats were selected because

of the diminishing sensitivity to 4-AP that occurs in mammalian myelinated axons during maturation (Bowe et al. 1985; Kocsis et al. 1983). The sural nerve (SN) was selected to study myelinated cutaneous afferents because it contains >90% sensory and autonomic fibers (Peyronnard and Charron 1982) and nearly all of myelinated sensory fibers are cutaneous afferents. To permit selective examination of muscle afferent axons we studied the muscle branch of the anterior tibial nerve (ATN) after ventral rhizotomy of L₄-L₆ (performed 10–12 days before ATN excision); this deafferented nerve is abbreviated dATN. We exposed the SN and ATN and excised a 1.5- to 3.0-cm segment of nerve distal to the sciatic notch. The nerves were desheathed and placed in a modified Krebs solution referred to as a normal electrolyte solution (NS). Only nerves that could be removed and desheathed with no apparent disruption were studied.

Solutions and drugs

The modified Krebs solution contained (in mM) 124 NaCl, 3.0 KCl, 1.3 NaH₂PO₄, 2.0 MgCl₂, 2.0 CaCl₂, 26.0 NaHCO₃, and 10.0 dextrose, saturated with 95% O₂-5% CO₂. Isotonic KCl solutions contained (in mM) 120 KCl, 7.0 NaCl, 1.3 NaH₂PO₄, 2.0 MgCl₂, 2.0 CaCl₂, 26.0 NaHCO₃, and 10.0 dextrose. The isotonic sucrose solution contained 320 mM sucrose. Test solutions containing TEA (10 mM), 4-AP (1 mM), or tetrodotoxin (TTX, 10 nM) were made by adding appropriate concentrations to the modified Krebs solution. The nerves were exposed to 4-AP and TEA for ≥20 min and no longer than 1 h.

Sucrose gap recording

Isolated SN and ATN segments were positioned across a sucrose gap chamber (Kocsis and Waxman 1983) partitioned into compartments by petroleum jelly seals. The center compartment was continuously washed with isotonic sucrose solution, the right compartment with modified Krebs solution to which blocking agents were added, and the left compartment with isotonic KCl solution. All solutions flowed at 1–2 ml/min. Experiments were carried out at 20°C.

The nerve segments were oriented with the distal end within the test compartment. The two outer compartments were connected to the inputs of a high-impedance DC-coupled differential electrometer (Axoprobe 1A; Axon Instruments) with silver–silver chloride wires embedded in agar bridge electrodes (3% agar in 1 M NaCl). The nerve was stimulated with a bipolar Teflon-coated stainless steel electrode cut flush and placed directly on the nerve segment in the test compartment. Stimulation pulses were delivered through an isolation unit and the timing of the pulses was controlled by a digital timing device. The high extracellular resistance in the middle (isotonic sucrose) compartment limited signal conduction through the axon cylinder. To allow for relatively homogeneous membrane activation and to minimize temporal dispersion, the number of active nodes was reduced by limiting the length of nerve segment positioned in the test well to 2–4 mm.

Intraaxonal recording

For intraaxonal recordings, nerves were placed in an in vitro submersion-type chamber. Intraaxonal recordings were performed with borosilicate electrodes pulled on a Brown-Flaming P-80 puller and filled with 4 M potassium acetate and 0.1 M KCl. The DC resistances of the microelectrodes ranged from 100 to 150 MΩ. Identification of intraaxonal recordings utilized criteria that have been discussed previously (Kocsis and Waxman 1982). Intraaxonal recordings with resting potentials larger than –50 mV and action potentials equal to or larger than resting potential were studied. Impalements were considered to be intracellular if passage of a constant hyperpolarizing current caused an increase in action potential amplitude compared with that recorded in the resting state (Barrett and Barrett 1982; Blight and Someya 1985; Kapoor et al. 1993; Kocsis and Waxman 1982). Whole-nerve stimulation pulses were delivered

through a bipolar Teflon-coated stainless steel stimulation electrode cut flush and placed directly on the nerve segment. Single axon stimulation pulses were delivered through the recording microelectrode and consisted of constant-current pulses (≤ 0.5 nA) ≤ 100 ms in duration provided by the step current command of the recording amplifier and monitored on a separate channel. An active bridge circuit was used to compensate for electrode and preparation resistance. Action potential recovery properties were evaluated during the presentation of paired stimuli delivered at varying interstimulus intervals. The sharp electrodes selected for larger axons and the relatively fast conduction velocities indicated that all axons studied were myelinated.

