Two Opposing Roles of 4-AP-sensitive K⁺ Current in Initiation and Invasion of Spikes in Rat Mesencephalic Trigeminal Neurons

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Running Head: Switching by IK₄-AP between two functional modes

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ABSTRACT

The axon initial segment plays important roles in spike initiation and invasion of axonal-spikes into the soma. Among primary sensory neurons, those in the mesencephalic trigeminal nucleus (MTN) are exceptional in their ability to initiate soma-spikes (S-spikes) in response to synaptic inputs, consequently displaying two kinds of S-spikes, one caused by invasion of an axonal-spike arising from the sensory receptor and the other initiated by somatic inputs. We investigated where spikes are initiated in such MTN neurons and whether there are any differences between the two kinds of S-spikes. Simultaneous patch-clamp recordings from the soma and axon hillock revealed a spike-backpropagation from the spike-initiation site in the stem axon to the soma in response to 1-ms somatic current pulse, which disclosed the delayed emergence of S-spikes after the current pulse offset. These initiated S-spikes were smaller in amplitude than S-spikes generated by stimulation of the stem axon; however, 4-AP (≤ 0.5 mM) eliminated the amplitude difference. Furthermore, 4-AP markedly shortened the delay in spike initiation without affecting the spike-backpropagation time in the stem axon, whereas it markedly prolonged the refractory period of S-spikes arising from axonal-spike-invasion without markedly affecting that of presumed axonal-spikes. These observations suggest that 4-AP-sensitive K⁺ currents exert two opposing effects on S-spikes depending on their origins; suppression of spike initiation and facilitation of axonal-spike-invasion at higher frequencies. Consistent with these findings, strong immunoreactivities for Kv1.1 and Kv1.6, among 4-AP-sensitive and low-voltage-activated Kv1 family examined, were detected in the soma but not in the stem axon of MTN neurons.
INTRODUCTION

The axon initial segment (IS) has long been known to play pivotal roles in the initiation of spikes as well as in the invasion of axonal spikes into the soma (Coombs et al. 1957). Recently, the spike-initiation site has directly been demonstrated to exist in the axon using dual patch-clamp recordings, as revealed by spike-backpropagation (Stuart and Sakmann 1994). More recently, it has also been reported that low-voltage-activated Na⁺ channels responsible for the spike initiation were localized in the axon far beyond the IS (Colbert and Johnston 1996; Colbert and Pan 2002). Further, it was proposed that the spike-initiation site is not necessarily a discrete site but rather a region typically expanding along the axon over 200-300 µm, and as long as a few millimeters in the most extreme case of myelinated axons (Colbert and Pan 2002; Gogan et al. 1983).

In primary sensory neurons (PSNs), such a spike-backpropagation has not been addressed. This is because impulses exclusively originate from the sensory ending of the peripheral axon, but not from the soma or IS of PSNs, which are located in the dorsal root ganglia (DRG) and are considered to receive no synaptic inputs with the exception of a very small number of DRG neurons (Kayahara et al. 1981). Under physiological conditions, series of afferent impulses arising from the sensory ending propagate along the peripheral axon to the junction with the central and the stem axon, and thereafter propagate along the two axons separately to the soma and the presynaptic terminal. Similar to antidromic spikes, afferent impulses invade the soma through the stem axon, suggesting that the stem axon may be specialized to safely conduct axonal spikes “retrogradely” to the soma. However, it is also known that PSNs can generate spikes under pathological conditions such as neuropathic pain (Amir et al. 1999; Wall and Devor 1983) or in limited physiological conditions (Sengupta et al. 2004).

PSNs in the mesencephalic trigeminal nucleus (MTN), which supply periodontal...
mechanoreceptors or jaw-closer muscle spindles are unique in receiving synaptic inputs (Hinrichsen and Larramendi 1970; Liem et al. 1992) onto various types of receptors in the soma (Copray et al. 1990; Ishii and Kang 2002; Lazarov 2002), thereby initiating spikes (Verdier et al. 2004). This is because their somata are exceptional in being located in the brainstem. MTN neurons are also unique in having a very long myelinated stem axon expanding over a few millimeters, which bifurcates into the peripheral and central axons (Shigenaga et al. 1988a; Shigenaga et al. 1988b), thereby forming the three-way junction.

Thus, these unique PSNs in the MTN would display two kinds of spikes, one resulting from invasion of the impulse arising from the sensory ending and the other being triggered by somatic inputs. Then, three important questions arise: (1) Where are spikes initiated in MTN neurons with specialized stem axons to secure the spike invasion? Is spike-backpropagation seen at spike initiation in MTN neurons? (2) Are there any differences between the two spikes recorded in the soma of MTN neurons? (3) If there are, would such differences reflect which one of the two kinds of impulses is forwarded to target synapses through the central axon via the three-way junction?

In the present study, we addressed these questions in MTN neurons by using single and dual patch-clamp recording methods together with an immunohistochemical technique.
METHODS

Whole-cell recordings.

Coronal slices 200-250 µm in thickness were made from the brainstem of Wistar rats (12-18 days postnatal). The standard extracellular solution had the following composition (in mM): 124 NaCl, 3 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 26 NaHCO₃, and 10 glucose. The internal solution had the following ionic composition (in mM): 123 K-gluconate, 18 KCl, 10 NaCl, 2 MgCl₂, 2 ATP-Na₂, 0.3 GTP, 0.2 EGTA, and 10 HEPES; pH 7.4 adjusted with KOH for whole-cell recordings. The liquid junction potential between the internal solution for the whole-cell recording (negative) and the standard extracellular solution was 10 mV. Membrane potential values given in the text were corrected for the junction potentials. The recording chamber, with a volume of 1.0 ml, was continuously perfused with the extracellular solution at a flow rate of 1.0-1.5 ml/min.

Using an Axopatch 1D, 200A and 200B and an Axoclamp 2B (Axon Instruments, Foster City, CA) (see Supplementary Information), single or dual whole-cell recordings were made on MTN neurons whose oval cell bodies were clearly visible under Nomarski optics (BX-50WI, Olympus, Tokyo, Japan). 0.1% Lucifer Yellow CH (Sigma-Aldrich, St. Louis, MO) was included in the internal solution to clearly visualize the location of the axon hillock from which the stem axon protrudes. Two patch pipettes for simultaneous whole-cell recordings were pulled using the same parameter configuration (P-97, Sutter Instruments, Novato, CA) to result in similar pipette resistances of 4-6 MΩ. The mean values of pipette resistance and capacitance were 5.5 MΩ and 5.0 pF, respectively. The series resistance was usually < 10 MΩ, and recordings in which the series resistance was > 10 MΩ were not included in the analysis. Therefore, the Axopatch 200A/B was used in the normal current-clamp mode; consequently, there was no virtual difference in the current-clamp performance between the Axopatch 1D and 200A/B (Magistretti et al. 1996) (see
Supplementary Information). The time constants given by the product of the series resistance and pipette shunting capacitance should range between 30-50 µs, which should act as a low-pass filter but without distorting the action potential that displayed a relatively slower time course compared to the time constant (see Supplementary Information). Since the series resistance compensation was disabled in the current-clamp mode of the Axopatch 1D and 200A or not used in the Axopatch 200B, only the recordings that showed no sign of an apparent bridge imbalance were included in the analysis. If the bridge balance is adequate, the electrotonic voltage responses should linearly increase at the offset of current pulses but not at the onset of current pulses with increasing current intensities, and the membrane potential at the onset of current pulses should remain at the holding potential. Moreover, because spikes were always triggered after the offset of current pulses, the artificial potential change created by inappropriate bridge balance should not affect the spike height.

Most of the recordings were made at room temperature (21-24°C). In some MTN neurons (n = 28), the effects of temperature on the peak level, the half duration and the maximum rate of rise of spikes were examined at between 22 and 34°C (see Fig. 9 in Supplemental Material) using a temperature control system (DTC-200, DIA Medical, Tokyo, Japan). The seal resistance was usually > 5 GΩ. Recordings of currents and voltages were low-pass filtered at 5-10 kHz (3-pole Bessel filter), digitized at a sampling rate of 40 kHz (Digidata 1332A, Axon Instruments) and stored on a computer hard disk. Under the cell-attached voltage-clamp mode, the patch potential was held at –70 mV to depolarize the patch membrane to about 0 mV for inactivation of the Na⁺ channels in the patch membrane. Following the spike generation in this condition, only the capacitative current can be recorded by the patch electrode. With a tungsten microelectrode (impedance; 1 MΩ at 5 kHz), microstimulation (intensity; 0.5-5.0 µA, duration; 60 µs) was applied to the stem axon at a
site 40-60 µm from the soma. 4-Aminopyridine (4-AP, Sigma-Aldrich) was bath-applied at concentrations of 0.2-0.5 mM. Data given in the text are presented as mean ± SD unless otherwise mentioned, and statistical significance was assessed using the two-tailed t-test and one-way ANOVA.

