Functional coupling between sodium-activated potassium channels and voltage-dependent persistent sodium currents in cricket Kenyon cells

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SODIUM-ACTIVATED POTASSIUM (K_{Na}) channels have been shown to be present in many excitable cell membranes (Bader et al. 1985; Bischoff et al. 1998; Dale 1993; Dryer 1991; Dryer et al. 1989; Egan et al. 1992; Haimann et al. 1992; Safronov and Vogel 1996; Schwindt et al. 1989) since their original discovery in guinea pig cardiomyocytes as high-conductance K^+ channels activated by high concentrations of internal Na^+ (Kameyama et al. 1984). Subsequently, considerable evidence has accumulated showing that K_{Na} channels play a role in the regulation of neuronal excitability (Schwindt et al. 1989), the modulation of action potential waveform (Dryer 1994), and the response of excitable cells to hypoxia and ischemia (Yuan et al. 2003). Recently, it was shown that K_{Na} channels are activated by Na^+ influx through TTX-sensitive persistent Na^+ channels in tufted/mitral cells of the olfactory bulb (Hage and Salkoff 2012), thus showing functional coupling between Na^+-activated K^+ channels and TTX-sensitive persistent Na^+ currents. K_{Na} channels are found in insect neurons such as cockroach DUM neurons (Grolleau and Lapied 1994) and in cultured Drosophila neurons (Saito and Wu 1991). In the cricket Gryllus bimaculatus, Aoki et al. (2008) showed the presence of K_{Na} channels in Kenyon cells, and they were postulated to be an important target molecule in olfactory learning circuits that mediate reward and punishment signals, which are regulated by octopamine and dopamine, respectively (Schwaerzel et al. 2003; Unoki et al. 2005, 2006). Recently, Inoue et al. (2014) suggested the presence of two types of voltage-dependent Na^+ currents in cricket Kenyon cells: the first Na^+ current (I_{Na}) was activated at $-40$ mV and half-inactivated at $-46.7$ mV, and the persistent Na^+ current (I_{NaP}) was activated at $-51$ mV and half-inactivated at $-30.7$ mV. TTX (1 $\mu$M) completely blocked I_{Na} and I_{NaP}, but 10 nM TTX blocked I_{NaP} more potently than I_{Na}. Cd^2+ (50 $\mu$M) potently blocked I_{Na} with little effect on I_{NaP}. On the basis of voltage- and current-clamp experiments, K_{Na} channels are suggested to play a role in generating membrane oscillations by coupling with TTX-sensitive Na^+ currents. However, it is still uncertain which type of Na^+ current may participate in the activation of K_{Na} channels in cricket Kenyon cells. Therefore, in this study, we aimed to determine the source of Na^+ influx for the activation of K_{Na} channels in Kenyon cells of the cricket G. bimaculatus. Our results showed that I_{NaP}, which is blocked by TTX (1 $\mu$M) or Cd^2+ (50 $\mu$M), may play a major role in the activation of K_{Na} channels.

METHODS

Animals. We used adult male crickets (G. bimaculatus) in all experiments. They were kept in a colony in the Department of Biology at 25–30°C with a relative humidity of 65–85% under a 12:12-h light-dark photoperiod. They were fed on an artificial diet for insects (Oriental Yeast) and given water. Before dissection, adult male crickets were anesthetized with CO_2. The cricket brain was removed carefully from the head capsule, taking care not to tear the alimentary tract. Mushroom bodies (MBs) were dissected out of the brains and placed in a silicone chamber (volume of 3 ml) filled with Ca^2+ -free normal saline and incubated for 15 min. The MBs were then transferred to a vial containing a dissociation solution (Sumitomo Nerve-Cell Culture medium; Sumitomo Bakelite). The isolated MBs were incubated in this medium for 30 min at 25°C. After incubation, the pooled MBs were rinsed with normal saline and dissociated by gentle trituration through a fire-polished pipette with an inner diameter of $-100$ $\mu$m. Freshly dispensed cells were allowed to settle on the flat glass bottom of a silicone chamber mounted on the stage of an inverted microscope (Nikon Diaphot).

Patch-clamp recording. Patch electrodes were pulled from capillary tubes (G1.5; Narishige) using a two-stage pipette puller (PP-83; Narishige) with a three-dimensional hydraulic micromanipulator (MHW-3; Narishige). The reference electrode was an Ag-AgCl wire
connected to the bath solution through a 100 mM KCl-agar bridge. Single-channel recordings were performed using the cell-attached configuration of the patch-clamp technique. Single-channel currents were measured in cell-attached patches by filling the patch pipettes (about 6–10 MΩ) with a high-K+ solution containing 140 mM KCl and 5 mM HEPES buffered to pH 7.4 with Tris-HCl. Voltage across the patch was controlled by setting the cellular membrane potential to 0 mV using a high-K+ extracellular solution containing 140 mM KCl, 74 mM glucose, and 2 mM HEPES buffered to pH 7.4 with Tris-HCl. For selective recordings of KNa-channel currents, 10 mM NaCl was added to the high-K+ extracellular bath solution. Currents were filtered at 2 kHz and digitized at 10 kHz. In all experiments, voltage-clamp was controlled with a List EPC-7 patch-clamp amplifier. The experiments were performed at room temperature (20–25°C).