Cell culture

DRG cultures were prepared according to the methods of Birch et al. (1992), with slight modification. Lumbar (L₄-L₅) ganglia of 5- to 7-wk-old female Wistar rats were excised after exsanguination under pentobarbital anesthesia (50 mg/kg) and freed of both nerve trunks and connective tissue sheath. Pooled ganglia were incubated with gentle agitation for 25 min in a solution containing a mixture of complete saline solution (CSS) and 1 mg/ml collagenase (Boehringer Mannheim Biochemical), 0.2 mg/ml cysteine, 1.5 mM CaCl₂, 0.5 mM ethylenedinitrilotetraacetic acid, disodium salt. The CSS contained (in mM) 137 NaCl, 5.3 KCl, 1 MgCl₂, 25 sorbitol, 3 mM CaCl₂, and 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), titrated to pH 7.2 with NaOH. This was followed by a 10-min incubation with 30 U/ml papain (Worthington Biochemical), then a 25-min incubation in the above CSS mixture. Ganglia were then transferred to culture media containing 1:1 Dulbecco's Modified Eagles' Medium, and Ham's F12 medium containing 10% fetal calf serum, 1.5 mg/ml trypsin inhibitor, 1.5 mg/ml bovine serum albumin, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (GIBCO). The ganglia were then gently triturated using a siliconized Pasteur pipette and plated on a polyornithine/laminin coated glass coverslip.

In whole-cell patch-clamp experiments, DRG cell bodies giving rise to cutaneous afferent fibers were identified by retrograde labeling with Fluoro-Gold (Schmued and Fallon 1986). A 4% solution of Fluoro-Gold mixed in distilled water was injected subcutaneously in the lateral plantar region 1 wk before sacrifice for culture preparation.

Solutions for voltage-clamp experiments

Before electrical recording the coverslips containing cultured DRG were rinsed in a protein-free saline containing (in mM) 25 NaCl, 110 tetramethylammonium (TMA) chloride, 3.0 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 0.1 CdCl₂, 1.0 4-AP, and 10 HEPES titrated to pH 7.4 with NaOH (this added an additional 5–6 mOsm of Na⁺ ion). Cells placed in a recording chamber maintained their morphological and electrical properties in this saline bath at 18–20 °C for ≥ 2 h. Recording pipettes were filled with (in mM) 140 CsCl, 2 MgCl₂, 1 ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 10 HEPES titrated to pH 7.2 with CsOH.

Without external cadmium (0.1 mM) cells exhibited additional components of noninactivating inward current, presumably carried by Ca²⁺ ions. TMA was used as a nonpermeant monovalent cation to replace external Na⁺ to reduce current amplitude and therefore errors caused by series resistance artifact. Cs⁺ ions, known to be impermeant to most conventional K⁺ channels, were used to replace internal K⁺. In some experiments (Fig. 9, *C* and *D*) a small amount of sustained outward current remained at the end of a strong depolarizing pulse, indicating either that not all K⁺ had been completely dialyzed from the internal milieu or that a Cs⁺-resistant component was present.

Voltage-clamp recording technique

The whole-cell patch-clamp method (Hamill et al. 1981) was used to record voltage-dependent Na^+ currents from the cultured DRG. Patch electrodes (Corning #7052 capillary glass) of 1.2–1.6 M Ω were constructed and fire-polished using a Narishige PP-83 vertical puller and MF-83 microforge. The electrodes were mounted on the headstage of a Medical Systems APC-8 patch-clamp amplifier using a 500-M Ω feedback resistor. The shunt capacitance between the pipette and bath was kept at a minimum by maintaining the bath level to ~10–20 μm above the cell being clamped. Capacity current was further reduced, along with linear leak currents, via P/4 pulse protocols (Bezánilla and Armstrong 1977).

The current was sampled and converted into (16 bit) digital form using an acquisition system (ITC-16, Instrutech) that interfaced the patch-clamp amplifier with an Apple Macintosh computer. The current was sampled at 50 kHz and low-pass filtered (4-pole Bessel filter) at 10 kHz. Generally, four sweeps were averaged at each test pulse.

RESULTS

Action potential waveforms of cutaneous afferent, normal, and deafferented muscle nerves recorded in NS

The compound action potential (CAP) and single fiber action potentials were similar in shape and time course for cutaneous, muscle, and deafferented muscle nerves. The control CAPs of the SN and the ATN recorded in the sucrose gap (Fig. 1, *A* and *B*) were similar in shape and waveform. The average CAP half-width for SN was 0.64 ± 0.13 (SD) ms ($n = 14$); for ATN it was 0.64 ± 0.07 ms ($n = 8$). Similarly, mean spike half-width calculated from single axon recordings in SN ($n = 16$), ATN ($n = 4$), and dATN ($n = 10$) were relatively comparable: 0.81 ± 0.24 , 0.75 ± 0.06 , and 0.74 ± 0.24 ms, respectively. Rarely was spontaneous impulse activity observed in any of the axon populations during recording in NS. Low-amplitude depolarizing afterpotentials were occasionally observed but were not common features of the action potentials.