**Immunohistochemistry.**

Adult Wistar rats of either sex were deeply anaesthetized with sodium pentobarbital (50-60 mg/kg, i.p.) and perfused transcardially with a 4% paraformaldehyde and 0.2% picric acid fixative dissolved in 0.1 M phosphate buffer (pH 7.3). The brains were immediately removed from the skull, postfixed with the same fresh fixative overnight at 4°C, and cryoprotected with 25% sucrose in 0.1 M phosphate buffer at 4°C. Coronal sections 30 µm thick were then cut serially on a freezing microtome and divided into six groups. Every sixth section through the MTN was processed for immunohistochemical staining with rabbit antibodies against 10 subtypes of K⁺ channels, Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kv2.1, Kv3.1, Kv3.2 and Kv3.4, (Alomone, Jerusalem, Israel). After washes in 0.1 M phosphate-buffered saline (PBS; pH 7.3) containing 1% skimmed milk, each group of sections 180 µm apart was incubated overnight with one of the primary antibodies (1:500 dilution) in 0.1 M PBS containing 1% normal horse serum (NHS), 0.05% NaN₃, and 0.25% carrageenan with or without Triton X-100 (0.3%). The optimal dilution for these primary antibodies was determined as 1:500 by testing the dilution ranged between 1:100 and 1:1,000. Subsequently, the sections were incubated with biotinylated goat anti-rabbit IgG antibody (1:200 dilution; Vector, Burlingame, CA) in the same fresh NHS-PBS incubation medium for 2 h, followed by ABC Elite (1:100 dilution; Vector) in 0.1 M PBS for 1 h. Finally, the sections were reacted for 10 min in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.04% diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan), 0.04% NiCl₂, and 0.002% H₂O₂. All immunohistochemical procedures were carried out at room temperature,
and the sections were rinsed three times (10 min each) in 0.1 M PBS between each of the steps. In control experiments, each of the primary antibodies was preabsorbed with the corresponding peptide and, also, replaced with normal IgG.

**Double fluorescence histochemistry.**

In adult Wistar rats tetramethylrhodamine dextran amine (RDA; M.W. 3,000; Molecular Probes, Eugene, OR; 10% solution) was applied to the masseteric nerve. After survival periods of 5-7 days, the rats underwent perfusion-fixation as described above. Coronal sections (30 µm thickness) through the MTN were obtained from the brains and incubated overnight with rabbit antibodies against Kv1.1, Kv1.6, and Kv2.1 (1:500 dilution). Subsequently, the sections were incubated with the biotinylated goat anti-rabbit IgG antibody (1:200) for 2 h, followed by streptavidin-HRP (1:100; NEN, Boston, MA) for 1 h and then FITC-tyramide (1:100; NEN) for 10 min. The sections were mounted onto gelatin-coated glass slides, air-dried, coverslipped with ProLong Antifade Kit (Molecular Probes), and then observed with a confocal laser-scanning microscope (TCS SP, Leica, Tokyo, Japan); two laser systems providing excitation light of 488 nm wavelength (emission 510-525 nm) and that of 543 nm wavelength (emission ≥ 570 nm) were used for FITC and RDA labeling, respectively. Digital images taken with the confocal microscopy were processed via Photoshop 7.0 (Adobe, San Jose, CA).

**SUPPLEMENTAL MATERIAL**

Supplementary Information includes data (partly illustrated in Fig. 9) obtained by using Axoclamp 2B to make sure that the difference in the current-clamp performance between the Axoclamp 2B and Axopatch series of amplifiers does not affect the present results. Effects of temperature on the spikes were also examined.
RESULTS

Spike-backpropagation revealed by a paired patch-clamp recording from the soma and AH

Whole-cell current-clamp (Fig. 1B) and cell-attached voltage-clamp recordings (Fig. 1C) were simultaneously obtained from the soma (S) and axon hillock (AH) of the same MTN neuron (Fig. 1A), respectively. In response to injection of a current pulse (* in Fig. 1B) into the soma at the resting membrane potential of –55 mV, an action potential was triggered during the decay phase of the electrotonic response (Fig. 1Ba). The temporal derivative of the spike potential (Fig. 1Bb) represents the rate of charging of the soma membrane, which is proportional to the capacitative transient current across the soma membrane (Magee and Johnston 1995). The simultaneously obtained cell-attached voltage-clamp recording at –70 mV (Fig. 1Cb) revealed two sequential negative peaks of capacitative current (Fig. 1Ca; see METHODS) in response to injection of a current pulse through the soma pipette. Since the latter was invariably larger than the former and slightly preceded the peak of the somatic capacitative current (S) (refer to vertical dotted line in Fig. 1Bb and Ca), the latter would reflect the spike generated at the AH, from which the cell-attached recording was made. Then, the former capacitative current is most likely to have been generated in the patch membrane, presumably following the IS-spike generation (Colbert and Johnston 1996). Thus, it is likely that, in response to the injection of current pulses through the soma pipette, the IS-spike was generated first, and this in turn backpropagated to sequentially trigger AH- and S-spikes. Similar observations were obtained from five paired recordings made on the soma and AH of MTN neurons. The time difference between the presumed IS and AH components of the capacitative currents recorded from the AH in response to the injection of current pulses through the soma pipette was 0.18 ± 0.03 ms (n = 5).
Two distinct spikes in the soma and AH originating from the aniso-potential nature in a round cell body of MTN neurons

Similar dual whole-cell current-clamp recordings were made from the soma and AH (Fig. 2Aa and Ab, respectively) of another MTN neuron (Fig. 2Ad). When short current pulses were applied through the soma pipette at a hyperpolarized membrane potential of –91 mV, S-spikes were triggered during the decay phase of the electrotonic responses (black traces in Fig. 2Aa) while AH-spikes were invariably triggered from the peak of the underlying subthreshold depolarization evoked at –85 mV whenever it reached the spike threshold (red traces in Fig. 2Ab). These observations indicate that the soma membrane is electrotonically more separated from the spike-initiation site than is the AH membrane. Nevertheless, the peak level of the S-spikes (+29.4 ± 8.3 mV) was significantly higher ($p < 0.02, n = 5$) than that of the AH-spikes (+18.1 ± 5.1 mV; compare the black and red horizontal dotted lines in Fig. 2Ab) when evoked at similar holding potentials (–82.8 ± 8.3 mV and –80.9 ± 7.1 mV for S- and AH-spikes, respectively). Furthermore, the maximum rate of rise of the S-spikes (325 ± 39 V/s) was also significantly ($p < 0.004, n = 5$) larger than that of the AH-spikes (259 ± 29 V/s). These features of backpropagation are in contrast to those of cortical pyramidal neurons (Stuart et al. 1997; Stuart and Sakmann 1994).

With an increase in the amplitude of the current pulses that increased the amplitude of the depolarizing electrotonic responses, the S-spike was triggered with less delay, but was decreased in amplitude slightly (refer to black horizontal dotted line in Fig. 2Aa). This decrease in spike amplitude was not seen when S-spikes were evoked at a depolarized membrane potential (> –60 mV, figure not shown), suggesting an involvement of a voltage-dependently inactivated $K^+$ current in shaping the S-spikes. In contrast, the amplitude of simultaneously recorded AH-spikes did not decrease with an increase in the underlying depolarization (refer to red dotted line in Fig. 2Ab). Thus, the simultaneously
recorded S- and AH-spikes were quite different from each other.

This was not expected, because the membrane potential is assumed to be isopotential anywhere in a round cell body. This electrotonic separation between the center of the soma and the AH was also evident from the amplitude attenuation (56 ± 6%, n = 5) and the lag time (0.45 ± 0.08 ms, n = 5) between the two peaks of subthreshold depolarizations in the soma and the AH (compare downward and upward arrowhead in Fig. 2Aa and Ab). Thus, the transient aniso-potentiality between the soma and AH was observed not only during the fast action potentials but also during the slow subthreshold responses. This indicates that the aniso-potentiality is not brought about artificially by the low-pass filtering, but likely by an unusually high internal resistance (R_i) between the soma and AH. To quantify the electrotonic separation, we calculated R_i by simulating the short-lived aniso-potentiality during the subthreshold responses (Fig. 2Bb). R_i was found to be unusually high (see DISCUSSION).

Furthermore, the simultaneous recordings disclosed two crucial differences between the S- and AH-spikes. Consistent with the observation shown in Fig. 1B and C, the generation of an S-spike was actually preceded by that of an AH-spike (black and red open arrowheads, respectively, in Fig. 2Ac) and the spike duration at half amplitude was significantly longer (p < 0.05, n = 8) in AH-spikes (0.80 ± 0.18 ms) than in S-spikes (0.71 ± 0.13 ms), as revealed in the superimposed traces of S- and AH-spikes (black and red traces, respectively, in Fig. 2Ac). Such a difference in the timing of the spike generation is also clearly seen in the temporal derivative traces (compare red and black arrows in the upper traces in Fig. 2Ac). The time difference between the two positive peaks, reflecting the AH- and S-spikes, was 0.04 ± 0.01 ms in 8 MTN neurons. Taken together, these data suggested that a spike was first initiated at the IS or the stem axon in response to the injection of current pulses at the soma, and then backpropagated to sequentially cause the AH- and S-spikes.
Therefore, by adding the backpropagation time from the spike-initiation site to AH (0.18 ± 0.03 ms, \(n = 5\)) to that from the AH to soma (0.04 ± 0.01 ms, \(n = 8\)), the total backpropagation time can be estimated as 0.22 ms.

