Bidirectional Interactions between H-Channels and $\text{Na}^+ - \text{K}^+$ Pumps in Mesencephalic Trigeminal Neurons

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The $\text{Na}^+ - \text{K}^+$ pump current (I_p) and the h-current (I_h) flowing through hyperpolarization-activated channels (h-channels) participate in generating the resting potential. These two currents are thought to be produced independently. We show here bidirectional interactions between $\text{Na}^+ - \text{K}^+$ pumps and h-channels in mesencephalic trigeminal neurons. Activation of I_h leads to the generation of two types of ouabain-sensitive I_p with temporal profiles similar to those of instantaneous and slow components of I_h , presumably reflecting Na^+ transients in a restricted cellular space. Moreover, the I_p activated by instantaneous I_h can facilitate the subsequent activation of slow I_h . Such counteractive and cooperative interactions were also disclosed by replacing extracellular Na^+ with Li^+ , which is permeant through h-channels but does not stimulate the $\text{Na}^+ - \text{K}^+$ pump as strongly as Na^+ ions. These observations indicate that the interactions are bidirectional and mediated by Na^+ ions. Also after substitution of extracellular Na^+ with Li^+ , the tail I_h was reduced markedly despite an enhancement of I_h itself, attributable to a negative shift of the reversal potential for I_h presumably caused by intracellular accumulation of Li^+ ions. This suggests the presence of a microdomain where the interactions can take place. Thus, the bidirectional interactions between $\text{Na}^+ - \text{K}^+$ pumps and h-channels are likely to be mediated by Na^+ microdomain. Consistent with these findings, hyperpolarization-activated and cyclic nucleotide-modulated subunits (HCN1/2) and the $\text{Na}^+ - \text{K}^+$ pump $\alpha 3$ isoform were colocalized in plasma membrane of mesencephalic trigeminal neurons having numerous spines.

Key words: H-channel; $\text{Na}^+ - \text{K}^+$ pump; bidirectional interactions; Na^+ -transient; microdomain; spines; excitability; mesencephalic trigeminal neurons

Introduction

The h-current (I_h) is characterized in various central neurons (Pape, 1996; Luthi and McCormick, 1998a; Moosmang et al., 2001) as well as in the cardiac sinoatrial node (Brown et al., 1979; Yanagihara and Irisawa, 1980). The I_h is produced by the activity of a hyperpolarization-activated channel (h-channel) or a hyperpolarization-activated and cyclic nucleotide-modulated (HCN) channel that is composed of four homomeric or heteromeric HCN subunit isoforms, HCN1–4 (Ludwig et al., 1998; Ishii et al., 1999; Santoro and Tibbs, 1999; Seifert et al., 1999). On the basis of findings of diverse localizations of HCN channels on various neuronal subcellular structures (Lorincz et al., 2002; Notomi and Shigemoto, 2004), the I_h has been implicated in diverse functions (Luthi and McCormick, 1998a,b; Seifert et al., 1999; Chen et al., 2001). The I_h is also involved in shifting the resting membrane potential to a more depolarized level (Banks et al.,

1993; Maccaferri et al., 1993; Pape, 1996; Janigro et al., 1997). Because the $\text{Na}^+ - \text{K}^+$ pump is an important determinant of the resting membrane potential (Senatorov and Hu, 1997; Biser et al., 2000; Lin et al., 2002) and because the I_h is persistently active at resting potential (Solomon and Nerbonne, 1993; Pape, 1996), the resting membrane potential may be regulated by some interaction between $\text{Na}^+ - \text{K}^+$ pumps and h-channels; however, it has never been addressed in neurons how $\text{Na}^+ - \text{K}^+$ pumps respond to a transient activation of I_h . This is probably because $[\text{Na}^+]_i$ is not likely to be changed easily or rapidly in response to activation of I_h . This is not necessarily the case, however, with such neurons that have a morphological specialization providing a restricted cellular space for Na^+ to enter and creating a Na^+ microdomain. We here demonstrate bidirectional interactions between $\text{Na}^+ - \text{K}^+$ pumps and h-channels in a presumed Na^+ microdomain of primary sensory neurons in mesencephalic trigeminal nucleus (MTN). The $\text{Na}^+ - \text{K}^+$ pump exhibited cooperative or counteractive response to I_h activation, depending on the degree of its activation. Consistent with these electrophysiological findings, HCN1/2 subunits and an $\alpha 3$ isoform of the $\text{Na}^+ - \text{K}^+$ pump were colocalized in the plasma membrane of somata and spines of MTN neurons. These results may provide a novel mechanism of the Na^+ microdomain for the control of excitability in primary sensory neurons in MTN.

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Figure 1. Pulse-AHP in MTN neurons. *A*, Rhodamine fluorescence (*a*) and Nomarski (*b*) images of the same MTN neuron (arrowhead). Scale bar: in *b* (*a*, *b*), 40 μm . *B*, Two well characterized features: (1) a prominent depolarizing sag potential in response to injection of hyperpolarizing current pulses; (2) single spiking (*a*, arrow) at the onset of long depolarizing current pulses when applied at a hyperpolarized membrane potential (e.g., -90 mV). A prominent pulse-AHP (*a*, asterisk) evoked at the offset of a depolarizing current pulse applied at -90 mV , but not at -65 mV . Current, voltage, and time calibrations in *b* also apply in *a*. A burst of spikes triggered in response to a depolarizing current pulse at a depolarized membrane potential (e.g., -65 mV).