Effects of 4-AP in action potential waveform of cutaneous afferents and normal and deafferented muscle nerves

Application of 4-AP led to different changes in muscle and sensory nerve responses. The ATN action potential was prolonged in duration, with a gradual and continuous return to baseline over several milliseconds (Fig. 1*B*). In contrast, the response recorded from the SN (Fig. 1*A*) displayed a delayed depolarization and characteristic “ripple” after the initial action potential waveform (Kocsis et al. 1983).

Intraaxonal recordings indicate that the differences in CAP waveforms are attributable to differences in 4-AP-induced membrane potential and burst-firing patterns. After exposure to 4-AP, the action potentials recorded in axons from intact ATNs and dATNs were broadened (Fig. 1, *D* and *F*). In contrast, application of 4-AP to SN resulted in a pronounced depolarization after the initial potential (the delayed depolarization) and individual axons responded to a single brief (100 μs) stimulus with bursts of action potentials arising from the delayed depolarization (Fig. 1*C*). Although some axons in SNs bathed in NS showed isolated “spontaneous” action potentials, a single stimulus never evoked spike burst activity.

A temporal correspondence was noted between the late component of the whole-nerve response (Fig. 1*A*, arrowhead) and the delayed depolarization with superimposed spike burst activity recorded intraaxonally (Fig. 1*C*) from the same SN. The initial action potential was more reliably elicited than the late spikes and the late spikes varied in latency and amplitude from sweep to sweep. The delayed depolarization often terminated in an afterhyperpolarization

(AHP), as shown in Fig. 2C; both the delayed depolarization and AHP occurred after whole-nerve or single axon stimulation. In Fig. 1E, the fiber was activated by passage of a constant-current depolarizing pulse through the recording microelectrode. Note the distinct hump (delayed depolarization) after the action potential.

Comparison of effects of 4-AP and TEA

To examine the role of TEA-sensitive K^+ current in muscle afferents and cutaneous afferents we studied SN and dATN before and after exposure to TEA. Application of TEA alone to either type of nerve had a relatively negligible effect on single action potential waveform (Fig. 2, A and B). However, striking effects were observed when TEA was applied to 4-AP-treated sensory nerves. In the presence of 4-AP a prominent AHP followed the delayed depolarization elicited by a single stimulus in SN (Fig. 2, C and E). The peak amplitude of the AHP shown in Fig. 2E is 5 mV and the duration is ~100 ms. Application of 10 mM TEA reduced the 4-AP-induced AHP (Fig. 2, D and F) and resulted in repetitive spike discharge. The combination of 4AP and TEA led to increased spontaneous action potential activity.

These results are in agreement with previous studies that demonstrated a TEA-sensitive AHP that is enhanced in the presence of 4-AP (Eng et al. 1988).

Recovery time of the action potential and the delayed depolarization in SN

We used paired stimuli, presented at varying interstimulus intervals, to examine the refractory period of the initial action potential and the delayed depolarization in the SN after 4-AP application. The refractory period of the delayed depolarization, as measured by the time course of amplitude recovery, was significantly greater than that of the initial action potential. The time courses of recovery of the action potential before and after 4-AP application are presented in Fig. 3, A and B, respectively. When a conditioning stimulus was presented at an interstimulus interval of 30 ms, the action potential was elicited, but the delayed depolarization was not observed (Fig. 3C). However, the delayed depolarization appeared with a longer interstimulus interval (200 ms) (Fig. 3D). The amplitudes of the test action potential and delayed depolarization in a SN were evaluated separately as a function of interstimulus interval (Fig. 4A); the increased recovery time of the delayed depolarization is evident. A discrepancy between the recovery times of the action potential and the delayed depolarization was also noted in assessments of the interstimulus interval required to attain 50% recovery of the amplitude compared with the control action potential (Fig. 4B). The half-recovery time was 3.06 ± 1.82 ms ($n = 12$) for control SN action potentials, 18.84 ± 9.13 ms ($n = 16$) for the initial SN action potential after exposure to 4-AP, and 99.06 ± 28.27 ms ($n = 15$) for the delayed depolarization.

