Function of the Hyperpolarization-Activated Inward Rectification in Nonmyelinated Peripheral Rat and Human Axons

PETER GRAFE, 1 STEFAN QUASTHOFF, 2 JULIAN GROSSKREUTZ, 2 AND CHRISTIAN ALZHEIMER 1

1Department of Physiology, University of Munich, D-80336 Munich; and 2Department of Neurology, Technical University of Munich, D-81675 Munich, Germany

Grafe, Peter, Stefan Quasthoff, Julian Grosskreutz, and Christian Alzheimer. Function of the hyperpolarization-activated inward rectification in nonmyelinated peripheral rat and human axons. J. Neurophysiol. 77: 421–426, 1997. The function of time-dependent, hyperpolarization-activated inward rectification was analyzed on compound potentials of nonmyelinated axons in the mammalian peripheral nervous system. Isolated rat vagus nerves and fascicles of biopsied human sural nerve were tested in a three-chambered, Vaseline-gap organ bath at 37°C. Inward rectification was assessed by recording the effects of long-lasting hyperpolarizing currents on electrical excitability with the use of the method of threshold electrotonus (program QTRAC, copyright Institute of Neurology, London, UK) and by measuring activity-dependent changes in conduction velocity and membrane potential. Prominent time-dependent, cesium-sensitive inward rectification was revealed in rat vagus and human sural nerve by recording threshold electrotonus to 200-ms hyperpolarizing current pulses. A slowing of compound action potential conduction was observed during a gradual increase in the stimulation frequency from 0.1 to 3 Hz. Above a stimulation frequency of 0.3 Hz, this slowing of conduction was enhanced during bath application of 1 mM cesium. Cesium did not alter action potential waveforms during stimulation at frequencies <1 Hz. Cesium-induced slowing in action potential conduction was correlated with membrane hyperpolarization. The hyperpolarization by cesium was stronger during higher stimulation frequencies and small in unstimulated nerves. These data show that a cesium-sensitive, time-dependent inward rectification in peripheral rat and human nonmyelinated nerve fibers limits the slowing in conduction seen in such axons at action potential frequencies higher than ~0.3 Hz.

INTRODUCTION

Inward rectification induced by membrane hyperpolarization has been demonstrated in myelinated and nonmyelinated axons of the mammalian peripheral nervous system and CNS (Baker et al. 1987; Birch et al. 1991; Eng et al. 1990; Marsh 1982; Waxman 1995). In addition, recordings of threshold electrotonus have provided evidence that inward rectification also occurs in human axons (Bostock and Baker 1988), and that its effects on excitability are more pronounced in sensory than motor axons (Bostock et al. 1994). Recent data from peripheral nerves in human diabetic neuropathy indicate that axonal inward rectification might be abnormal in pathophysiological conditions (Horn et al. 1996).

The inward rectifier in axons is activated relatively slowly by hyperpolarization and is blocked by Cs+; the underlying ion channels are permeable to both Na+ and K+ ions (for review see Waxman 1995). It resembles in all aspects the hyperpolarization-activated cation current Ih found in the somata of neurons (Pape 1996). It has been suggested that this conductance may maintain membrane potential at appropriate levels during high-frequency firing when electrogenic pump activity otherwise might result in excessive hyperpolarization. In fact, inhibition of inward rectification by extra-cellular application of Cs+ has been found to reduce excitability of myelinated peripheral axons during stimulation frequencies >50 Hz (Baker et al. 1987).

Nonmyelinated axons are more sensitive than thick myelinated axons to repetitive activation. The rise of intraxon sodium and/or calcium (Lüscher et al. 1996) concentrations and the subsequent membrane hyperpolarization due to activation of electrogenic sodium extrusion are much more prominent in small, nonmyelinated fibers compared with thick, myelinated ones (Ritchie 1995). These mechanisms presumably contribute to the slowing of conduction seen, for example, in human C fibers at stimulation frequencies as low as 1 Hz (Schmelz et al. 1995; Torebjörk 1974). The extent of membrane hyperpolarization should be controlled by the activation of inward rectification, which has been reported in nonmyelinated axons (Marsh 1982), but the importance of this conductance for the excitability of C fibers has not previously been explored. The results of the present study demonstrate that a hyperpolarization-induced inward rectification helps to maintain the excitability and conduction velocity of nonmyelinated axons, or part of them, conducting impulses at frequencies of ≥1 Hz. Parts of the data have been presented in abstract form (Grafe et al. 1996).