Materials and Methods

Whole-cell recordings. Coronal slices of 200–250 μm thickness were made from the brain stem of Sprague Dawley (SD) rats (14–21 d postnatal). The standard extracellular solution had the following composition (in mM): 124 NaCl, 1.8 KCl, 2.5 CaCl_2 , 1.3 MgCl_2 , 26 NaHCO_3 , 1.2 KH_2PO_4 , 10 glucose. Using Axopatch-200A (Axon Instruments, Union City, CA), whole-cell recordings were made from MTN neurons with oval cell bodies that were clearly visible under Nomarski optics (BX-50WI, Olympus, Tokyo). Some of the MTN neurons were identified by retrograde labeling with rhodamine-dextran injected into the masseter muscle. The internal solution of patch pipettes had the following ionic composition (in mM): 123 K-gluconate, 18 KCl, 10 NaCl, 3 MgCl_2 , 2 ATP- Na_2 , 10 HEPES, 10 BAPTA or 0.2 EGTA; pH 7.4 adjusted with KOH. The liquid junction potential between the internal solution for the whole-cell recording (negative) and the standard extracellular solution was $\sim 10\text{ mV}$ when measured by using a wide-bore patch pipette filled with 3 M KCl as the bath ground (Neher, 1992). The recording chamber with a volume of 1.0 ml was perfused continuously with the extracellular solution at a flow rate of 1.0–1.5 ml/min. To isolate I_h , whole-cell patch-clamp recordings were made in the presence of tetrodotoxin (1 μM), 4-aminopyridine (4-AP) (1 mM), Ni^{2+} (0.2 mM), Ba^{2+} (0.3–1.0 mM), and tetraethylammonium (30 mM) (Tanaka et al., 2003). For the Na^+ ion substitution experiment, 124 mM NaCl was replaced with equimolar lithium chloride (Li^+/Cl^-). Membrane potential values given in the text were corrected for the junction potential. Ouabain and ZD 7288 were obtained from Sigma (St. Louis) and Tocris Cookson (Ellisville, MO), respectively. All recordings were made at room temperature (21–24°C). The seal resistance was usually $>5\text{ G}\Omega$. The series resistance was usually $<15\text{ M}\Omega$. When the series resistance was $>15\text{ M}\Omega$, the records were discarded. The series resistance was compensated by $\sim 70\%$. Whole-cell currents were low-pass filtered at 5–10 kHz (three-pole Bessel filter), digitized at a sampling rate of 2 kHz (DigiData 1322A, Axon Instruments), and stored on a computer hard disk. Data given in the text are presented as mean $\pm$ SD unless stated otherwise. The statistical significance was assessed using the two-tailed *t* test or one-way ANOVA. The null current level (0 pA) is indicated by interrupted lines in the figures.

Double immunofluorescence. Male SD rats (postnatal day 19) were deeply anesthetized with pentobarbital (50 mg/kg body weight) and perfused through the aorta with 25 mM PBS for 1 min, followed by an ice-cold fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 15% saturated picric acid made up in 0.1 M phosphate buffer (PB), pH 7.4, for 15 min. The brains were immediately removed and cut into several blocks. For fluorescence double-labeling experiments, coronal sections of the brain stem were cut on a freezing microtome at a thickness of 40 μm after cryoprotection in 30% sucrose and then incubated at 4°C with guinea pig antibody (Ab) (1.0 $\mu\text{g}/\text{ml}$) for HCN2 or HCN1 (Notomi and Shigemoto, 2004) and mouse monoclonal Ab for the $\alpha 1$ isoform ($\alpha 6$, Developmental Studies Hybridoma Bank, University of Iowa) or rabbit Ab (1.0 $\mu\text{g}/\text{ml}$) for the $\alpha 2$ or $\alpha 3$ isoform of Na^+/K^+

pump (Upstate Biotechnology, Lake Placid, NY) in PBS containing 0.1% Triton X-100, 0.25% λ -carrageenin, and 0.5% normal goat serum (NGS). After several washes in PBS, the sections were incubated with Alexa594-conjugated goat anti-guinea pig IgG (1:500; Molecular Probes, Eugene, OR) and Alexa488-conjugated goat anti-mouse or anti-rabbit IgG (1:500; Molecular Probes). All images were obtained using an Olympus Fluoview confocal microscope (FV300-IX). For control experiments, one of the primary antibodies was omitted or replaced with normal IgG or serum. No specific immunofluorescence for the omitted or replaced Ab was detected. For electron microscopy, coronal sections (50 μm thick) were prepared with a microslicer (DTK-1000; Dosaka, Kyoto, Japan) and washed several times in 0.1 M PB. PBS containing 10% NGS and 0.05% Triton X-100 was used to block nonspecific binding at room temperature for 1 hr. The sections were then incubated in the guinea pig Ab (2.0 $\mu\text{g}/\text{ml}$) for HCN2 or rabbit Ab (2.0 $\mu\text{g}/\text{ml}$) for the $\alpha 3$ isoform of the Na^+/K^+ pump diluted in PBS containing 10% NGS. After washes, the sections were incubated with 0.8 nm gold-coupled secondary antibodies (1:100; Aurion, Wageningen, The Netherlands) for immunogold reaction and then reacted with R-Gent SE-EM (Aurion). After treatment with 1% OsO_4 in 0.1 M PB, the sections were stained with uranyl-acetate, dehydrated, and flat embedded in Durcupan resin (Fluka, Buchs, Switzerland). Ultrathin sections were prepared (Ultracut S; Leica, Nussloch, Germany) and examined with a 1200EX electron microscope (JEOL, Tokyo, Japan).

Results

Primary sensory neurons in the MTN (Fig. 1*A*) display prominent depolarizing sag potentials mediated by I_h in response to injection of hyperpolarizing current pulses (Fig. 1*Ba*). MTN neurons are also characterized by a 4-AP-sensitive A-like K^+ current (Del Negro and Chandler, 1997). MTN neurons display only one spike at the onset of long depolarizing current pulses when applied at -90 mV (Fig. 1*Ba*, arrow); however, a burst of spikes was triggered at -65 mV in response to a depolarizing current pulse, presumably caused by partial inactivation of the A-like K^+ current (Fig. 1*Bb*), as documented previously (Del Negro and Chandler, 1997; Yoshida and Oka, 1998). In addition to these features, we noticed the generation of a prominent afterhyperpolarization (AHP), namely pulse-AHP (Fig. 1*Ba*, asterisk), at the offset of a depolarizing current pulse applied at membrane potentials around -90 mV , but not at -65 mV (Fig. 1*Bb*). The pulse-AHP was characterized by its brief time course (half duration = $23.7 \pm 4.9\text{ msec}$; $n = 13$) and high negative peak level ($-138.4 \pm 7.9\text{ mV}$; $n = 13$). The sharp peak was far beyond the equilibrium potential for K^+ ions (-97 mV). This type of response has never been demonstrated in any central or peripheral neurons.