Since the amplitude of the S-spikes, but not that of the AH-spikes, decreased with an increase in the current pulse intensity (Fig. 2Aa), a rapidly activating K\(^+\) current involved in shaping spikes may be larger in the soma than in the AH. Therefore, effects of 4-AP on the spike duration difference were examined. As shown in Fig. 2Ba and Bb, the difference in the duration between the S- and AH-spikes was completely abolished by applying 4-AP (\(n = 3\)) (black and red traces, respectively), leaving the spike amplitude difference almost unchanged. Thus, it is likely that a 4-AP-sensitive K\(^+\) current involved in shaping spikes was larger in the soma than in the AH. This possibility was further investigated in the next series of experiments.

*Involvement of 4-AP-sensitive K\(^+\) current in shaping S-spikes*

Whole-cell current-clamp recordings were made from either the soma or the AH alone in the following series of experiments. The resting membrane potential was –67.8 ± 4.7 mV (\(n = 76\)) in the soma and –70.0 ± 5.1 mV (\(n = 13\)) in the AH. Similar to that seen in Fig. 2Aa, the S-spikes decreased in amplitude with an increase in the membrane depolarization level reached at the current pulse-end at a holding potential of –75 mV (Fig. 3Ac), but not at –60 mV (Fig. 3Ab). When the peak potential of the S-spikes (\(p\) in Fig. 3Ab) was plotted against the membrane depolarization level reached at the current pulse-end (\(v\) in Fig. 3Ab), \(p\) decreased with an increase in \(v\) for the S-spikes evoked at –75 mV (filled circles in Fig. 3Af), whereas \(p\) remained almost constant for those at –60 mV (open circles Fig. 3Af). The difference in \(p\) between the S-spikes evoked at –60 and those at –75 mV became apparent at \(v > –50\) mV, and increased with an increase in \(v\) (Fig. 3Af). The S-spikes evoked at –75 mV...
were also shorter in duration than those at –60 mV, as revealed by superimposed traces of the
two respective S-spikes shown with asterisks in Fig. 3Ac and Ab (compare continuous and
interrupted traces in Fig. 3Ad). Furthermore, the application of 0.5 mM 4-AP increased the
\( p \) values of the respective S-spikes evoked with different \( v \) values to the same level,
abolishing the difference in the spike amplitude (refer to horizontal dotted line in Fig. 3Ae,
and see also filled squares in Fig. 3Af). Thus, a 4-AP-sensitive \( K^+ \) current that is largely
inactivated at –60 mV and activated at potentials > –50 mV is likely to be involved in
modulating the amplitude and duration of the S-spikes.

**Involvement of 4-AP-sensitive \( K^+ \) current in delays in spike initiation**

It was also noticed that the delay in evoking S-spikes, i.e., the spike initiation delay
plus the spike-backpropagation delay, was shortened by 4-AP (Fig. 3Ae), suggesting the
involvement of \( K^+ \) currents in increasing the apparent electrotonic distance from the soma to
the spike-initiation site. As shown in Fig. 3C, when the time to the maximum rate of rise
(MRR) of the S-spikes measured from the timing of the current pulse offset (\( t \) in Fig. 3Ab)
was plotted against \( v \), \( t \) decreased nonlinearly with an increase in \( v \). The \( t \) in evoking the
S-spikes from –75 mV (filled circles) was larger than that from –60 mV (open circles), when
compared at the same \( v \). This difference became apparent at \( v > –50 \text{ mV} \), and increased with
an increase in \( v \) (Fig. 3Ad and C). Thus, the \( K^+ \) current that largely inactivates at –60 mV
and is active at potentials > –50 mV is likely to be involved in causing the delay in evoking
S-spikes at –75 mV. In fact, the \( v-t \) relationship as a measure of the delay in evoking
S-spikes was shifted negatively by applying 4-AP, as illustrated in Fig. 3C (compare filled
squares with filled circles). In the \( v-t \) relationships obtained from 5 MTN neurons examined
at holding potentials between –80 and –75 mV, the \( t \) value at the same \( v \) ranging between –50
and –30 mV was significantly shortened by 4-AP from 1.45 ± 0.23 to 0.76 ± 0.12 ms \((n = 5, p \)
The voltage dependencies of K+ current shaping the S-spikes were very similar to those causing a delay in evoking the S-spikes (Fig. 3Af and C), and the shortening of the voltage-dependent delay in the evoking S-spikes by 4-AP was always accompanied by the elimination of the voltage-dependent shaping of the S-spikes (Fig. 3Ae). Therefore, the K+ current characterized by (1) sensitivity to 0.5 mM 4-AP, (2) large steady-state inactivation at –60 mV and (3) activation at potentials > –50 mV, is likely to be involved in shaping the S-spikes as well as in elongating the electrotonic distance from the soma to the spike-initiation site. This presumed 4-AP-sensitive K+ current is hereafter termed $I_{K_{4-AP}}$.

On the other hand, when whole-cell recordings were obtained from the AH (Fig. 3Ba), the AH-spike amplitude did not decrease with an increase in the current pulse intensity even at a holding potential of –77 mV (continuous traces in Fig. 3Bb). Furthermore, 4-AP did not affect the delay in evoking the AH-spikes while increasing the spike amplitude similarly (interrupted traces in Fig. 3Bb). Subsequently, the $v$-$t$ relationship of the AH-spikes was not shifted negatively by 4-AP (compare open and filled triangles in Fig. 3C). These observations suggest that the presumed $I_{K_{4-AP}}$ was not involved in causing the delay in evoking the AH-spikes. Similar observations were obtained in whole-cell recordings made from the AH of the 5 MTN neurons examined at holding potentials between –90 and –75 mV. There was no significant difference ($n = 5$, $p > 0.1$) in the $t$ value at the same $v$ ranging from –60 to –40 mV, between the AH-spikes obtained before and after the application of 4-AP ($0.57 \pm 0.16$ and $0.54 \pm 0.15$ ms, respectively).

Since 4-AP shortened the delay in evoking S-spikes by $0.69 \pm 0.25$ ms ($n = 5$) while leaving that in evoking AH-spikes almost unchanged ($0.04 \pm 0.03$ ms, $n = 5$), 4-AP likely shortens the electrotonic distance from the soma to the spike-initiation site without markedly affecting that from the AH to the spike-initiation site (see DISCUSSION). These observations
strongly suggest that the distribution of 4-AP-sensitive K\(^+\) channels differs between the soma and stem axon.

**Input-dependent difference in the amplitude of S-spikes**

In the same paired recordings as shown in Fig. 2A, a current pulse injection into the AH caused a larger S-spike (black continuous trace in Fig. 4Aa) in comparison with that evoked by a current pulse injection into the soma (black interrupted trace in Fig. 4Aa). The amplitude of the S-spikes triggered by the AH injection (97.4 ± 11.6 mV) was invariably and significantly (\(p < 0.02\), \(n = 5\)) larger than that of the S-spikes triggered by soma injection (92.9 ± 10.4 mV), when compared at the same holding potential ranging between –73 and –91 mV (Fig. 4Aa). Such an input-dependent difference in the amplitude of S-spikes may indicate an input-dependent differential involvement of the presumed \(I_{K_{4-AP}}\). In contrast, there was no significant difference (\(p > 0.7\), \(n = 5\)) in the amplitude between AH-spikes evoked by current pulse injections into the AH and those by current pulse injection into the soma (81.8 ± 8.8 mV and 81.6 ± 9.3 mV, respectively; compare red continuous and interrupted traces in Fig 4Ab), when compared at the same holding potential ranging between –70 and –85 mV.

Since somata of MTN neurons can display two distinct types of S-spikes arising from somatic inputs and peripheral sensory inputs, a similar input-dependent difference might be seen between the two types of S-spikes. Therefore, in the next experiments, the characteristics of the S-spike originating from invasion of axonal spikes (abbreviated as inv-S-spike) were compared with those of the S-spike initiated by somatic inputs (abbreviated as init-S-spike).
Differential effects of 4-AP on init- and inv-S-spikes

As seen in Fig. 4Ba, recording and stimulating electrodes were placed on the soma and on the stem axon of an MTN neuron, respectively. A spike was evoked almost in an all-or-nothing manner by stimulation of the stem axon with a threshold intensity of 1.0 µA, and was identified as an inv-S-spike based on the abrupt rising phase of the spike at a latency of 0.25 ms (Fig. 4Bb).