METHODS

Preparation

The experiments were performed on isolated rat vagus nerves and specimens of human sural nerves. Male Wistar rats weighing 250–500 g were anesthetized with urethan (1.5 g/kg ip, supplemented as required) for exposure of the vagus nerve as described by Brown and Marsh (1978). The experiments on isolated human sural nerves (from 9 patients) have been carried out in accordance with requirements of the ethics committee of the Technical University, Munich, Germany, and were performed after informed written consent was obtained from the patients. After biopsy, single fascicular segments 20–25 mm in length were prepared as previously described in detail (Quasthoff et al. 1995).

Experimental setup

After preparation, single fascicles of human sural nerves and desheathed nerve trunks of the cervical rat vagus nerve were tested in an experimental organ bath (Marsh chamber; Hugo Sachs Elek-
stimulus. The starting time of the polarizing currents was stepped from 2 ms after the test stimulus to 398 ms before it, over a period of 10–15 min.

Solutions

The standard solution contained (in mM) 118 NaCl, 3.4 KCl, 0.8 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, and 5 glucose, pH 7.4, bubbled with 95% O₂-5% CO₂. Cesium chloride was purchased from Sigma (Deisenhofen, Germany) and added to the standard bathing solution.

RESULTS

Membrane excitability during long-lasting hyperpolarization is cesium sensitive

In our first series of experiments, computer-controlled tracking of threshold electrotonus (see METHODS) was used to follow changes in membrane excitability of C fibers in nine isolated rat vagus nerves during long-lasting hyperpolarizing currents (200 ms). In the examples illustrated in Fig. 2, A and B, the level set as threshold was 40% of the maximal C fiber peak amplitude. A time window was set to exclude any contribution of fast-conducting A fibers to the recording. The alterations in current threshold seen during the 200-ms hyperpolarizing pulses (i.e., threshold electrotonus) resemble what has previously been observed in extracellular recordings of membrane potential changes (i.e., electrotonus) in isolated rat vagus nerve (Marsh 1982).

Membrane excitability during long-lasting hyperpolarization is cesium sensitive

After the initial subexcitability, membrane excitability slowly recovered toward the baseline level. This is similar to the behavior of membrane potential, which slowly returns toward the resting level during a constant hyperpolarizing pulse (Marsh 1982). This voltage- and time-dependent recovery of membrane excitability was found to be cesium sensitive. After addition of 1–3 mM CsCl to the bathing solution, more and more subexcitability was found during the entire period of the current pulse (Fig. 2B).

Cesium-induced slowing of action potential conduction is frequency dependent

In the next series of experiments on isolated rat vagus nerves, bath application of cesium (1–3 mM) was used as a pharmacological tool to explore the function of the hyperpolarization-activated axonal inward rectification of C fibers. First we observed that cesium induced a slowing of action potential conduction. This effect was strongly frequency dependent. Examples of recordings are illustrated in Fig. 3. Figure 3A shows the continuous recording of the latency to 50% of maximum peak of the C fiber compound action potential in a rat vagus nerve. The stimulation frequency was altered gradually from 0.1 to 3 Hz. This resulted in slowing of conduction, as is well known for C fibers (Schmelz et al. 1995; Shin and Raymond 1991; Thalhammer et al. 1994; Torebjörk 1974). During each period of stimulation with a fixed frequency, CsCl (3 mM) was then added to the bathing solution. At frequencies of 0.1 and 0.3 Hz, cesium had little effect on the nerve, but at frequencies of ≥1 Hz, it strongly and reversibly slowed conduction, as indicated by the increases in latency, amplifying the effects of the increase in frequency. Figure 3B shows the rising...
phases of C fiber compound action potentials taken from a different rat vagus nerve. Superimposed are recordings in the normal bathing solution and after addition of CsCl (1 mM). A frequency-dependent effect of cesium is revealed by the strong slowing of conduction seen during 3 Hz, a much smaller effect at 1 Hz, and a lack of effect of cesium at a stimulation frequency of 0.1 Hz. A statistical analysis of changes in latency by repetitive stimulation and/or addition of CsCl (1 mM) to the bathing solution is given in Fig. 3C. The data are from five different isolated rat vagus nerves on which experiments similar to the ones shown in Fig. 3, A and B, have been performed (mean ± SE). On average, a latency shift of 0.5 ms along a nerve segment of 5 mm (distance between the stimulating and recording electrodes) was induced by addition of 1 mM CsCl to the bathing solution during stimulation with 3 Hz.

We also tested for effects of cesium on activity-dependent slowing of A fibers in the rat vagus nerve. CsCl (3 mM) added to the bathing solution did not show an effect on the latency of A fibers stimulated at frequencies of up to 10 Hz (not illustrated).