To study ionic mechanisms of the pulse-AHP, we first examined the possible involvements of Ca^{2+} -dependent K^+ currents and A-like K^+ current. There was no significant difference ($p > 0.2$) in the amplitude between the pulse-AHPs recorded with patch pipettes containing 10 mM BAPTA ($-137.4 \pm 6.9\text{ mV}$; $n = 7$) and 0.2 mM EGTA ($-139.5 \pm 9.6\text{ mV}$; $n = 6$). Furthermore, neither apamin (0.3 μM ; $n = 4$) nor Co^{2+} (1 mM; $n = 3$) affected the pulse-AHP appreciably (data not shown). Thus, small- or large-conductance Ca^{2+} -activated K^+ channels seem to be irrelevant to generating the pulse-AHP. Similarly, the pulse-AHP was still observed in the presence of 4-AP ($-133.6 \pm 5.4\text{ mV}$; $n = 5$). There was no significant difference ($p > 0.2$) in the amplitude between the pulse-AHPs obtained in the absence and presence of

4-AP. Therefore, A-like K^+ current is also unlikely to be involved in the generation of the pulse-AHP.

Effects of ZD 7288 and ouabain on the pulse-AHP

Next, we examined whether a steady activation of I_h at -90 mV is essential for the generation of the pulse-AHP using the I_h blockers, ZD 7288 and Cs^+ . ZD 7288 ($10 \mu\text{M}$) attenuated the depolarizing sag potential (Fig. 2*Aa, Ab*, arrows) and suppressed the pulse-AHP (Fig. 2, compare *Aa, Ab*, asterisk). Similar results were obtained by using Cs^+ (1 – 5 mM). The peak amplitude of pulse-AHP was attenuated by $64.7 \pm 11.8\%$ ($n = 3$) and $56.8 \pm 7.4\%$ ($n = 5$) by ZD 7288 and Cs^+ , respectively. Thus, I_h seems to be involved in the generation of the pulse-AHP; however, because the I_h cannot underlie the membrane hyperpolarization, the pulse-AHP should be generated by a process secondary to activation of I_h .

During the depolarizing current pulse, deactivation of I_h might be progressive, leading to an increase in the whole-cell input resistance. Because the membrane potential level during the depolarizing current pulse remained almost constant (Figs. 1*Ba, 2A, B*), however, the whole-cell input resistance is considered to be constant during the pulse. Then, it is not possible for the offset of the depolarizing current pulse to electrotonically cause such a large pulse-AHP. Because the negative peak level of pulse-AHP far exceeded the equilibrium potential for K^+ ions (-97 mV), the pulse-AHP is most likely to be accounted for by the activity of the $\text{Na}^+ - \text{K}^+$ pump, the reversal potential of which is more negative than -150 to -200 mV (Chapman et al., 1983; Gadsby and Nakao, 1989; Trotier and Doving, 1996). To test this possibility, we applied ouabain, an inhibitor of the $\text{Na}^+ - \text{K}^+$ pump.

The pulse-AHP increased in amplitude with depolarization during current pulses, almost reaching -150 mV in some cases (Fig. 2*Ba, D*). Ouabain (20 – $50 \mu\text{M}$) attenuated the pulse-AHP by $46.6 \pm 9.4\%$ ($n = 5$) (Fig. 2, compare *Ba, Bb*; see *D*), without alteration of the I – V relationship measured just after the onset or just before the offset of the current pulse (Fig. 2*C*). This suggests that the decrease of pulse-AHP by ouabain is caused by a reduction in the activity of the $\text{Na}^+ - \text{K}^+$ pump rather than by alterations in passive membrane properties.

Cs^+ - and ouabain-sensitive outward current

Under voltage-clamp conditions, the effects of Cs^+ and ouabain on the possible outward current responsible for evoking the pulse-AHP were also examined. A series of negative test voltage pulses from -90 to -150 mV at a 10 mV step were applied, with or without a positive prepulse stepped to -20 from -90 mV (Fig. 3*Aa, Ba*).

Subtraction of the currents obtained by these procedures in the presence of Cs^+ (Fig. 3*Ab, Bb*, black traces) from those in the absence of Cs^+ (control) (Fig. 3*Ab, Bb*, gray traces) reveals Cs^+ -sensitive currents (Fig. 3*Ac, Bc*). Thus, obtained Cs^+ -sensitive inward current can be regarded as I_h ; however, the Cs^+ -sensitive I_h

Figure 2. Effects of h-channel blockers and ouabain on the pulse-AHP. *A*, Simultaneous abolishment of sag potential (arrow) and pulse-AHP (*) by an h-channel blocker, ZD 7288. Current, voltage, and time calibrations in *a* also apply in *b*. *B*, Sag potentials and pulse-AHPs recorded before (*a*) and after (*b*) application of ouabain ($50 \mu\text{M}$). Note a marked attenuation of pulse-AHP without appreciably affecting sag potential. Also note an injection of an additional direct hyperpolarizing current of -250 pA to bring back the holding potential to -90 mV after applying ouabain (compare the holding current levels in *a* and *b*). Current, voltage, and time calibrations in *a* also apply in *b*. *C*, No marked changes in the I – V relationships measured just after the onset (circles) and just before the offset (squares) of the current pulses, before (open symbols) and after (filled symbols) application of ouabain. *D*, The amplitude of pulse-AHP plotted against the membrane potential level just before the offset of depolarizing current pulses, before (open triangles) and after (filled triangles) application of ouabain. Ouabain decreased the amplitude of the pulse-AHP by $\sim 40\%$ at respective membrane potentials.

obtained by applying the test pulses with the prepulse (Fig. 3*Ac*) was quite different from that obtained without the prepulse (Fig. 3*Bc*). As revealed by the subtraction of the latter from the former (Fig. 3*C*), the presence of depolarizing prepulse produced Cs^+ -sensitive fast outward current, which increased almost linearly with membrane hyperpolarization (Fig. 3*D*).