Effects of membrane potential on delayed depolarization

The magnitude of the delayed depolarization is affected by membrane potential. Shifts in the holding potential in the depolarizing direction were accompanied by a comparable amplitude decrement in the delayed depolarization in the SN (Fig. 5). Conversely, when the holding potential was shifted in the hyperpolarizing direction the delayed depolarization became larger. Holding potentials positive to -40 mV eliminated the response. These data suggest that the mechanism underlying the delayed depolarization is an active, voltage-dependent process that is sensitive to the membrane potential before stimulation.

Effects of Ca^{2+} and Na^+ channel blockade on the 4-AP-induced delayed depolarization of cutaneous afferents

The effects of 4-AP on cutaneous sensory nerves were not dependent on the presence of extracellular Ca^{2+} . Nerves were bathed for 1 h in a superfusion solution in which $CaCl_2$ was

removed and replaced by a solution containing 2.0 mM MgCl₂ and 1 mM EGTA. Introduction of 4-AP in this Ca²⁺-free environment was still effective in eliciting a delayed depolarization (Fig. 6A). Application of Cd²⁺ (1 mM), a Ca²⁺ channel blocker (Hagiwara and Byerly 1981) to the superfusate did not significantly alter the action potential waveform. It also did not block the 4-AP-induced burst activity (Fig. 6B) or the delayed depolarization and AHP (Fig. 6C). Phosphates were excluded from the solutions to prevent Cd²⁺ precipitation. The Na⁺ channel blocking toxin TTX (10 nM) completely blocked both the early and late depolarization induced by 4-AP after whole-nerve stimulation (Fig. 7A). Responses to intraaxonal passage of a constant-current depolarizing pulse were also inhibited by TTX (Fig. 7B). The delayed depolarization appeared to require the preceding strong depolarization of the initial spike for activation.

Na⁺ currents on DRG neuronal cell bodies

Whole-cell patch-clamp recordings were obtained from either randomly selected DRG neurons 44–50 μm diam or those of the same size range identified by retrograde labeling with Fluoro-Gold (see METHODS). Only neurons without neurites were selected to minimize problems associated with space clamp. An example of a Fluoro-Gold-labeled DRG neuron is shown in Fig. 8. Of the two neurons observed with bright field microscopy (Fig. 8A), only one is retrogradely labeled by subcutaneous injection of Fluoro-Gold, indicating that it is a cutaneous afferent neuron. Of the population of 27 randomly selected size-matched neurons (i.e., a group containing muscle and cutaneous afferents), 18 (67%) had a singular fast Na⁺ current of the form seen in the neuron of Fig. 9, C and D; 2 had kinetically separable (fast and slow) Na⁺ currents, 5 had only a single slow current, and 2 were indeterminate.

The Na⁺ currents shown in Fig. 9, C and D, were recorded from a nonlabeled, randomly selected 48-μm-diam neuron. In Fig. 9C the neuron was held at –60 mV, preconditioned at –120 mV for 150 ms, then depolarized to test potentials from –40 to +20 mV in steps of 10 mV as shown in Fig. 9A. The inward current, which follows after a variable delay, exhibits voltage-dependent kinetics, spontaneous decrease in the current after a maximum, and reversal near that expected for Na⁺ ion (+50 mV under these conditions). The current traces in Fig. 9D were recorded from the same cell but using the protocol shown in Fig. 9B. In this protocol the 150-ms conditioning potential was varied between –140 and –40 mV in steps of 10 mV and followed by a test potential to +20 mV (to simplify the figure, not all sweeps are shown). The kinetics of each trace are the same, but the peak amplitude decreases as the conditioning potentials become more positive and eventually approaches 0 at –40 mV. Thus this neuron had a uniform population of Na⁺-selective channels that exhibited rapid, voltage-dependent activation and inactivation, and steady-state inactivation properties.

In 14 Fluoro-Gold-identified cutaneous afferent neurons in the same size range, 6 had kinetically separable (fast and slow) Na⁺ currents, another 6 had a single relatively slow current, and the remaining 2 were relatively slow but indeterminate as to whether or not more than one current was present. Although it might be argued that Fluoro-Gold labeling could have affected channel expression and/or current kinetics, neurons with either an isolated slow variety or with multiple current components were present in unlabeled preparations. Figure 9, E and F, shows voltage-dependent inward currents recorded from a neuron that had kinetically separable currents. Using the same stimulation protocols as in Fig. 9, C and D, the identified cutaneous afferents revealed a more complex inward current that appeared to be the result of activation of two types of Na⁺ selective channels. In Fig. 9E, weak depolarizations elicited a prolonged, inward current that showed little sign of inactivation after 12 ms. At –20 mV a small bump in the record (*) indicates the emergence of a faster kinetic component. The peak of this faster component was best observed at strong depolarizations of +10 to +20 mV (Fig. 9E) and occurred at ~1.5 ms. The singular fast current of Fig. 9C had a peak current at 0.9–10 ms at

the same depolarization. At +20 mV the net current of Fig. 9E takes the form of a sharp peak followed by a ramplike decay that does not conform to a simple exponential decay, as would occur if a uniform population of channels were inactivating. Taken together, the slow component of current, when compared to the fast component, appeared to activate at test potentials 10–20 mV more negative.