Such S-spikes generated by stimulation of the stem axon were compared with those initiated by injection of current pulses in the same MTN neurons. When a current pulse injection was applied in combination with stimulation of the stem axon at –61 mV, there was no marked difference in the amplitude between the init- and inv-S-spikes (Fig. 4Bc). However, when the combined stimulation was applied at –76 mV, the amplitude of the init-S-spike (* in Fig. 4Bc) was smaller than that of the inv-S-spike (** in Fig. 4Bc). With membrane hyperpolarization, the peak potential level of the init-S-spikes (filled circles in Fig. 4Bd) markedly decreased while that of the inv-S-spikes (open circles in Fig. 4Bd) decreased only slightly. Thus, the difference in the amplitude between the init- and inv-S-spikes emerged voltage-dependently. When inv- and init-S-spikes were evoked at the same holding potential ranging between –80 and –75 mV, the amplitude of the inv-S-spikes (99.0 ± 8.0 mV) was invariably and significantly (p < 0.01, n = 5) larger than that of init-S-spikes (89.2 ± 7.0 mV). However, the differences in the amplitude between the inv- and init-S-spikes at various holding potentials were completely eliminated by applying 4-AP (open and filled triangles in Fig. 4Bd, and Fig. 4Ca and Cb). These observations clearly indicate that the involvement of the presumed IK_{4-AP} in modulating the spike amplitude is larger in the init-S-spike than in the inv-S-spike. This may be consistent with the observation that the amplitude of the S-spike evoked by injection of a current pulse at the AH, which may be comparable to an inv-S-spike, is larger than that evoked by injection of a
current pulse into the soma as the init-S-spike (Fig. 4A).

Consistent with those seen in Fig. 3 Ae and C, 4-AP shortened the delay in spike initiation, as revealed in the superimposed traces in Fig. 4Cc. By contrast, the delay or latency to spike invasion seemed not to be shortened as revealed in the superimposed traces, obtained after subtraction of the nothing responses from the inv-S-spikes in the respective conditions (Fig. 4Cd). There was no significant \( p > 0.1, n = 5 \) change in the latency to the abrupt rising of the inv-S-spikes obtained before and after the application of 4-AP (0.20 ± 0.03 and 0.23 ± 0.05 ms, respectively). These observations suggest another aspect of the differential involvement of the presumed \( IK_{4-AP} \) between spike initiation and invasion. 4-AP might have shortened the electrotonic separation from the soma to the spike-initiation site while leaving the length constant in the stem axon almost unchanged. This is consistent with the differential effects of 4-AP on the delay in evoking S- and AH-spikes (Fig. 3). Provided that the spike-initiation site is located in the stem axon, the shortening of the delay by 4-AP would largely result from the electrotonic separation between the soma and AH, but not from the electrotonic separation between the AH and the spike-initiation site. The validity of these notions would strongly depend on the presence or absence of 4-AP-sensitive K\(^+\) channels in the soma and stem axon, respectively. This issue was further examined in the next experiment.

**Differential effects of 4-AP on the refractory period between inv-S-spikes and axonal spikes**

Since 4-AP prolonged the duration of S-spikes, the refractory period of S-spikes should also be prolonged. Then, the sensitivity of axonal spikes to 4-AP can be indirectly accessed by examining its effects on the refractory period of inv-S-spikes. As shown in Fig. 5Aa, when the interstimulus interval (ISI) of paired stimulation applied to the stem axon was decreased, the second inv-S-spike displayed only a slight decrease in amplitude but
disappeared suddenly at 3.1 ms ISI (open arrowhead in Fig. 5Aa), which was also illustrated by plotting the relative amplitude (RA) of the second to the first S-spike against the ISI (minimum RA = 0.96, open circles and arrow in Fig. 5Ca). Since the failure of the second S-spike occurred without a marked attenuation of amplitude, the failure should have occurred at the stimulated site of the stem axon. Therefore, the possible refractory period of the impulse caused at the stimulated site of the stem axon (approx. 3.1 ms) should be longer than that of the inv-S-spike itself. As seen in Fig. 5Ab, when 4-AP was applied, the second S-spike displayed a progressive attenuation of its amplitude with a decrease in the ISI to 3.6 ms (minimum RA = 0.75, filled circle and arrow in Fig. 5Ca), which led to the failure of the second S-spike at 3.5 ms ISI (filled arrowhead in Fig. 5Ab). However, it was not clear whether the failure of the second inv-S-spike occurred due to the refractoriness of the S-spike itself, or to that of the impulses evoked at the stem axon. To distinguish between the two possibilities, paired current pulses were injected into the soma in the presence of 4-AP. When the interval between the two current pulses was decreased in the presence of 4-AP, the minimum RA of the init-S-spikes (0.67, Fig. 5B; see also open triangles in Fig. 5Cb) was smaller than that of the inv-S-spikes (0.75, compare Fig. 5Ab and B, and Fig. 5Ca and Cb). Therefore, the failure of the second inv-S-spike seen in Fig. 5Ab would be due to the refractoriness of the impulse evoked at the stimulated site, but not due to the refractoriness of the inv-S-spike itself. Thus, 4-AP prolonged the possible refractory period of the presumed impulse in the stem axon from 3.1 to 3.5 ms (Fig. 5Aa, Ab and Ca).

When the progress of the attenuation was plotted against the inter-peak interval between the first and second spikes, the second inv- and init-S-spikes (filled circles and open triangles, respectively, Fig. 5Cb) seen in the presence of 4-AP displayed similar attenuation curves irrespective of their origins. The progress of attenuation of the init-S-spike obtained in the presence of 4-AP could be fitted by a double exponential curve with a fast (0.64 ms)
and slow (5.30 ms) time constants (open triangles and continuous line in Fig. 5Cb). Using the same two time constants with the proportion of the fast phase increased 27 times, the progress in the attenuation of the inv-S-spikes in the absence of 4-AP could also be fitted (open circles and dotted line in Fig. 5Cb). Therefore, 4-AP might have prolonged the refractory period by decreasing the proportion of the fast to slow component of the time-dependent recovery from the refractoriness of the spike potential. A similar bi-exponential recovery from inactivation has been reported in Na⁺ channels (O'Reilly et al. 2000), K⁺ currents (Catacuzzeno et al. 2003) and L-type Ca²⁺ current (Workman et al. 1999), and also a similar modulation of the proportion of the two time constants of recovery from inactivation in an L-type Ca²⁺ current has been reported in heart myocytes (Workman et al. 1999).

With a decrease in the interval of paired current pulses especially beyond 4.0 ms in the presence of 4-AP, the RA of the init-S-spikes decreased to less than 0.70, leading to a failure of the second init-S-spike (Fig. 5B and open triangles in Fig. 5Cb), while the inter-peak interval remained almost constant at 4.0 ms (open triangles in Fig. 5Cc). Therefore, the absolute refractory period of the init-S-spikes in the presence of 4-AP can be regarded as such an inter-peak interval that remains almost unchanged in spite of decreases in the interpulse interval. At this minimum inter-peak interval, i.e., the absolute refractory period, the RA was less than 0.70. Subsequently, the minimum inter-peak intervals of 3.3 and 4.2 ms between the paired inv-S-spikes obtained before and after 4-AP application, respectively, can be regarded as the absolute refractory periods of presumed impulses in the stem axon that would cause the RA of 0.70 (open and filled diamonds, respectively, in Fig. 5Cb). Using the same two time constants but assuming the proportion of the two components arbitrarily between the two values of proportions for the inv-S-spikes in the absence and presence of 4-AP, the attenuation curves were reconstructed for the two
refractory periods of the presumed impulse in the stem axon in the absence and presence of 4-AP (interrupted lines attached to open and filled diamonds, respectively, in Fig. 5Cb). Thus, from the regression analyses, the attenuation curve of the presumed axonal spikes seemed to be less affected by 4-AP than that of the S-spikes (see Fig. 5Cb).

Similar differential effects of 4-AP on the refractory period between an S-spike and a presumed impulse in the stem axon were observed in 4 MTN neurons examined. Due to the presumed $I_{K_{4-AP}}$, the absolute refractory period of an inv-S-spike was invariably shorter than that of the presumed impulse in the stem axon ($3.15 \pm 0.43 \text{ ms}, n = 4$) as revealed by the large minimum RA ($0.92 \pm 0.04, n = 4$) of the inv-S-spikes. Indeed, 4-AP prolonged the refractory period of the inv-S-spike to a degree similar to that of the presumed impulse in the stem axon ($4.05 \pm 0.27 \text{ ms}, n = 4$), as revealed by the small minimum RA ($0.71 \pm 0.05, n = 4$) of the inv-S-spikes. This observation also indicates that there was a prolongation of the refractory period of the presumed impulses in the stem axon from $3.15 \pm 0.43 \text{ ms}$ to $4.05 \pm 0.27 \text{ ms} (n = 4$), which would be much smaller than that of the refractory period of the inv-S-spikes in view of the large change in RA. This smaller prolongation may, however, not be necessarily caused by a direct effect of 4-AP. The broadening of inv-S-spikes by 4-AP could indirectly cause a prolongation of the refractory period of the presumed impulses in the stem axon due to the electrotonic continuity between the soma and the stem axon.

These electrophysiological observations strongly suggested that the distribution of 4-AP-sensitive $K^+$ channels differs between the soma and stem axon of MTN neurons. Therefore, in the next experiment, the distribution of $K^+$ channels was directly investigated using an immunohistochemical technique.