Similarly, ouabain-sensitive currents were also obtained by applying the same test pulses with and without the prepulse (Fig. 4*Aa, Ba*). In response to the test pulses with the prepulse (Fig. 4*Aa*), the ouabain-sensitive currents were characterized by the fast-transient outward component followed by the slowly developing sustained outward component (Fig. 4*Ab, Ac*). Both the fast and slow outward currents increased with an increase of the negative command pulse, contrary to the behavior expected from the I – V relationship of the I_p (Chapman et al., 1983; Gadsby and Nakao, 1989; Trotier and Doving, 1996). In contrast, in response to the test pulses without the prepulse (Fig. 4*Ba*), the fast components became very small, whereas the slowly developing sustained outward currents were still dominant (Fig. 4*Bb, Bc*). Thus, the fast ouabain-sensitive outward current (Fig. 4*C*) was evoked by the presence of the depolarizing prepulse and therefore may be responsible for evoking the pulse-AHP.

The pulse-AHP was suppressed by I_h blockers (Fig. 2*Aa, Ab*), and the Cs^+ -sensitive fast outward current (Fig. 3*C*) was quite similar to that sensitive to ouabain (Fig. 4*C*). Therefore, the ouabain-sensitive pulse-AHP or the ouabain-sensitive outward current underlying the pulse-AHP would be generated by a process secondary to activation of I_h . To further confirm this possibility, we obtained the ouabain-sensitive currents in the presence of Cs^+ , which inhibits I_h but activates I_p (Glitsch, 2001).

As shown in Figure 4, *Ea1* and *Ea2*, at the offset of depolarizing prepulse, there was no ouabain-sensitive outward current in the

Figure 3. Cs⁺-sensitive outward currents indirectly underlying the pulse-AHP. *Aa, Ba*, A series of hyperpolarizing test voltage pulses from -90 to -150 mV at a -10 mV step either preceded by a depolarizing prepulse stepped from -90 to -20 mV (*Aa*) or without the prepulse (*Ba*). *Ab, Bb*, Superimposed current traces obtained before (gray traces) and after (black traces) applying 2.5 mM Cs⁺, respectively. Current responses corresponding to the part of the command pulses marked with a horizontal bar in *Aa* and *Ba* were shown in *Ab* and *Bb*, respectively, on a faster time scale. *Ac, Bc*, Cs⁺-sensitive currents isolated by subtraction of currents obtained after application of Cs⁺ from the control. Note the contamination of *I_h* by "fast outward current" at the offset of depolarizing prepulse (*Ac*). Also note that uncompensated capacitive currents seen in *Ab* and *Bb* were totally canceled out after subtraction in *Ac* and *Bc*. *C*, Effects of the prepulse evaluated by subtraction of *Bc* from *Ac*. Note the generation of Cs⁺-sensitive fast outward current by the presence of prepulse. *D*, Plots of peak amplitudes of Cs⁺-sensitive fast outward currents in *C* against test voltages.

presence of Cs⁺ (5 mM). In the five cells examined, ouabain-sensitive outward currents were consistently blocked by Cs⁺. Thus, the ouabain-sensitive fast and slow outward currents, evoked by negative command pulses, are probably generated in response to activation of *I_h*. This could be the reason why the ouabain-sensitive current shows a linear increase (Fig. 4*D*), rather than a decrease, by membrane hyperpolarization.

Indeed, a closer look revealed a deactivation or reduction of ouabain-sensitive steady currents in response to negative command pulses applied at -50 mV in the presence of Cs⁺ (5 mM) (Fig. 4*Eb1, Eb2*). The amplitude of these currents decreased almost linearly with hyperpolarization (Fig. 4*Ec*). This relation is consistent with the *I-V* relationship of Na⁺-K⁺ pump currents (Chapman et al., 1983; Gadsby and Nakao, 1989; Trotier and Doving, 1996). The mean amplitude of such a steady *I_p* at -50 mV obtained in the presence of 5 mM Cs⁺ in addition to 3 mM K⁺ was 47.8 ± 11.6 pA ($n = 5$).

The presence of depolarizing prepulse apparently facilitates the generation of the fast outward current. This suggests that a transient deactivation of *I_h* during the depolarizing prepulse or an increase in the driving potential for the *I_h* at the offset of the prepulse, or both, may be involved in the generation of the fast *I_p*.

Voltage-dependent nature of fast *I_p*

To examine the possible involvement of *I_h* deactivation in generating the fast *I_p*, negative command pulses were applied at three different holding potentials: -90, -50, and -40 mV (Fig. 5). Considering the steady-state voltage-dependent activation of *I_h* (Maccafferri et al., 1993; Pape, 1996; Cardenas et al., 1999), h-channels are highly active at -90 mV, whereas they are deactivated substantially at -50 mV and almost completely at -40 mV. When *I_h* was evoked from -90 mV, ouabain increased both the instantaneous and slow-rising components of *I_h* (Fig. 5*Aa*),

disclosing the fast and slow components of ouabain-sensitive outward currents (Fig. 5*Ab*). In contrast, when *I_h* was evoked from -50 mV, ouabain increased the instantaneous component, but the slow-rising component of *I_h* was decreased (Fig. 5*Ba*, asterisk) or increased (Fig. 5*Ba*, double asterisk), depending on the extent of *I_h* activation. Thus, the ouabain-sensitive currents showed the fast outward component followed by a slow inward component or by a biphasic slow inward and outward component (Fig. 5*Bb*). The fast outward current appears to be curtailed by an inward-going h-like current (see next section). This inward-going h-like current also appears to be further curtailed by an outward component with additional increases in the amplitude of negative command pulses.

At -90 and -50 mV, the amplitude of the fast *I_p* increased almost linearly with an increase in the hyperpolarizing command pulse (Fig. 5*Ac, Bc*, open circles). Because the instantaneous component of *I_h* also increases linearly with an increase in the hyperpolarizing command pulse (Fig. 3), there may be a strong correlation between the amplitudes of the instantaneous *I_h* and the fast *I_p*. This leads to a possibility that

the fast *I_p* may be generated by the activation of instantaneous *I_h*. The generation of instantaneous *I_h* should theoretically depend both on the steady-state conductance of the h-channel (*G_h*) at the holding potential and on the driving potential. Then, the fast *I_p* would not be produced when *I_h* was activated at a depolarized holding potential (e.g., -40 mV) where there is no steady-state *G_h*. Consistent with this idea, only the slow *I_p* but not the fast *I_p* was detected when *I_h* was activated from -40 mV (Fig. 5*Ca, Cb*).