It is unlikely that the slow currents we identified on DRG cell bodies are a result of artifact related to unfavorable space-clamp conditions for two reasons. First, the neurons recorded from were 44–50 μm diam with a corresponding surface area of 6,000–8,000 μm^2 and a theoretical capacitance of 60–80 pF. Neurons without neurites were selected for these studies and the measured capacitances tended to confirm the absence of neurites. The capacitive currents we observed had time constants of 0.1–0.3 ms, corresponding to series resistance, in the worst case, of $\sim 4\text{ M}\Omega$. This would tend to impose a lower limit on the kinetic measurements of the faster variety of current such as that shown in Fig. 9, C and D. Second, the presence of a combination of fast and slow currents in cells of equivalent size and therefore capacitance favors the idea that two separate channel families underlie these currents. The current plots depicted in Fig. 9E indicate the presence in a single cell of kinetically separable components. It is not clear whether the faster component, which occurs in combination with a slow component, corresponds to the fast Na^+ channel that was observed in isolation in other sensory neurons (Fig. 9, C and D). The fast current that occurs in isolation peaks earlier than the fast Na^+ current that occurs in combination with a slower one in other cells under the same stimulating conditions. The presence of multiple types of Na^+ channels in 44- to 50- μm DRG neurons is consistent with previous observations (Caffrey et al. 1992; Roy and Narahashi 1992).

In Fig. 9F, the two kinetic components show different sensitivity to the conditioning potential. At -140 mV conditioning potential the current reaches a peak (*) and then follows a ramplike decay. With progressively more positive conditioning potentials, the sharp peak gives way to a more smoothly contoured peak whose maximum amplitude (#) occurs later than the sharp peak. This result conforms with those of Caffrey et al. (1992) and of Roy and Narahashi (1992), and with our data (not shown) in which the steady-state inactivation of the slower form of Na^+ conductance, when studied in isolation, is incomplete and is shifted to more positive potentials (Rizzo et al. 1993).

DISCUSSION

Mammalian myelinated axons express several pharmacologically and kinetically distinct ion channel types that influence the shape and patterning of action potentials (Baker et al. 1987; Eng et al. 1988; Kocsis et al. 1986, 1987; Roper and Schwartz 1989). It is now appreciated that individual channel types are regionally distributed along the myelinated axon in a nonuniform fashion. Sodium channels cluster in high density at the node of Ranvier (Black et al. 1990; Brismar 1980; Chiu and Ritchie 1981; Chiu et al. 1979; Waxman 1977; Waxman and Ritchie 1993). The distribution of K^+ channels in mammalian axons is more complex in that at least two types of K^+ currents have been identified: a fast 4-AP-sensitive current and a slower TEA-sensitive K^+ current (Baker et al. 1987; Kocsis et al. 1986; Roper and Schwartz 1989; see Black et al. 1990 for review). Recent work suggests that the TEA-sensitive current is present at the node and that the 4-AP-sensitive current has a greater representation in the paranodal or internodal axon membrane (Baker et al. 1987; Eng et al. 1988). Additionally, an inwardly rectifying current, which utilizes both Na^+ and K^+ as charge carriers, is present on both peripheral (Baker et al. 1987; Birch et al. 1991) and CNS axons (Eng et al. 1990).

Despite generally similar morphological features at both the light and electron microscopical level (Bowe et al. 1992; Fields et al. 1986), different types of mammalian myelinated axons subserve

specialized physiological functions and it is reasonable to suspect that their selective physiological properties may be subserved by distinctive distributions of various ion channels. It has been suggested that sensory fibers may have kinetically fast and slow Na^+ channels. This possibility was recognized in studies using intraaxonal recording techniques on sciatic nerve fibers (Kocsis et al. 1983). A subpopulation of axons gave rise to a prominent, Ca^{2+} - independent, delayed depolarization after the action potential after application of 4-AP (Bowe et al. 1985). This potential was present on sensory (dorsal root) and not motor (ventral root) fibers (Bowe et al. 1985, 1992; Kocsis et al. 1986). It should be pointed out that relatively young animals were used in the present study because the effects of 4-AP on axons attenuate during maturation (Kocsis et al. 1983). However, on demyelination of adult nerves 4-AP elicits effects on sensory and motor axons similar to those of immature rats, including the delayed depolarization (Targ and Kocsis 1986). The results of the present study further demonstrate that the delayed depolarization is not a general property of sensory axons, as has been previously suggested, but rather is specific for cutaneous afferents.