**Differential subcellular localization of 4-AP-sensitive vs. -insensitive $K^+$ channels**

In comparison with the Kv1 family, the Kv4 family is known to be less sensitive to
4-AP (IC\textsubscript{50} of 4-AP = 1.5 to 10 mM; Coetzee et al. 1999; Faivre et al. 1999; Fiset et al. 1997; Tseng et al. 1996). Since a low concentration of 4-AP (≤ 0.5 mM) completely abolished the voltage-dependent modulations of init-S-spikes (Figs. 2C, 3 and 4C), it is unlikely that the Kv4 family is involved in these voltage-dependent modulations of S-spikes. Therefore, the following 10 subtypes of K\textsuperscript{+} channels that are sensitive to relatively lower concentrations of 4-AP (Coetzee et al. 1999; North 1995) were examined in the present immunohistochemical analysis; Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kv2.1, Kv3.1, Kv3.2, and Kv3.4. Of these 10 subtypes, three subtypes, Kv1.1, Kv1.6 and Kv2.1, were predominantly expressed in MTN neurons. Kv1.1 (Fig. 6A and B) and Kv1.6 (Fig. 6C and D), which are highly sensitive to 4-AP with IC\textsubscript{50} values of 0.16-1.0 (Christie et al. 1989; Stuhmer et al. 1989; Stuhmer et al. 1988) and 0.3-1.5 mM (Grupe et al. 1990; Kirsch et al. 1991), respectively, were localized in the somata alone. A large number of such immunostained neurons were observed throughout the entire rostrocaudal extent of the MTN.

On the other hand, Kv2.1 (Fig. 6E and F), which is much less sensitive to 4-AP with IC\textsubscript{50} values of 6-18 mM (Baranauskas et al. 1999; Belevych et al. 2002; Smirnov et al. 2002) and is relatively high-voltage-activated (activation threshold ≈ −30 to −20 mV, half-maximal activation at potentials ≈ +10 mV; Coetzee et al. 1999) in comparison with the Kv1 family, was distributed not only in the somata but also in the stem axons of MTN neurons (filled arrowheads in Fig. 6E and F). These Kv2.1-positive neurons were found mainly at the caudal level of the MTN. In addition, weak immunostaining with anti-Kv1.2, -Kv1.3, -Kv1.5, -Kv3.2, and -Kv3.4 antibodies occurred only in the somata of MTN neurons. No MTN neurons were immunostained with anti-Kv1.4 or -Kv3.1 antibody.

Consistent with these observations, the present double fluorescence histochemistry revealed that only the somata, but not the stem axon, of MTN neurons retrogradely labeled with RDA were immunostained for Kv1.1 (Fig. 7A) and Kv1.6 (Fig. 7B), while both the
somata and the stem axons of the RDA labeled MTN neurons were immunostained for Kv2.1 (Fig. 7C). The immunoreactivity for each Kv subtype in the somatic region was located not only along the plasma membrane but also in the cytoplasm, probably in the endoplasmic reticulum, as previously reported in nodose ganglion neurons (Glazebrook et al. 2002). Of particular interest was that the AH region of MTN neurons was virtually devoid of Kv1.1 or Kv1.6 immunoreactivity (asterisks in Fig. 7Ab and Bc), consistent with the less involvement of the presumed IK4-AP in AH-spikes in comparison with S-spikes (see Fig. 2Ac and compare Fig. 3Ae and Bb).

Thus, only the soma, but not the stem axon, of MTN neurons strongly expressed Kv1.1 and Kv1.6 among the low-voltage-activated (activation threshold < –50 to –40 mV, half-maximal activation at potentials < –30 to –20 mV; Bertoli et al. 1994; Stuhmer et al. 1989; Swanson et al. 1990) and relatively low-concentration 4-AP-sensitive Kv1 family.
DISCUSSION

Differential involvement of 4-AP-sensitive K⁺ current between spike initiation and spike invasion

Irrespective of patch-clamp amplifiers, current follower (Axopatch series, Fig. 4B and C) or voltage follower (Axoclamp 2B, see Fig. 9A and B in Supplementary Information), the amplitude of the inv-S-spike was invariably and significantly larger than that of the init-S-spike, when compared in the same MTN neuron. Furthermore, the spike amplitude difference was abolished by 4-AP application (Fig. 4Bd and C). Therefore, it was strongly suggested that 4-AP-sensitive K⁺ channels were involved in differentially shaping the S-spikes, in a manner dependent on whether the S-spike was initiated (backpropagated) or generated by the invasion of axonal spikes.

The K⁺ current that was previously identified as having half-maximal activation and inactivation at –34.7 and –62.0 mV, respectively, in MTN neurons was sensitive to a low concentration (0.5 mM) of 4-AP (Del Negro and Chandler 1997). Since the activation of Na⁺ current is much faster than that of 4-AP-sensitive K⁺ currents (Patlak 1991; Rudy 1988), the involvement of the presumed IK₄-AP would be smaller when an S-spike is evoked by the rapid invasion of an axonal spike than when an S-spike was activated with a delay during the decay phase of the depolarizing response to current pulses into the soma (Fig. 4Ca). The electrotonic separation of the soma from the spike-initiation site may allow time for backpropagated S-spikes to be shaped by the presumed IK₄-AP. Thus, due to the differential involvement of the presumed IK₄-AP, the amplitude and duration of the init-S-spikes were distinct from those of inv-S-spikes. 4-AP shortened the delay in evoking S-spikes without affecting the delay in evoking AH-spikes (Fig. 3), indicating that the presumed IK₄-AP increases the delay in the initiation of S-spikes without increasing the backpropagation time from the spike-initiation site in the stem axon to the AH. Consistent with this observation,
4-AP did not affect the latency of inv-S-spikes (Fig. 4Cd), and the refractory period of presumed axonal spikes was not markedly prolonged by 4-AP (Fig. 5C). These observations strongly suggest the differential distribution of 4-AP-sensitive K⁺ channels in the soma and stem axon.

Kinetics of IK₄-AP vs. Kv1.1 and Kv1.6

The present immunohistochemical examinations demonstrated that Kv1.1, Kv1.6 and Kv2.1 channel subunits were localized in the somata of MTN neurons, whereas only the Kv2.1 subunit was expressed in the stem axon (Figs. 6 and 7). The possible inactivation kinetics of the presumed IK₄-AP seen in the present study (Figs. 3Ab-c, Af, C and 4Bc-d) seems to be inconsistent with those of a possible K⁺ current if these subunits form homomultimeric channels. The homomultimeric channels composed of these Kv subunits display little time-dependent and voltage-dependent inactivation even at potentials > –40 mV, as reported in an expression system (Christie et al. 1989; Stuhmer et al. 1989). However, the inactivation kinetics of those Kv channels can be enhanced and their voltage-dependence may be shifted negatively by various mechanisms, including the formation of heteromultimeric channels (Isacoff et al. 1990; Ruppersberg et al. 1990), the coexpression with Kvβ subunit (Heinemann et al. 1996; Rettig et al. 1994), and the post-translational processing of Kvα/β subunit protein (Kwak et al. 1999; Nitabach et al. 2002). Therefore, the electrophysiological findings in this study are not necessarily inconsistent with the inactivation kinetics of these Kv channels reported previously. Furthermore, the presumed IK₄-AP was activated from potentials as low as –50 to –40 mV (Figs. 3 and 4) and was sensitive to a low concentration of 4-AP (≤ 0.5 mM). These properties of the presumed IK₄-AP were consistent with those of the homomultimeric channels of these Kv subunits (Christie et al. 1989; Swanson et al. 1990). The presumed IK₄-AP is likely to correspond to
the slow transient outward current isolated in MTN neurons previously, which was largely
inactivated at –60 mV and was largely sensitive to 0.5 mM 4-AP (Del Negro and Chandler
1997).

Functional differences among the peripheral, stem and central axons

In a simulation study (Amir and Devor 2003), it was argued that neither the
excitability in the IS nor in the soma affects the through-conduction from the peripheral to the
central axon. Nevertheless, whenever an impulse arising from the peripheral axon failed to
invade the stem axon, it also failed to invade the central axon, as demonstrated in frog DRG
neurons (Stoney 1990). Taken together with the present findings on the spike initiation
mechanism in the stem axon, it is likely that impulses arising from the peripheral axon trigger
impulses in the stem axon before they reach the branching point, and then invade into the
soma and simultaneously propagate forward through the central axon (Fig. 8Aa). If this is
the case, the Na⁺ channel density of the axonal membrane around the three-way junction
would be relatively low. Then, without triggering impulse in the stem axon, there would be
no through-conduction from the peripheral to the central axon. Indeed as reported
previously, the central axon of MTN neurons displayed a pause of impulse activity whenever
soma spiking was inhibited during jaw-opening phase (Kolta et al. 1995; Westberg et al.
2000). Taken together, it is most likely that no impulses would be seen in the central axon
whenever impulses arising from the muscle spindles fail to invade the stem axon and/or soma,
as originally suggested by a series of previous studies (Kolta et al. 1995; Westberg et al.
2000). On the other hand, when a spike was initiated in the stem axon in response to
synaptic action, it would invade the central axon, and also propagate backward into the soma
(Fig. 8Ab).