The apparent facilitation of the fast *I_p* by a brief depolarizing prepulse may be attributed to an increase in the driving potential for *I_h* at the offset of the prepulse or by some changes in the steady interaction between h-channels and Na⁺-K⁺ pumps (see Discussion). The temporal profile of ouabain-sensitive *I_p* was almost identical to that of *I_h* at -40 and -90 mV. Therefore, Na⁺-K⁺ pumps may be activated directly by the influx of Na⁺ ions through h-channels during the pulse, thereby displaying a temporal profile of activity identical to that of h-channels at -40 and -90 mV. These observations suggest a unidirectional interaction from h-channels to Na⁺-K⁺ pumps; however, the temporal profile of ouabain-sensitive *I_p* at -50 mV (Fig. 5*Bb*) was complex and distinct from that of *I_h* at -50 mV (Fig. 5*Ba*), suggesting an additional interaction between *I_h* and *I_p*.

Facilitatory effects of fast *I_p* on the slow *I_h*

As seen in Figure 5, *Ab* and *Ac* (open triangles), the amplitude of ouabain-sensitive current measured just before the offset of hyperpolarizing command pulses increased almost linearly with an increase in the hyperpolarizing command pulses at -90 mV; however, this relation was nonlinear at -50 mV (Fig. 5*Bb, Bc*, open triangles). As mentioned above, this complex change appears to be caused by the presence of ouabain-sensitive inward-going h-like current. Thus, ouabain seems to suppress the slow *I_h* (Fig. 5*Ba*, compare the third gray and black traces marked with an

asterisk). Then, it is possible that the activity of the Na^+/K^+ pump during the pulse facilitates the activation of I_h . This unusual facilitatory effect on the slow-rising I_h was most apparent when the amplitude of slow-rising I_h was smaller than the half-maximal amplitude. In fact, steady-state voltage-dependent activation of I_h ($V_h = -100.4 \pm 9.2$ mV; $n = 4$) was significantly ($p < 0.01$) shifted in the hyperpolarizing direction by ouabain ($V_h = -107.3 \pm 9.0$ mV) (Fig. 5*Bd*), without significant changes in the slope factor ($k = 9.7 \pm 2.5$ and $k = 12.5 \pm 3.8$; $p > 0.05$). Such a facilitation of I_h was never observed when activated at a holding potential of -90 mV ($n = 5$) or -40 mV ($n = 4$) (Fig. 5*Ab,Cb*). Moreover, there was no significant ($p > 0.5$) difference in the V_h or the slope factor between the steady-state G_h values at -40 mV obtained before ($V_h = -101.1 \pm 4.8$ mV; $k = 9.6 \pm 1.7$) and after ($V_h = -100.5 \pm 4.7$ mV; $k = 9.9 \pm 1.4$) application of ouabain, as shown in Figure 5*Cd*.

Thus, Na^+/K^+ pumps and h-channels can interact cooperatively or counteractively, suggesting bidirectional influences. Because such bidirectional interactions seemed to be mediated by Na^+ ions, we examined whether the interaction is affected by decreasing Na^+ influx through h-channels in the following experiments.

Substitution of extracellular Na^+ with Li^+

Li^+ is permeant through h-channels (Ho et al., 1994; Maruoka et al., 1994) but does not stimulate the Na^+/K^+ pump as strongly as Na^+ ions (Foley, 1984; Chen et al., 1989; Rasmussen et al., 1989). As shown in Figure 6*Aa*, when extracellular Na^+ ions were substituted with Li^+ ions, the apparent I_h was depressed or enhanced depending on the amplitude of test pulses applied at -50 mV ($n = 5$). The I - V relationship of fast and slow components of Li^+ -sensitive currents (Fig. 6*Ab,Ac*) was similar to that of ouabain-sensitive currents (Fig. 5*Bb,Bc*). When I_h was evoked at -40 mV, there was no fast-rising component in the Li^+ -sensitive outward current (data not shown). This also suggests that Na^+ influx through h-channels is responsible for the bidirectional interactions between Na^+/K^+ pumps and h-channels.

Effects of decreasing $[\text{K}^+]_o$ on I_p

The presence of extracellular K^+ is crucial for activation of Na^+/K^+ pumps (Glitsch, 2001). When $[\text{K}^+]_o$ is reduced to 0.1 mM, the I_p is expected to be suppressed to a large extent even if Na^+ influx is present (Glitsch, 2001). In fact, as shown in Figure 6*B*, there was no outward ouabain-sensitive current when I_h was evoked in 0.1 mM $[\text{K}^+]_o$ at a holding potential of -40 mV. On the contrary, ouabain-sensitive currents were consistently inward-going at -80 to -150 mV, and the maximum amplitude was very small (-108.4 ± 28.7 pA at -100 or -110 mV; $n = 5$), in contrast to the prominent ouabain-sensitive outward current (620.3 ± 204.2 pA at -150 mV; $n = 4$) in 3 mM $[\text{K}^+]_o$ evoked at

Figure 4. Ouabain-sensitive outward currents underlying the pulse-AHP. *Aa, Ba*, A series of hyperpolarizing test voltage pulses from -90 to -150 mV at a -10 mV step either preceded by a depolarizing prepulse stepped from -90 to -20 mV (*Aa*) or without the prepulse (*Ba*). *Ab, Bb*, Superimposed gray and black current traces obtained before and after applying $50 \mu\text{M}$ ouabain, respectively. Current-responses corresponding to the part of command pulses marked with a horizontal bar in *Aa* and *Ba* were shown in *Ab* and *Bb*, respectively, on a faster time scale. *Ac, Bc*, Ouabain-sensitive currents isolated by subtraction of currents obtained after application of ouabain from the control. Note the presence of fast outward currents caused by the presence of a depolarizing prepulse. The fast outward current was much less prominent without the prepulse. Both the fast and slow outward currents increased as the test voltage was hyperpolarized, contrary to that expected from the I - V relationship of I_p . Also note that the uncompensated capacitive currents seen in *Ab* and *Bb* were totally canceled out after subtraction in *Ac* and *Bc*. *C*, Effects of prepulse evaluated by subtraction of *Bc* from *Ac*. Note the generation of ouabain-sensitive fast outward current by the presence of the prepulse. A filled circle indicates the peak of the respective currents. *D*, Peak amplitudes of the fast outward currents in *C* plotted against test voltages. *E*, Ouabain-sensitive currents in the presence of 5 mM Cs^+ . Note no outward current (*a2*) at the offset of prepulse (*a1*). On the contrary, negative command pulses (*b1*) revealed the deactivation of ouabain-sensitive pump currents (*b2*) when I_h was blocked by 5 mM Cs^+ . I - V relationship (*c*) measured at the time indicated with a filled circle in *b2*, displaying a well known property of the Na^+/K^+ pump current.