**Frequency-dependent membrane hyperpolarization by cesium**

It is well known that nonmyelinated fibers in the mammalian vagus nerve hyperpolarize during trains of action potentials (Ritchie and Straub 1956, 1957). The following experiments were performed to explore whether repetitive activity can produce a membrane hyperpolarization sufficient to activate axonal inward rectification. For this purpose, effects of Cs⁺ on the extracellular direct current potential were tested at different frequencies of C fiber stimulation. Such experiments are illustrated in Fig. 4. Superimposed are averages of the extracellular direct current potential induced by application of cesium during repetitive stimulation of three vagus nerves with stimulation frequencies of 0.1, 1, and 3 Hz. The membrane hyperpolarization was greater during stimulation at higher frequencies. These data are consistent with a cesium-sensitive membrane conductance activated by membrane hyperpolarization.

**Function of inward rectification in isolated human sural nerve**

In another series of experiments, computer-controlled tracking of threshold electrotonus (see METHODS) was used...
conduction. Under control conditions, latency to 50% of maximum peak height was delayed by ~0.4 ms after 3 min of stimulation with 3 Hz. In the presence of cesium, however, slowing of conduction was more prominent and resulted in a latency shift of ~0.8 ms. It can be also seen in the recording that addition of cesium to the bathing solution during resting conditions (stimulation frequency 0.3 Hz) did not effect action potential conduction. This finding is in accordance with the observations made on rat vagus nerve and indicates that the cesium-sensitive membrane conductance has a function at or above a stimulation frequency of ~1 Hz. Results similar to the data illustrated in Fig. 6 were obtained in the other four human sural nerves tested.

**DISCUSSION**

**Repetitive stimulation of nonmyelinated fibers**

Repetitive stimulation of nonmyelinated C fibers produces several alterations in the ionic homeostasis and in the electrophysiological parameters of these axons. First, there are activity-dependent changes in extracellular ion activities, such as a rise in extracellular K⁺ activity (Endres et al. 1986), biphasic changes in extracellular pH (Endres et al. 1986), a rise in free intracellular Ca²⁺ concentration (Lüscher et al. 1996), and very likely, although not yet measured directly, a rise in intracellular Na⁺ concentration. Second, several authors have described an activity-dependent membrane hyperpolarization of C fibers induced by low-frequency stimulation such as 1 Hz (Ritchie and Straub 1956, 1957; Robert and Jirounek 1994). Third, hyperpolarization results in the activation of an inwardly rectifying ion conductance (Marsh 1982). This conductance is blocked by low concentrations of extracellular cesium (Mayer and Westbrook 1983). Consequently, effects of nerve excitability seen in the presence of Cs⁺ will be interpreted as a result of inhibition of axonal inward rectification. However, there is also the possibility that cesium interferes with the alterations in extra- and/or intracellular ion concentrations. In fact, cesium-sensitive inwardly rectifying ion

**Fig. 4.** Effects of cesium on membrane potential. Superimposed are averaged recordings of the extracellular direct current (d.c.) potential taken from 3 isolated rat vagus nerves. The preparations were stimulated at frequencies between 0.1 and 3 Hz. Application of cesium to the bathing solution resulted in membrane hyperpolarization that was most pronounced at higher frequencies. Illustrated are relative changes in direct current potential; the potential levels before application of cesium were normalized as baselines. Also, fast changes in membrane potential were filtered by low pass set to 0.1 Hz.

![Graph of extracellular d.c. potential](image)

**Fig. 5.** Plots of threshold electrotonus from C fiber component in fascicles from nine different isolated human sural nerves. Long-lasting hyperpolarizing currents (200 ms) induced changes in membrane excitability similar to the observations made on rat vagus nerve (see Fig. 2). A typical example of recordings from human sural nerve is illustrated in Fig. 5A. A voltage-dependent activation of a time-dependent sag in the hyperpolarizing threshold response was observed. This sag was virtually eliminated by cesium ions (Fig. 5B). These data indicate that a time-dependent, hyperpolarization-activated inward rectification is also present in nonmyelinated fibers of human sural nerve. The function of this conductance for signal transmission during low-frequency stimulation was examined in five experiments such as illustrated in Fig. 6. The stimulation frequency of the isolated human sural nerve was altered regularly between 0.3 and 3 Hz. This resulted in progressive slowing of C fiber action potential conduction.
channels have been found in Schwann cells (Konishi 1990, 1994) and it has been discussed that inhibition of inward rectification in Schwann cells enhances activity-dependent accumulation of extracellular potassium in rabbit vagus nerve (Robert and Jironenek 1994). However, two kinds of experimental data suggest that effects of cesium seen in the present study cannot be due to an enhancement of activity-dependent K⁺ accumulation. First, the rise in extracellular K⁺ concentration induced in the isolated rat vagus nerve during repetitive stimulation of up to 4 Hz at 37°C is <0.5 mM (Förstl et al. 1982) and passive elevation of extracellular K⁺ concentration even by 2 mM did not increase the latency of the C fiber compound potential (Endres et al. 1986). Second, effects of cesium on action potential latency were accompanied by membrane hyperpolarization (see Fig. 4), an observation opposite to what would be expected if accumulation of extracellular K⁺ concentration were a key factor.