Thus, provided that there is no through-conduction, either somatic inputs or impulses
arising from the sensory ending that trigger spikes in the stem axon first could be forwarded through the central axon to the target synapse (Fig. 8Aa and Ab). Then, S-spikes shaped differentially by the presumed $I_{K4-AP}$ would reflect which one of the two inputs, arising from the soma or sensory ending, is forwarded to the target synapses (Fig. 8Aa and Ab).

Voltage-dependent switching between two functional modes presumed in MTN neurons

Masticatory movement has been considered to be mediated by the central pattern generator (CPG), from which MTN neurons receive synaptic inputs and thereby act not only as PSNs but also as interneurons independent of the activities of the peripheral receptors (Fig. 8B). In addition, it is well established that the central axon of MTN neurons can act independently of the activities of somata through the mechanism of GABA-mediated primary-afferent depolarization (Kolta et al. 1995; Verdier et al. 2003; Westberg et al. 2000). Thus, MTN neurons can display multiple functional modes.

At depolarized membrane potentials in the somata (Fig. 8Ba) where 4-AP-sensitive K$^+$ currents might be largely inactivated, MTN neurons display oscillatory firing activity either spontaneously (Pedroarena et al. 1999; Wu et al. 2005; Wu et al. 2001) or triggered synaptically (Verdier et al. 2004; Yamuy et al. 2000). In contrast, at hyperpolarized membrane potentials in the soma of MTN neurons (Fig. 8Bb) where the inactivation of 4-AP-sensitive K$^+$ currents is largely removed, 4-AP-sensitive K$^+$ currents would facilitate spike invasion arising from sensory endings (Fig. 8Ca), but prevent MTN neurons from initiating spikes in response to fast synaptic inputs (Fig. 8Cb). However, as suggested by the previous studies (Kolta et al. 1995; Westberg et al. 2000), a further hyperpolarization in the soma might prevent the spike-initiation site from being activated by impulses arising from the peripheral axon, provided that the safety factor for the saltatory conduction from the peripheral axon to the spike-initiation site in the stem axon is relatively low due to the low
Na⁺ channel density around the three-way junction.

Thus, MTN neurons can act either as PSNs or as interneurons depending on the membrane potential levels (Fig. 8B). Our study would provide a rationale for exploring the slow modulatory actions underlying the membrane depolarization and hyperpolarization, which have not been clarified yet. Serotonin and dopamine receptors which are expressed in MTN neurons (5-HT₁₂: Kolta et al. 1993; Lazarov 2002; D₂: Lazarov 2002; Lazarov et al. 1998), may be strong candidates for inducing the slow membrane depolarization (Beique et al. 2004; Zhang 2003) and hyperpolarization (Einhorn et al. 1991; Lacey et al. 1987), respectively.

Electrotonic separation in round somata of MTN neurons

In a neuron having a round cell body, the membrane potential across the soma area is considered to be isopotential, as has been assumed in the equivalent cylinder model with a single lumped soma compartment (Segev 1992). However, the round cell body of MTN neurons was not isopotential during subthreshold slow depolarizations or during fast spiking (Fig. 2A and B, and Fig. 4A). Although this aniso-potentiality was short-lived, both the amplitude attenuation (56 ± 6%, n = 5) and the lag time (0.45 ± 0.08 ms, n = 5) between the two peaks of subthreshold depolarizations in the soma and the AH (compare downward and upward arrowhead in Fig. 2Aa and Ab) clearly indicate the unusual electrotonic separation within the spherical soma. The electrotonic separation is inversely proportional to the length constant (\( \sqrt{\frac{R_m}{R_i}} \)). Therefore, the internal resistance (\( R_i \)) between the two sites was estimated from the following simultaneous differential equations for the two compartments model.
\[ C_S \frac{dV_S}{dt} + \frac{V_S}{R_{m-S}} + \frac{V_S - V_{AH}}{R_i} - I = 0 \]

\[ \frac{V_S - V_{AH}}{R_i} - C_{AH} \frac{dV_{AH}}{dt} - \frac{V_{AH}}{R_{m-AH}} = 0 \]

where \( I \) is the injected current, \( V_S, C_S \) and \( R_{m-S} \) are the membrane potential, capacitance and resistance in the soma compartment, respectively, and so too are \( V_{AH}, C_{AH} \) and \( R_{m-AH} \) in the AH compartment.

Since the cell size varies from neuron to neuron, we have estimated \( R_{m-AH}/R_i \) and \( R_i \cdot C_{AH} \), rather than the absolute value of \( R_i \). For simplification, the two compartments of the soma and AH were assumed to have the same capacitance and membrane resistance. \( R_{m-AH}/R_i \) and \( R_i \cdot C_{AH} \) were estimated to be approximately 4 and 0.75 ms, respectively (Fig. 2Bb). Even if \( R_{m-AH} \) is as low as 15 MΩ, which was estimated by using 1-ms current pulses (Fig. 4Ab) and thereby reflects only a small fraction of \( R_{m-AH}, R_i \) is calculated to be 3.7 MΩ. This is over 150 times larger than that of the internal resistance (21 kΩ) between the two points separated by 30 µm in an axon having 30 µm diameter (50 Ω cm; Katz 1966).

Because of this unusual electrotonic separation, MTN neurons having round somata could display the backpropagation of an IS-spike to the soma through the AH. The nature of the active backpropagation is well reflected in the amplitude and the rate of rise of backpropagated S-spikes, which were larger than those of the preceding AH-spike (Fig. 2Aa and Ab, see also upper traces in Fig. 2Ac), unlike the case of cortical pyramidal cells (Stuart and Sakmann 1994).
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FIGURE LEGENDS

FIG. 1. Active spike-backpropagation.

A: A fluorescence photomicrograph showing the location of the two patch electrodes used for a paired recording from the soma (S; B) and axon hillock (AH; C) of a round MTN neuron with a thick stem axon.

B: A delayed activation of a soma spike by a current pulse injection (*) into the soma at the resting membrane potential (V_r) of –55 mV (a), under whole-cell current-clamp recording from the soma. The positive peak of the temporal derivative of the spike potential indicated with an arrow labeled ‘S’ (b). Time calibration in (B) also applies in (C).

C: Two successive negative components of capacitative current (a) under cell-attached voltage-clamp mode from the AH at –70 mV (b) recorded simultaneously with the S-spike (Ba). The smaller first and larger second peaks would reflect spiking at the initial segment (IS) and AH, respectively, as indicated with ‘IS’ and ‘AH’. The latter AH peak preceded the S peak, suggesting a backpropagation of the S-spike from IS through AH.

FIG. 2. Two distinct spikes in the soma and AH.

A: Simultaneous whole-cell current clamp recordings made from the soma (a) and AH (b) of an MTN neuron (d). The distance between the two recording sites was 30 µm. Black horizontal dotted line in (b) indicates the peak level of the largest S-spike. Note the difference in the time to peak between the subthreshold responses recorded at the soma and AH (compare downward and upward arrowheads in a and b). Temporal derivative of voltage, voltage and time calibrations in (a) also apply in (b). Superimposed traces of the two spikes indicated with asterisks in (a) and (b) showing the differences in the spike occurrence timing and in the spike duration (c). The latter ones of the two spikes were scaled to have the same amplitude (downward arrow, lower traces in c).
amplitude differences between the two positive peaks of the temporal derivatives of the AH- and S-spikes (red and black downward arrows, respectively, upper traces in c). Black and red traces represents the responses recorded from soma and AH, respectively. \( V_r \) at the soma and AH were \(-67\) and \(-69\) mV, respectively. Direct currents (DC) of \(-0.26\) and \(-0.09\) nA were injected into the soma and AH, respectively, to maintain the baseline membrane potentials at \(-91\) and \(-85\) mV in the soma and AH, respectively.

**B:** The transient aniso-potentiality between the soma (black traces) and AH (red traces), in recorded (a) and simulated subthreshold responses (b) to current pulse injections into the soma (compare downward and upward arrowheads in a and b).

**C:** Superimposed traces of simultaneously recorded S-spike (black traces) and AH-spike (red traces) evoked by injection of current pulses into the soma, obtained before (a) and after the application of 4-AP (b). 4-AP abolished the spike duration difference (open arrowheads). Voltage and time calibrations in (a) also apply in (b). \( V_r \) in the soma and AH; \(-72\) and \(-71\) mV, respectively. \(-0.22\) and \(-0.30\) nA DC injections into the soma and AH, respectively, to maintain baseline membrane potentials at \(-90\) and \(-91\) mV in the soma and AH, respectively.

*Fig. 3.* Involvement of 4-AP-sensitive K\(^+\) current in shaping S-spikes generated by somatic current pulses.