the same holding potential (Fig. 5*C*). Outward I_p was never obtained in 0.1 mM $[\text{K}^+]_o$. Thus, the outward I_p caused at a holding potential of -40 mV by Na^+ influx through h-channels was suppressed to a large extent by lowering $[\text{K}^+]_o$, consistent with the activity of the Na^+/K^+ pump.

Colocalization of h-channels and Na^+/K^+ pumps

In support of the electrophysiological findings, double immunofluorescence confocal microscopy revealed the spatially close relationship between HCN subunits and the $\alpha 3$ isoform of the Na^+/K^+ pump in MTN neurons. Immunoreactivity for the $\alpha 3$ isoform (Fig. 7*Aa*) and HCN2 (Fig. 7*Ab*) was colocalized (Fig. 7*Ac*) in somata and spines of large-diameter neurons in the MTN. Immunoreactivity for HCN1 was also colocalized with that for the $\alpha 3$ isoform (Fig. 7*Ad*). At the electron microscopic level, immunogold particles for the $\alpha 3$ isoform and HCN2 were distributed diffusely along the plasma membrane of somata and spines of MTN neurons in a similar manner (Fig. 7*Ba,Bb*). Immunoreactivity for the $\alpha 1$ and $\alpha 2$ isoforms of the Na^+/K^+ pump was not detected in MTN neurons expressing these HCN subunits (data not shown).

Na^+ microdomain

Na^+ influx through h-channels into a restricted cellular space (microdomain) will easily increase the local $[\text{Na}^+]_i$ and conse-

Figure 5. Three different patterns of ouabain-sensitive currents depending on the holding potentials. *Aa–Ca*, Superimposed gray and black traces of I_h , obtained before and after applying ouabain (100 μ M), respectively, evoked by negative command pulses applied at -90 , -50 , and -40 mV, respectively, in three different MTN neurons. *Ab–Cb*, Ouabain-sensitive currents isolated by subtraction of I_h obtained after applying ouabain from the control. Fast I_p was followed by either slow I_p at -90 mV (*Ab*) or inward-going h-like current at -50 mV (*Bb*), whereas there was no fast I_p but slow I_p at -40 mV (*Cb*). Time calibration in *Ab–Cb* also applies in *Aa–Ca*. *Ac–Cc*, I - V relationship of ouabain-sensitive currents at respective command potentials measured at the onset (open circles) and just before the offset (open triangles) of negative command pulses. Note the dual effects of ouabain at -50 mV: suppression (*Ba*, asterisk) and enhancement (*Ba*, double asterisk) of slow I_h depending on the voltages of command pulses, in contrast to the sole enhancement of slow I_h at -90 mV (*Aa*) and -40 mV (*Ca*). *Bd*, A significant ($p < 0.01$; ANOVA) hyperpolarizing shift of V_h by ouabain from -100.4 ± 9.2 mV to -107.3 ± 9.0 mV ($n = 4$) for I_h evoked from -50 mV. The normalized conductance–membrane potential relationship was fitted by a Boltzmann equation of the form $G/G_{\max} = (1 + \exp((V_m - V_h)/k))^{-1}$, where $G_{\max}$ is the maximal membrane conductance, V_h is the voltage at half-maximal conductance, and k is the slope factor. The reversal potential for h-channels was -40 mV. *Cd*, No significant ($p > 0.5$; ANOVA) shift of the steady-state activation curve by ouabain for I_h evoked from -40 mV. Vertical bars in *Bd* and *Cd* represent SE.

quently activate I_p (Glitsch, 2001). In turn, the activation of I_p would rapidly reduce the local $[Na^+]_i$, resulting in a sharp decline of I_p . Therefore, Na⁺ influx through h-channels into a microdomain might produce a transient I_p caused by a transient accumulation of Na⁺ ions. In fact, a transient fast I_p (537.2 ± 207.5 pA, $n = 8$) could be evoked at the offset of positive prepulses (Fig. 8*Ab*, *2) or at the onset of hyperpolarizing pulses (Figs. 4*Ac*, 5*Bb*). On the other hand, a smaller fast I_p (141.6 ± 47.8 pA; $n = 8$) was also evoked at the onset of positive pulses stepped from -90 to -20 mV in the same cell (Fig. 8*Ab*, *1). This transient I_p may result from the increased pump current at the onset of depolarization (Fig. 4*Ec*) in 8 mM $[K^+]_o$. At -90 mV, a larger steady I_p would be produced in such a relatively high $[K^+]_o$ than in 3 mM $[K^+]_o$ or in 5 mM $[Cs^+]_o$ in addition to 3 mM $[K^+]_o$ (Fig. 4*E*). This larger steady I_p at -90 mV might lead to the generation of the transient I_p at the onset of depolarization to -20 mV; however, in the presence of Cs^+ , such a transient I_p was not observed at either end of the positive pulse (Fig. 8*Ac*). Thus, the fast I_p strongly suggests the presence of a Na⁺ transient in the microdomain where h-channels and Na⁺-K⁺ pumps can interact.