Mechanism underlying the delayed depolarization

The delayed depolarization we described in cutaneous afferents is distinct from the depolarizing afterpotential previously reported (Barrett and Barrett 1982; Blight and Someya 1985; Bowe et al. 1987). The latter can be explained on the basis of passive discharge of the internodal capacitance through the myelin resistance. The delayed depolarization in cutaneous afferent axons was observed only after blockade of the 4-AP-sensitive K^+ channel. Several lines of evidence have been presented that indicate that the delayed depolarization results from activation of a Na^+ -selective channel that is distinct from the channel that underlies the action potential. First, the delayed depolarization can be selectively inactivated and its amplitude is directly dependent on the holding potential (Fig. 5). These observations support the idea that the delayed depolarization is due to membrane permeability changes to an ion whose reversal potential is positive to the resting potential. An alternative candidate to Na^+ would be Ca^{2+} , but this is unlikely to be the charge carrier because the delayed depolarization is present in the absence of external Ca^{2+} and could not be inhibited by Cd^{2+} .

It is well established that both TTX-sensitive and TTX-resistant Na^+ channels are present on mammalian DRG neurons (Caffrey et al. 1992; Elliot and Elliot 1993; Kostyuk et al. 1981; McLean et al. 1988; Roy and Narahashi 1992). Such a demonstration has not been made for mammalian axons. In our experiments on axons both the action potential and the delayed depolarization were blocked by TTX. This does not, however, prove that the delayed depolarization results from the activation of a TTX-sensitive current because with our recording configuration the delayed depolarization may be activated by the action potential. Definitive characterization of the kinetics and pharmacology of the current underlying the delayed depolarization will require a voltage-clamp analysis of the nodal and possibly paranodal axonal membranes in cutaneous afferent fibers. To date, such an analysis of these relatively small myelinated axons has not been carried out. However, our patch-clamp studies on the cell bodies of identified cutaneous afferents indicate that a relatively large proportion of these neurons express on their membranes at least two populations of Na^+ channels distinguishable both by kinetics and sensitivity to the conditioning potential. Taken together these results are consistent with the idea that the slow Na^+ channels are present on the axons of cutaneous afferents.

An alternative and more traditional explanation to account for the delayed depolarization is that a single Na^+ channel type can support late currents over a narrow voltage domain in which neither of the steady-state activation (m_∞) and inactivation (h_∞) parameters are 0 (Hodgkin and Huxley 1952; McAllister et al. 1975). If the m_∞ - h_∞ overlap occurred after a single action potential, this could account for a prolonged, weakly depolarizing inward current. However,

the presence of kinetically fast Na^+ spikes generated by and superimposed on the delayed depolarization make this explanation unlikely. Furthermore, this rationale cannot account for the amplitude in the delayed response during strong depolarizations when the membrane potential is positive to the overlapping voltage domain.

Another possibility is that a fraction of a single population of Na^+ channels enters a gating mode, which allows delayed openings and slower inactivation kinetics (Alzheimer et al. 1993; Armstrong and Bezanilla 1977; Patlak and Ortiz 1986; Sigworth 1981). These descriptions generally do not apply to what in our case would correspond to a significant fraction (>50%) of the channels, and the slow inactivation time constants are 1 order of magnitude faster than what we observed in our preparations. The ratio of the amplitude of slow versus fast components of Na^+ channel inactivation (Neumcke and Stämpfli 1982) in large rat sciatic nerve fiber was found to be <0.4 for depolarizations positive to -50 mV (in the presence of 6 nM TTX). For weak depolarizations (-38 mV) the slow inactivation time constant was 1.23 ms, ≥ 1 order of magnitude faster than the delayed depolarization observed in our preparation. These early studies were performed on large myelinated nerve fibers, and it appears unlikely that the fast Na^+ currents of our sensory axons are capable of an as yet undiscovered kinetically slow gating mode that could support such a large and prolonged inward current.