**A:** Superimposed Nomarski and fluorescence images showing the position of a patch electrode on the soma of an MTN neuron (a). S-spikes evoked by current pulse injection into the soma at \(-60\) (b) and \(-75\) mV (c). \( p \) and \( v \) represent the spike peak potential and the potential level reached at the pulse-end, respectively. \( t \) represents the time to the maximum rate of rise (MRR) of S-spikes (upper traces, b), measured from the pulse-end (b). Superimposed traces of two respective spikes, shown in (b) and (c) with asterisks, indicating that both the delay and spike amplitude are dependent on the holding potential (d).
Continuous and interrupted traces showing S-spikes obtained at −75 mV before and after application of 4-AP, respectively (e). Voltage and time calibrations in (b) also apply in (c-e). Plotting of peak potential of S-spikes ($p$) against membrane potential level ($v$) reached at the end of current pulses (f). Note the decrease in the peak level of the S-spikes evoked at −75 mV (filled circles) with an increase in pulse-end depolarization, but not at −60 mV (open circles). Such decreases at −75 mV were abolished by 4-AP (filled squares). $V_r$; −60 mV (b). −0.18 nA DC injection into the soma to maintain at −75 mV (c).

B: Superimposed Nomarski and fluorescence images showing a patch pipette positioned on the AH of another MTN neuron (a). Continuous and interrupted traces showing AH-spikes obtained at −75 mV before and after the application of 4-AP, respectively (b). $V_r$; −66 mV (b). −0.26 nA DC injection into the soma to maintain baseline membrane potential at −75 mV (c).

C: Plotting of time to MRR of S- and AH-spikes ($t$) against membrane potential level ($v$) reached at the end of current pulses. $t$ was larger in S-spikes evoked from −75 mV (filled circles) than in those from −60 mV (open circles), when compared at the same $v$. 4-AP application markedly decreased $t$ in S-spikes evoked from −75 mV (filled squares). There was no marked difference between the $v$-$t$ relationship of AH-spikes obtained in the absence and that of those obtained in the presence of 4-AP (open triangles and filled diamonds, respectively).


A: A larger S-spike evoked by injection of current pulses into the AH (black continuous trace) than that evoked by current pulse injection into the soma (black interrupted trace) (a), obtained in the same paired recordings as in Fig. 2A. No apparent difference between
AH-spikes evoked by current pulse injections into the AH and soma (red continuous and interrupted traces, respectively) (b). Voltage and time calibrations in (a) also apply in (b).

B: Superimposed Nomarski and fluorescence images showing the positions of a patch pipette and a stimulating tungsten electrode in relation to the soma and the stem axon of an MTN neuron (a). Superimposed traces of an inv-S-spike and a failure response (continuous and interrupted traces, respectively) to stimulation of the stem axon with a threshold intensity of 1.0 µA (b). The init-S-spikes showed a smaller amplitude in comparison with that of the inv-S-spikes when successively evoked at –76 mV (lower trace in c), but not at –61 mV (upper trace in c). With membrane hyperpolarization, the peak potential level of the init-S-spikes decreased in contrast to that of the inv-S-spike (d). Open and filled circles represent the peak level of inv- and init-S-spikes, respectively, in the absence of 4-AP. Open and filled triangles represent the peak level of inv- and init-S-spikes, respectively, in the presence of 4-AP. V_r; –62 mV. –0.04 (b), +0.02 (upper trace, c), and –0.25 nA (lower trace, c) DC injections into the soma to maintain baseline membrane potentials at –64, –61, and –76 mV, respectively.

C: Superimposed continuous and interrupted traces showing S-spikes evoked by stimulation of the stem axon and by injection of a current pulse, respectively, in the same MTN neuron as in (B), before (a) and after the application of 4-AP (b). Note the larger amplitude of the inv-S-spike in comparison with that of the init-S-spike in the control condition (a), but this is no longer seen after the 4-AP application (b). Horizontal arrow and dotted line indicate the peak level of the inv-S-spike before 4-AP application. Superimposed interrupted traces of init-S-spikes obtained before and after 4-AP (c). Superimposed continuous traces of inv-S-spike obtained before and after 4-AP (d). Each trace of the inv-S-spike was obtained after subtracting the nothing response from the spike potential. Upward arrowhead indicates the time of axonal stimulation. Note the differential effects of 4-AP on spike initiation and
spike invasion. Voltage and time calibrations in (a) also apply in (b–d). –0.30 nA DC injection into the soma to maintain baseline membrane potential at –78 mV.

FIG. 5. Differential effects of 4-AP on the absolute refractory period of inv- and init-S-spikes.

A: Superimposed traces of 1st and 2nd inv-S-spikes evoked by paired stimulation of the stem axon at varying intervals, obtained before (a) and after the application of 4-AP (b). Voltage and time calibrations in (Aa) also apply in (Ab) and (B). Vr; –62 mV. –0.38 (a) and –0.30 nA (b and B) DC injections into the soma to maintain baseline membrane potentials at –80 and –77 mV, respectively.

B: Superimposed traces of 1st and 2nd init-S-spikes evoked by paired current pulses at varying intervals injected into the soma of the same MTN neuron as in (A), obtained after 4-AP application. Dotted line with an arrow indicates the minimum peak level of the second init-S-spikes.

C: Plots of the relative amplitudes (RAs) of the second to the first spike against the interstimulus (a) and inter-peak interval (b). Open and filled circles (a and b) represent RAs in respective pairs of inv-S-spikes obtained before (Aa) and after the application of 4-AP (Ab), respectively. Open triangles (b) represent RAs in respective pairs of init-S-spikes obtained after the application of 4-AP (B). Bi-exponential fitting of the progress of attenuation of the init-S-spike obtained in the presence of 4-AP with fast (0.64 ms) and slow (5.30 ms) time constants (open triangles and continuous line in b). A similar bi-exponential fitting of the progress in the attenuation of inv-S-spikes in the absence of 4-AP, using the same two time constants with the proportion of the fast phase increased 27 times (open circles and dotted line in b). Open and filled diamonds represent the minimum inter-peak intervals of the presumed paired impulses in the stem axon whose RA was assumed to be 0.70, before and
after the 4-AP application, respectively. Attenuation curves (two interrupted lines attached to open and filled diamonds in b) were drawn by the double exponential function with the same two time constants as those for the S-spikes (see text). Note the smaller effects of 4-AP on the refractoriness of the presumed impulses in the stem axon. Relationships between the interstimulus (or interpulse) interval and inter-peak interval in respective pairs of S-spikes (c). Note the almost constant inter-peak interval at 4.0 ms in spite of the decreases in the interstimulus interval beyond 4.0 ms in the presence of 4-AP, in contrast to the linear decrease in the inter-peak interval in the absence of 4-AP.

FIG. 6. Differential subcellular localization of 4-AP-sensitive K+ channels in MTN neurons. 4-AP-sensitive K+ channels, Kv1.1 (A and B) and Kv1.6 (C and D), are expressed in somata alone, while K+ channels less sensitive to 4-AP, Kv2.1 (E and F), are expressed in both somata and stem axons. Arrows in (A), (C), and (E) point to the same neurons in (B), (D), and (F), respectively. Scale bars, 50 µm for (A), (C), and (E), 30 µm for (B), (D), and (F).

FIG. 7. Double fluorescence images showing differential subcellular localization of Kv channels in MTN neurons. MTN neurons labeled retrogradely with RDA applied to the masseteric nerve (Aa, Ba, Ca), and immunoreactivities for Kv1.1 (Ab), Kv1.6 (Bb), and Kv2.1 (Cb) visualized with FITC. Merged images of RDA and immunoreactivities for Kv channels (Ac, Bc, Cc). Arrowheads indicate the stem axons of MTN neurons. Note that immunoreactivities for Kv1.1 and Kv1.6 were seen only in the somata (A, B), but not in the stem axon (arrowheads in A and B) or the AH region (asterisks in Ab and Bc), whereas those for Kv2.1 were seen both in the somata and in the stem axons (C). Scale bar in (Aa) represents 20 µm, and also applies for all panels.
FIG. 8. Voltage-dependent switching between two functional modes presumed to exist in MTN neurons.

A: Peripherally arising impulses (a, spike invasion) or somatic inputs (b, spike initiation), whichever trigger spikes in the stem axon first, are forwarded to the central axon, as reflected by differentially shaped S-spikes in the soma.

B: The central pattern generator (CPG) for masticatory movements sends synaptic inputs not only to motoneurons but also to MTN neurons, thereby using MTN neurons as interneurons (IN). At depolarized membrane potentials, MTN neurons can display spontaneous oscillatory firing activity, thereby acting as IN (Ba). At hyperpolarized membrane potentials, MTN neurons can act as primary sensory neurons due to removal of the inactivation of $I_{K_{4AP}}$ (Bb). The caudal part of the central axon of MTN neurons can generate antidromic spikes by GABA-mediated primary-afferent depolarization (PAD; see DISCUSSION).