If the microdomain mediates the mutual interaction, an inhibition of the Na⁺-K⁺ pump would easily result in a prolonged accumulation of Na⁺ ions in the microdomain. Then, the prolonged accumulation of Na⁺ ions would be expected to produce

a negative shift of the reversal potential for I_h . This could be detected as a decrease in the amplitude of tail I_h when the holding potential was set closely to the expected reversal potential. In fact, despite an enhancement of I_h itself, a marked reduction of tail I_h could be seen when extracellular Na⁺ ions were replaced with Li⁺ (Fig. 8*Ba,Bb*). This clearly indicates an accumulation of Li⁺ ions in microdomain. Thus, both the generation of transient fast I_p and the reduction of tail I_h by Li⁺ substitution strongly suggest the presence of Na⁺ microdomain in a restricted cellular space, where h-channels and Na⁺-K⁺ pumps can interact.

Discussion

Na⁺ influx-dependent activation of Na⁺-K⁺ pump

In the present study, we found unexpected functional coupling between h-channels and Na⁺-K⁺ pumps in the MTN neuron. Under voltage-clamp conditions, hyperpolarizing voltage steps activated instantaneous and subsequent slow-rising sustained components of I_h . The instantaneous I_h might rapidly stimulate the Na⁺-K⁺ pump, as reflected in the generation of ouabain-sensitive fast outward current (Figs. 4, 5, 8). In fact, the amplitude of fast I_p invariably and linearly increased with an increase in the amplitude of negative command pulses (Fig. 5*Ac,Bc*), similar to the case for the instantaneous I_h (Fig. 3). Na⁺-K⁺ pumps were also slowly activated as reflected by the time course of the slow component of I_h . Na⁺ influx-dependent activation of Na⁺-K⁺ pumps was also demonstrated by Na⁺ ions substitution experiments (Fig. 6*A*). These observations strongly suggest that the I_p must be strictly reflecting a possible Na⁺ transient in a microdomain where Na⁺-K⁺ pumps and h-channels can interact, as if the Na⁺-K⁺ pump were activated directly by the Na⁺ influx through h-channels. Consistent with the functional coupling between Na⁺-K⁺ pumps and h-channels, colocalization of the $\alpha 3$ isoform of the Na⁺-K⁺ pump and HCN1/2 subunits of h-channels was revealed by the immunocytochemical method (Fig. 7*A*).

Na⁺ microdomain in spines

The cell surface of rat MTN neurons is covered by numerous spines of 0.9 – 2.6 μ m in length and 0.4 μ m in width (Liem et al., 1991). A similar observation has been made on cat MTN neurons (Honma et al., 2001). In our confocal images, many spines were discernible (Fig. 7*Ac*, inset). Colocalization of HCN subunits and Na⁺-K⁺ pumps was evident in these spines as well as in somatic membrane. A Na⁺ microdomain may be created in such a spine (Fig. 7*Ac*, inset). The spine in MTN neurons is very similar to microvilli or perikaryal projections, which are widely seen in various sensory ganglion neurons as the specialization of the perikaryal surface (Pannese et al., 1983, 1994; Pannese, 2002), although their functional roles remained unknown.

The time course of fast I_p (Fig. 8*Ab*) and the reduction of tail I_h (Fig. 8*Ba,Bb*) strongly suggest the presence of Na⁺ microdomain. First, rapidly rising fast I_p reflects a sharp rise of [Na⁺]_i that can be achieved only in the restricted cellular space. Second, the rapid decay of fast I_p reflects the activity of the Na⁺–K⁺ pump in an extremely restricted cellular space. Third, as revealed by the reduction of tail I_h , an accumulation of Li⁺ ions brought about through h-channels also suggests the interaction in the restricted cellular space. Thus, the bidirectional interactions between Na⁺–K⁺ pumps and h-channels are likely to be mediated by a Na⁺ microdomain, presumably created by the morphological specialization of spines in primary sensory neurons in the MTN.

Functional and physiological significance of the interactions

The finding of the pulse-AHP led to a disclosure of the interactions. The h-channel on the spine can act more functionally through the bidirectional interactions. More importantly, the interactions revealed the presence of the Na⁺ microdomain, which is of great physiological significance for the local Na⁺ homeostasis. The property of the Na⁺ microdomain in MTN neurons is well reflected in the pulse-AHP. The property of the Na⁺ microdomain is crucial in regulating the behavior of any cation channels that are located on spines or small dendrites, such as AMPA or NMDA receptor channels and noninactivating Na⁺ channels, in addition to h-channels. Because the reversal potential for nonselective cation channels or Na⁺ channels would be affected strongly by the local Na⁺ homeostasis, the current intensity would also be affected to a large extent by the property of the Na⁺ microdomain. Thus, the behavior of any cation channels on spines or small dendrites would be governed by the property of the Na⁺ microdomain that is involved in the regulation of the local Na⁺ homeostasis.

Although the functional consequence of the present findings in MTN neurons as primary sensory neurons is not clear, the presence of the cooperative and counteractive interactions is implicated in the control of excitability at the somatic membrane. This further suggests an involvement of the cell body of MTN neurons in the control of its firing activity together with the integration of various synaptic inputs (Coprav et al., 1990; Kolta et al., 1993), independent of impulses arising from peripheral mechanoreceptors. This is in contrast to the function of the cell body of other primary sensory neurons that is involved in the regulation of axon growth during development (Zhang et al., 1994; Snider and Silos-Santiago, 1996) and in regeneration or maintenance of axons (Bergman et al., 1999; Donnerer, 2003), through activation of neurotrophin receptors.

Cooperativity between h-channels and Na⁺–K⁺ pumps

The suppression of slow-rising sustained I_h by ouabain (Fig. 5*Ba,Bb*) is unexpected and somewhat puzzling. Because the ouabain-sensitive h-like current is always preceded by a fast I_p , rapid activation of the Na⁺–K⁺ pump by instantaneous I_h (Fig. 9*A*) may subsequently facilitate the voltage- and time-dependent activation of

Figure 6. Effects of replacing extracellular Na⁺ with Li⁺ and lowering [K⁺]_o on I_p . *A*, Superimposed gray and black traces of I_h before and after replacing extracellular Na⁺ with Li⁺, respectively. *a*, Li⁺-sensitive currents isolated by subtraction of I_h obtained after replacing extracellular Na⁺ with Li⁺ from the control (*b*), quite similar to those sensitive to ouabain (Fig. 5*Bb*). *I*–*V* relationship of Li⁺-sensitive currents measured at the onset (open circles) and just before the offset (open triangles) of negative command pulses (*c*), quite similar to that of ouabain-sensitive currents (Fig. 5*Bc*). *B*, Superimposed gray and black traces of I_h evoked from –40 mV in 0.1 mM [K⁺]_o before and after applying ouabain, respectively. *a*, I_p was very small and inward-going (*b*). *I*–*V* relationship of the I_p (*c*).