Unique Na^+ current on cutaneous afferent neurons

The present results support the idea that DRG neurons corresponding to cutaneous afferent neurons have a population of Na^+ channels that is unique and distinguishes them from afferents that arise from noncutaneous receptors. DRG cells giving rise to the cutaneous afferents are more likely to have a relatively slow Na^+ channel or more than one population of Na^+ channels when compared to all size-matched, randomly selected neurons. Moreover, when cutaneous afferents were selectively examined, none had rapidly activating and inactivating channels. In contrast, in a randomly chosen population of DRG cells containing muscle as well as cutaneous afferent neurons, 67% of the cells exhibited a singular “fast” current. The relative distribution of these currents in randomly selected and identified cutaneous afferent neurons is summarized in Figure 10. These data suggest that most noncutaneous afferent neurons express a uniform population of rapidly activating and inactivating Na^+ channels. In contrast, cutaneous afferent neurons have a greater representation of kinetically slower Na^+ channels, often with multiple types on the same neuron. These results are consistent with the idea that cutaneous afferent axons may have kinetically distinct and multiple Na^+ channels.

Functional role of the delayed depolarization

The functional role of the delayed depolarization on cutaneous afferents is not certain. Similar responses have been suggested to be associated with pathological responses, i.e., abnormal repetitive impulse generation after blockade of fast K^+ channels (Kocsis and Waxman 1987; Kocsis et al. 1983). One possibility is that slow Na^+ channels along the trunks of cutaneous afferent axons play a role in high-frequency discharge. Alternatively, it is possible that the functionally relevant site for a kinetically slow Na^+ channel on cutaneous afferents is at the sensory ending in the periphery, where this channel may participate in transduction or regulation of incoming sensory impulse activity. The deposition of slow Na channels along the axonal trunk, i.e., along the course of their transport from the site of synthesis in the cell body to peripheral sensory endings, may be unimportant for normal impulse activity. The localization of fast K^+ currents at the paranodal and internodal region would prevent activation of the ectopic Na^+ currents under normal conditions, thereby minimizing evolutionary pressure for the development of a more selective targeting mechanism to route these channels exclusively to the sensory ending.

We propose that blockade of the 4-AP-sensitive K^+ current allows the delayed activation of the kinetically slower Na^+ current that underlies the delayed depolarization. The delayed depolarization was not generated when the 4-AP-sensitive K^+ current was not blocked. This observation suggests that a possible functional role of the 4-AP-sensitive K^+ current in cutaneous afferents is to limit generation of the delayed depolarization and the associated spike burst activity.

The use of ionic channel blocking agents in the treatment of patients with a variety of neurological disorders has further elucidated the potential functional importance of specific ionic conductances. Potassium channel blockade with 4-AP has been examined in clinical studies in patients with Eaton-Lambert syndrome (Lundh et al. 1977; Murray and Newsom-Davis 1981) and multiple sclerosis (Davis et al. 1990; Jones et al. 1983; Stefoski et al. 1987) in an attempt to enhance the release of synaptic transmitters and to overcome conduction block, respectively. Paresthesias and dysesthesias have been reported as side effects of 4-AP, whereas distinctive motor afferent or efferent complications have not been noted (Lundh et al. 1979; Murray and Newsom-Davis 1981). These observations further emphasize the specificity of 4-AP action with regard to the induction of burst firing in cutaneous sensory fibers.

In conclusion, the results reported here demonstrate a fundamental difference in the electrophysiological organization of cutaneous versus muscle afferent myelinated axons. In particular, cutaneous afferent axons give rise to a prolonged depolarizing potential after the action potential after blockade of fast 4-AP-sensitive K^+ channels. Several lines of evidence, including whole-cell patch-clamp experiments on the cell bodies of the cutaneous afferent axons, suggest that a kinetically slow Na^+ channel underlies the depolarizing after-potential. These properties of cutaneous afferent axons provide a basis for pharmacologically distinguishing them from motor fibers and from muscle afferent fibers. Moreover, the expression of the delayed depolarization on cutaneous afferents predicts a plausible mechanism for the generation of ectopic impulse activity that may be specific for cutaneous afferents.

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FIG. 1.

Superimposed action potentials recorded from the sural nerve (SN), anterior tibial nerve (ATN), and deafferented ATN (dATN) before and after superfusion with 4-aminopyridine (4-AP) (1 mM). Compound action potential recorded in the sucrose gap chamber from SN (A) and ATN (B). Intraaxonal recordings from SN (C), ATN (D), and dATN (F) are also shown. An action potential elicited by long depolarization pulse applied into a single axon in the SN through the recording microelectrode after 1 mM 4-AP application (E). Arrowheads: response after 4-AP application.