C: $I_{K_{4AP}}$ would facilitate successive spike invasion by shortening the refractory period (Ca), but suppress spike initiation by increasing the threshold (Cb).
Supplementary Information

Effects of series resistance on spike height, spike half-duration and the maximum rate of rise

When action potentials are recorded using glass pipettes, only lower frequency components of the action potential will be filtered depending on the time constants formed by the series resistance and pipette transmural capacitance (Purves 1981). Then, it is possible that the differences in the spike height, the half-duration and the maximum rate of rise between the soma and AH spikes observed in the present study resulted from a difference in the low-pass filtering property between the two recording pipettes, if they had different series resistances. We performed a simulation on an equivalent circuit of whole-cell recordings to calculate the effects of low-pass filtering on the spike height, the spike half-duration and the maximum rate of rise, by assuming that the two patch pipettes have the same transmural capacitance of 5 pF, but have different series resistances of 10 and 15 MΩ, as an extreme case. The difference in the spike amplitude between the two electrodes was calculated to be < 3%, and there was no appreciable difference in the spike duration and in the maximum rate of rise between the two spikes. Thus, a possible difference in the low-pass filtering of spike potentials can neither fully explain the differences in the spike height (12% attenuation) nor account at all for the differences in the spike duration (13% prolongation) and in the rate of rise (17% slow down) between the simultaneously recorded S- and AH-spikes seen in the present experiments.

On the other hand, patch-clamp amplifiers working in the current-clamp mode are known to introduce two voltage errors generated by the absorption of an error current ($I_\text{E}$) (Magistretti et al. 1996). One is a ‘resistive error’ ($V_R$) due to a voltage drop across the series resistance ($R_S$): $V_R = I_\text{E}R_S$, and the other is a ‘capacitative error’ ($V_C$) due to an alteration of the capacitative current charging the membrane capacitance ($C_M$): $V_C = C_M\int I_\text{E}dt$. These errors distort the absolute voltage signals, and therefore it could be suggested that differences between
the S- and AH-spikes may be due to differences in the current-clamp performance. In the present study, however, the dual whole-cell current-clamp recordings were made by two of the three Axopatch series amplifiers that have similar clamp performance unless the fast current-clamp mode is used in the Axopatch 200A/B (Magistretti et al. 1996). In fact, whichever two amplifiers were combined for simultaneous recordings from the soma and AH, the size relations in the height, duration and maximum rate of rise between the soma and AH spikes were consistent. Thus, a difference in low-pass filtering or in the preamplifier would not be able to account for the differences between the S- and AH-spikes seen in the present study.

**Comparison between Axopatch 200B and Axoclamp 2B**

We also made path-clamp recordings using a “bridge” amplifier (Axoclamp 2B), to compare between S-spikes recorded with the current-follower and those with the voltage-follower amplifier. The most notable difference was the amplitude of the injected current pulse. Due to the current absorbance in the current-follower amplifier (Magistretti et al. 1996), the amplitude of the current pulses required to initiate S-spikes was about ten times larger in the Axopatch 200B than in the Axoclamp 2B. There were also differences between the S-spikes recorded by the Axoclamp 2B and those by the Axopatch 200B. The amplitude (94.9 ± 3.5 mV, \( n = 16 \)) and the maximum rate of rise (269 ± 31 V/s, \( n = 16 \)) of the S-spikes recorded by the Axoclamp 2B were significantly (\( p < 0.001 \) and \( p < 0.01 \), respectively) smaller than the amplitude (110.6 ± 7.7 mV, \( n = 16 \)) and the maximum rate of rise (342 ± 65 V/s, \( n = 16 \)) of the S-spikes recorded by the Axopatch 200B. These observations may indicate that the current-clamp performance of the Axoclamp 2B is better. However, there was no significant (\( p > 0.5 \)) difference in the half-duration between the S-spikes recorded by the Axoclamp 2B (0.66 ± 0.09 ms, \( n = 16 \)) and those by the Axopatch 200B (0.64 ± 0.11 ms, \( n = 16 \)). Furthermore, there was no significant (\( p > 0.6 \)) difference in the \( \nu-t \) relationship between the Axopatch 200B (0.47 ±
0.04 ms at the pulse offset potential of $-45.7 \pm 7.7$ mV, $n = 10$) and the Axoclamp 2B (0.47 \pm 0.03 ms at the pulse offset potential of $-46.6 \pm 4.7$ mV, $n = 10$), when the S-spikes were evoked at holding potentials < $-75$ mV. Thus, when the Axopatch 200B was used to record the S-spikes, among the factors reflecting the differential involvement of 4-AP-sensitive K$^+$ current in the initiation and invasion of spikes, the spike height difference between the init- and inv-S-spikes may not be accurate, whereas the $v$-$t$ relationship and the half-duration could be accurate. However, a similar spike-height difference between the init- and inv-S-spikes was also observed when using the Axopatch 2B (Fig. 9Ab). Therefore, there appeared to be no marked difference between the Axopatch 200B and Axoclamp 2B, as far as the differential involvement of 4-AP-sensitive K$^+$ current in initiation and invasion of the S-spikes was concerned.

**Differential effects of temperature increases on init- and inv-S-spikes**

Using the Axoclamp 2B whether the differential involvement of 4-AP-sensitive K$^+$ current in the initiation and invasion of spikes is affected by raising the temperature to 33°C from room temperature was examined. In order to compare the init- and inv-S-spikes directly, the inv-S-spike was evoked 30 ms after evoking the init-S-spike. As partly shown in the superimposed traces of the init- and inv-S-spikes evoked at 23°C and 33°C (black and red traces, respectively, in Fig. 9A), when the temperature of the recording chamber was gradually raised to 33°C (Fig. 9Ba), the peak levels of both the init- and inv-S-spikes linearly decreased (Fig. 9Bb). Similarly, the spike durations at the half control amplitude of the init- and inv-S-spikes also decreased linearly (Fig. 9Bd). In contrast, the maximum rate of rise of the init- and inv-S-spikes remained almost constant (Fig. 9Be). Since both the S-spike amplitude and duration were largely modulated by 4-AP sensitive K$^+$ currents (Figs. 2C, 3A and 4C), these observations suggest that the $Q_{10}$ of 4-AP-sensitive K$^+$ channels is larger than that of Na$^+$.
channels. Furthermore, as clearly seen in Fig. 9Bc, the difference in the amplitude between the two S-spikes increased almost two fold over the control difference. Thus, the amplitude difference between the init- and inv-S-spikes obtained at room temperature was invariably and significantly ($p < 0.05, n = 5$) increased by $162 \pm 100\%$ by raising the temperature from 23 to $33^\circ C$ in 5 MTN neurons examined. Since the amplitude difference itself was attributed to the differential involvement of the 4-AP-sensitive K$^+$ current, this observation is also consistent with the hypothetical larger $Q_{10}$ of Shaker K$^+$ channels kinetics in comparison with those of Na$^+$ channels kinetics.

The membrane potential level at the pulse-end that is required to initiate S-spikes with a similar delay (time-to-MRR) was more positive at $33^\circ C$ than at $23^\circ C$ (Fig. 9Cc), as partly seen in the superimposed traces of the init-S-spikes recorded at 23 and $33^\circ C$ (Fig. 9Ca). Subsequently, the $v$-$t$ relationship shifted positively when the temperature increased (Fig. 9Ca and Cb). As 4-AP shifted the $v$-$t$ relationship negatively (Fig. 3C), this observation is also consistent with the larger $Q_{10}$ of 4-AP-sensitive K$^+$ currents. Indeed, the $Q_{10}$ values of the activation time constant (3.3-4.0, Russ and Siemen 1996; 3.14, Nobile et al. 1997) and conductance (> 4, Rodriguez et al. 1998; 2.95, Nobile et al. 1997) in Shaker K$^+$ channels were relatively larger than those of the activation time constant (2.3-3.1, Russ and Siemen 1996; 2.54, Jonas 1989) and conductance (1.3, Kay et al. 1998; 1.28, Nagy et al. 1983) in Na$^+$ channels. Thus, when MTN neurons are acting as primary sensory neurons at hyperpolarized membrane potentials, they can act more accurately under a more physiological condition.
References


Fig. 9. Differential effects of temperature increase on init- and inv-S-spikes.

A: Superimposed traces of the init- and inv-S-spikes evoked at 23 and 33°C (black and red traces, respectively, in b and c). Resting membrane potential, –72 mV. Direct currents of +8 pA (black traces in b and c) and +2 pA (red traces in b and c) were injected into the soma to maintain the baseline membrane potential at about –70 mV at 23 and 33°C, respectively.

B: Plotting of the various parameters of the init- (filled circles) and inv-S-spikes (open circles) against temperature. The peak levels (b) and spike durations (d) at the half control amplitude of the two spikes linearly decreased with the increase in temperature (a). The maximum rate of rise remained almost constant (e). Note increases in the difference in the amplitude between the two spikes (c).

C: Effects of temperature increase on the threshold for spike invasion. A larger membrane depolarization level was required to initiate S-spikes with a similar delay at higher temperatures (a, see also c). The resting membrane potential, –66 mV. A direct current of –15 pA was injected into the soma to maintain the baseline membrane potentials at –80 mV. Note the positive shift of the v-t relationship following the temperature increase from 23 to 33°C (b). Membrane depolarization levels required to init-S-spikes with a time-to-MRR of 0.6 ms at 23 and 33°C (c).