h-channels. One might ask then how the enhancement of I_h is brought about by rapid activation of Na⁺–K⁺ pumps under voltage-clamp conditions. If the rapid activation of Na⁺–K⁺ pumps causes an additional hyperpolarization in the microdo-

Figure 7. Colocalization of the Na⁺–K⁺ pump $\alpha 3$ isoform and HCN2/HCN1 subunits. *Aa–Ac*, Immunoreactivity for the Na⁺–K⁺ pump $\alpha 3$ isoform (*a*; green) and HCN2 (*b*; red) in MTN neurons. Immunofluorescence for the Na⁺–K⁺ pump $\alpha 3$ isoform (green) and HCN2 (red) were highly colocalized (*c*; yellow; overlaid images of *a* and *b*) and restricted to the plasma membrane of MTN neurons in a 0.5 μ m confocal optic section. An area enclosed with a rectangle is enlarged in the inset (*c*). Arrows in the inset shows spines. *Ad*, A similar colocalization is observed for immunofluorescence for the Na⁺–K⁺ pump $\alpha 3$ isoform and HCN1 in MTN neurons. Scale bar: (in *Ad*) *Aa–Ac*, 50 μ m. *B*, Immunogold particles for the Na⁺–K⁺ pump $\alpha 3$ isoform (*a*) and HCN2 (*b*) are observed along the plasma membrane of MTN neurons (S). Scale bar represents 0.2 μ m. An arrow indicates a somatic spine labeled for HCN2.

Figure 8. Microdomain revealed by transient I_p and local accumulation of cations. *Aa*, *Ab*, Two types of transient I_p (*Ab*, *1 and *2) evoked at the onsets of depolarization and hyperpolarization (*Aa*) in 8 mM $[\text{K}^+]_o$ and 145 mM $[\text{Na}^+]_o$. The amplitude of the latter (*2) was larger than that of the former (*1), as shown on the expanded time scale. *Ac*, Two types of transient I_p were not evoked in the presence of 5 mM Cs^+ . *Ba*, Superimposed gray and black traces of I_h evoked by a negative pulse to -150 mV obtained before and after replacing extracellular Na^+ with Li^+ , respectively. Note a prominent reduction of tail I_h despite an enhancement of I_h itself. *Bb*, The encircled part of tail I_h in *Ba* was reproduced on the expanded current and time scales.

main even under voltage-clamp conditions, this local hyperpolarization may further activate h-channels in the same microdomain, as in a positive feedback manner (Fig. 9*B*). In such a case, the microdomain must be electrotonically separated from the surrounding soma membrane, just as the case of spines, so that the microdomain can act independently of the clamped membrane potential.

Alternatively, it may be possible that decreases in $[\text{Na}^+]_i$ caused by a rapid activation of I_p would increase the Na^+ gradient for I_h . This is unlikely, however, because the reversal potential of I_h is hardly affected by decreases in $[\text{Na}^+]_i$. In the most extreme case, even if $[\text{Na}^+]_i$ is reduced to zero, the reversal potential can be estimated to shift positively only by <1 mV, from the Goldman–Hodgkin–Katz equation under the present experimental condition.

Limitation of functional coupling

At a holding potential of -50 mV, the facilitatory effect of Na^+/K^+ pumps on the generation of slow I_h was most apparent when the extent of slow I_h activation was less than half-maximal, and it was never observed when the slow I_h was maximally activated by negative pulses to -150 mV (Fig. 5*B*). When h-channels are rapidly and strongly activated by a large negative command pulse, the cooperativity between h-channels and Na^+/K^+ pumps is masked. Instead, the counteraction becomes apparent. On the other hand, when I_h was activated from a holding potential of -90 mV, the fast I_p was not followed by inward-going h-like currents (Fig. 5*A*). In this case, there is no facilitatory effect of the fast I_p on the I_h . This situation may result from two possible conditions. First, a steady activation of Na^+/K^+ pumps by the steady activation of I_h at -90 mV reduces the instantaneous availability of Na^+/K^+ pumps for generating the fast I_p . Second, at -90 mV, I_h is already partially activated and may already be facilitated substantially, so that little h-channels remain to be facilitated. Thus, the facilitatory functional coupling between

Figure 9. A hypothetical functional coupling between Na^+/K^+ pumps and h-channels in a spine. After the offset of the positive pulse (Figs. 1–4), all Na^+/K^+ pumps will take either the free E_1 -ATP or the $\text{P-E}_2(\text{Na}_3)$ conformation. As soon as instantaneous I_h is activated by the offset of the positive pulse, Na^+ influx through the h-channel stimulates the pump of E_1 -ATP conformation, and Na^+ ions are simultaneously released from the $\text{P-E}_2(\text{Na}_3)$ conformation (*A*), resulting in the generation of the fast I_p , which in turn brings more hyperpolarization to their neighboring h-channels, leading to a positive feedback activation of h-channels (*B*). The Na^+/K^+ pump essentially exists in two conformations, E_1 and E_2 , which may be phosphorylated (E_1 -P, P-E_2) or dephosphorylated.

h-channels and Na^+/K^+ pumps may also be limited by the mutual steady-state interaction.

Such a limitation or the balance between the cooperative and counteractive interactions would be affected by the temperature, however, because of the temperature dependence of both Na^+/K^+ pumps and voltage-gated ion channels. The temperature coefficient (Q_{10}) of Na^+/K^+ pumps has been reported to be ~ 2 between 20 and 37°C (Glitsch and Pusch, 1984; Thompson and Prince, 1986; Nakamura et al., 1999). This value is similar to those of voltage-gated ion channels (Beam and Donaldson, 1983; Nobile et al., 1990), although the exact value of Q_{10} for h-channels is not known yet. It may not be unreasonable to assume that the bidirectional interactions are much more stringent under *in vivo* condition than under the present experimental condition at room temperature.

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