FIG. 2.

Intraaxonal recordings of action potentials from SN and dATN with and without tetraethylammonium (TEA) (10 mM). Superimposed action potentials recorded from SN (A) and dATN (B) before and after 10 mM TEA superfusion. C: in the presence of 4-AP a single whole-nerve stimulus leads to a delayed depolarization in SN. A prominent afterhyperpolarization (AHP) follows the delayed depolarization, as shown in C and E (longer time base). D: when TEA is applied in combination with 4-AP (D, and at a longer time base in F) the AHP is eliminated and a single stimulus induces repetitive firing.

FIG. 3.

Single fiber action potentials recorded from SN. A: series of action potential responses to double shock stimulation at varying interstimulus intervals before (A) and after (B–D) superfusion with 4-AP. When conditioned by another stimulus (interstimulus interval, 30 ms) the delayed depolarization is obliterated but the initial spike is not (C). However, the delayed depolarization appears with a longer interstimulus interval (200 ms) (D).

FIG. 4.

Graphic presentation of amplitude recovery vs. interstimulus interval in single fiber examined under various conditions as described above in Fig. 3. Peak amplitude (percent of control value) of initial spike and the delayed depolarization of single fiber action potential response plotted vs. interstimulus interval before and after 4-AP application (*A*). Note the increased refractoriness of the delayed depolarization. *B*: comparison of the interstimulus interval required to attain 50% recovery of the amplitude for each group. Error bars: SD.

FIG. 5.

Relationship between the magnitude of the delayed depolarization and the resting membrane potential. When spikes arose from the delayed depolarization the peak was measured from an extrapolated waveform without spikes. Data were recorded at various resting membrane potentials (-80 to -20 mV). Selected responses (-40 , -55 , and -75 mV) are shown.

FIG. 6. Effects of Ca²⁺-free and Cd²⁺ solutions on 4-AP-induced delayed depolarization and bursting activity. Intraaxonal recordings of action potentials recorded from SN without Ca²⁺ [with 1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)] generate both a delayed depolarization and burst activity (A). Cd²⁺ (1 mM) did not block burst activity (B) or the delayed depolarization (C). A–C were obtained from different axons.

FIG. 7. 10 nM tetrodotoxin (TTX) blocks the delayed depolarization and bursting activity as well as initial spike. Superimposed intraaxonal recordings after presentation of a single stimuli in SN before and after TTX application (A). B: both the initial spike and the delayed depolarization elicited by long depolarization pulse applied into the axon through the recording microelectrode are also blocked by 10 nM TTX. The initial action potential and the delayed depolarization do not recover even if stronger depolarizing pulse is applied after TTX application.

FIG. 8. Bright field (A) and fluorescence images of dorsal root ganglion (DRG) neurons selected for voltage-clamp experiments. The labeled neuron in B was identified after subcutaneous injection of Fluoro-Gold and therefore considered to be a cutaneous afferent neuron. Calibration in A is 20 μm .

FIG. 9.

Whole-cell patch-clamp recordings from 2 DRG neurons after 24 h in culture. Two stimulus protocols were applied. *A*: from a holding potential of -60 mV the cells were conditioned at -120 mV for 100 ms, then depolarized to -40 to $+20$ mV in steps of 10 mV. *B*: cells were conditioned for 150 ms at -140 to -40 mV in steps of 10 mV, then depolarized to $+20$ mV. *C* and *D*: voltage-dependent Na^+ currents recorded from a randomly chosen medium-sized neuron using protocols from *A* and *B*, respectively. *D*: not all current records are displayed for clarity. Note that a small amount of sustained outward current remained during the test pulse indicating that internal cesium had not adequately diffused into the cell from the pipette. *E* and *F*: currents were recorded from a cutaneous afferent neuron identified via retrograde Fluoro-Gold labeling. Stimulus protocols from *A* and *B* were applied to *E* and *F*, respectively. External and internal solutions and recording techniques are described in **METHODS**. Temperature: 19°C . *F*: record obtained from a holding potential of -20 mV was subtracted from each record. Traces in *E* are marked with test potential for clarity.

FIG. 10.

The relative distribution of “singular fast,” “combination of fast and slow,” “singular slow,” and “indeterminate” currents in randomly selected DRG neurons 44–50 μm diam and cutaneous afferent neurons of the same size range identified by retrograde labeling with Fluoro-Gold. Note that randomly selected neurons utilize a large population of singular fast Na^+ current. In contrast, cutaneous afferent neurons have a greater representation of kinetically slower Na^+ current, often with multiple types.