The effects of cesium seen in the present study during tetanic stimulation of nonmyelinated fibers resemble observations made during replacement of external chloride by the relatively impermeant anion isethionate (Ritchie 1973). Posttetanic hyperpolarization was found to be strongly enhanced in this condition and it was concluded that nonmyelinated fibers are highly permeable to chloride (Ritchie 1973). In fact, chloride channels have been found in single-channel current recordings from axons, although in low numbers only (Strupp and Grafe 1991; Wu and Shrager 1994). An alternative interpretation for the effects of isethionate has been suggested by recent studies that have shown that the hyperpolarization-activated, cesium-sensitive cation current Iᵦ is substantially reduced by isethionate, although the channel is not permeable to chloride (Pape 1996).

**Function of inward rectification**

It is well known that nonmyelinated fibers undergo slowing in conduction at low-frequency stimulation (Schmelz et al. 1995; Shin and Raymond 1991; Thalhammer et al. 1994; Torebjörk 1974). The present data indicate that inwardly rectifying ion currents help to limit this slowing by counteracting membrane hyperpolarization. This effect was observed in nonmyelinated rat and human C fibers active at frequencies of ≳1 Hz. This frequency range is almost 2 orders of magnitude below the frequency at which inward rectification has been revealed in myelinated rat fibers (Baker et al. 1987). The most likely explanation for this difference is the magnitude of membrane hyperpolarization reached by the different fiber types during repetitive stimulation. Thin fibers, with their higher surface-to-volume ratio, undergo much greater changes in transmembrane ion concentrations compared with thick myelinated fibers, and consequently they incur more electrogenic ion transport and membrane hyperpolarization. Alternatively, nonmyelinated fibers might have a higher density of channels involved in the whole cell inwardly rectifying ion current. However, neither the biophysics of the single channel current nor the density of such ion channels in the axonal membrane is known, although many other axonal membrane currents have been analyzed recently on the basis of their single-channel current behavior (for review see Vogel and Schwarz 1995).

An explanation for the lack of single-channel current recordings in axons might be a very low single-channel conductance (<1 pS). Such a low conductance was observed for single cation channels underlying hyperpolarization-induced inward rectification in rabbit sinoatrial node (DiFranco and Mangoni 1994).

A cesium-sensitive, time-dependent inward rectification (Iᵦ) has been observed in many recordings from neuronal somata. Several suggestions have been made as to the function of this conductance (for review see Pape 1996), such as assistance to integrative behavior near rest, contribution to overshoots in membrane voltage, and support of rhythogenesis. The present data indicate that Iᵦ may also make an important contribution to the regulation of membrane potential and excitability in axons of central mammalian neurons. Not only does Iᵦ allow conduction velocity to be maintained much better during repetitive activation, but the changes of conduction failure at sites of reduced safety factor, such as branch points, are presumably much reduced. Primary afferent neurons have been shown to be heterogeneous with respect to expression of Iᵦ (e.g., Ingram and Williams 1996). Such a mechanism might contribute to differences in activity-dependent modulation of conduction seen in functionally characterized single cutaneous afferents (Thalhammer et al. 1994). The data in the present study are from compound C fiber potentials and do not allow inferences to the behavior of single fibers.

Finally, our data suggest that abnormal axonal inward rectification could result in functional deficits in axons. Reduction of inward rectification in myelinated fibers has been found in human diabetic neuropathy (Horn et al. 1996). A similar lack of inward rectification in autonomic nerve fibers would disturb the capability of such fibers to transmit signals during continuous action potential activity and could there-
fore contribute to the autonomic dysfunction seen in diabetes.

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Address for reprint requests: P. Grafe, Dept. of Physiology, University of Munich, Pettenkoferstr. 12, D-80336 Munich, Germany.

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