Analysis of single-channel currents. Analysis of unitary currents was performed with pCLAMP 9.0 software (Axon Instruments). Current amplitude histograms based on the analysis of current amplitudes in single-channel recordings provided the clearest demonstration of multiple current levels. The total number of functional channels (N) in a patch was estimated by observing the number of peaks in the current amplitude histograms. The open probability (Po) of single-channel currents was expressed as NPo, and calculated with the following equation: NPo = (A0 + 2A1 + 3A2 + ... + nAn)/(A0 + A1 + A2 + ... An), where A0 is the area under the curve of an all-point amplitude histogram corresponding to the current in the closed state and A1-An represents the histogram area reflecting the different open-state current levels for 1–n channels present in the patch. Histogram parameters were obtained from multiple least-squares Gaussian fits of the data with Clampfit 9.2 software (Axon Instruments).

Drugs. The following drugs were used: TTX (Wako) as a voltage-dependent Na+ channel blocker; 2-amino-6-trifluoromethoxy benzothiazole (riluzole; Sigma) as a TTX-sensitive persistent Na+ channel blocker; cadmium chloride as a TTX-sensitive persistent Na+ channel blocker; and nickel chloride, cobalt chloride, and lithium chloride (all from Wako). All drug solutions were prepared freshly before use and were perfused continuously at a rate of ~2 ml/min.

Statistical analysis. Values are given as the means ± standard error, with n representing the number of cells. For comparisons between two groups, Student’s t-test, paired or unpaired, was used for statistical analysis, with P values <0.05 considered statistically significant.

RESULTS

Selective recording of KNa currents. In this study, we selectively recorded KNa-channel activity in a high-K+ (140 mM) bath solution with the addition of 10 mM NaCl according to previously reported methods (Aoki et al. 2008; Nakamura and Yoshino 2013). The average single-channel conductance of the KNa channels at full state calculated from the slope of the current-voltage (I-V) curve over the −60 to +60 mV range was 111 pS (n = 12; Fig. 1A).

Effect of mechanical influence on KNa-channel currents. Single-KNa-channel activity was recorded while stopping drug delivery by bath perfusion to avoid a mechanical effect on single-channel activity. To check whether perfusing a bath solution exerted a mechanical effect on channel activity, we compared the Po of KNa channels before and after perfusion of the bath solution from a cell-attached patch membrane (Fig. 1B). The Po of single KNa channels before and after perfusion of the bath solution was 0.0566 and 0.063, respectively. The average value of the relative Po examined from nine different cells was 1.0 before perfusion and 1.06 ± 0.063 after perfusion (Fig. 1D). No significant difference was observed between them, indicating that we could exclude a mechanical effect on the activity of single KNa channels by perfusing the bath solution. Therefore, subsequent experiments were performed by recording single-channel currents at the resting condition before and after perfusion of the test drugs.

Effect of increasing Na+ concentration in a bath solution on KNa-channel current. Previously, we reported that KNa-channel activity was regulated by Na+ ions crossing the cell membrane through TTX-sensitive Na+ channels (Nakamura and Yoshino 2013). To verify further the functional coupling between KNa channels and Na+ currents, we first examined the effects of increasing Na+ concentrations on KNa-channel activity. In these experiments, we increased Na+ concentrations to 30 and 50 mM from 10 mM by simply adding 20 and 40 mM NaCl in a bath solution, respectively. Therefore, the osmolarity of the external solution changed by those treatments. When we changed 10 to 50 mM NaCl, the osmolarity changed by 80 mosM. Therefore, we first tested whether the addition of 80 mM mannitol into a bath solution caused a change in Po of KNa channels. The Po before and after perfusion of 80 mM mannitol to the bath solution was 0.0348 and 0.0259, respectively (Fig. 1C). The average value of the relative Po examined from three different cells was 1.0 before and 0.882 ± 0.105 after (Fig. 1E). No significant difference was observed between them, indicating that 80 mosM change has no effect on KNa-channel activity. Therefore, the subsequent experiments were performed by simply adding NaCl to the bath solution.

Figure 2A shows the typical example showing the effect of increasing Na+ concentrations to the activity of KNa channels. Single-channel activity of KNa channels was recorded with a control bath solution containing 10 mM Na+ from a cell-attached patch membrane (Fig. 2A). When the concentration of Na+ was increased to 30 mM, the Po of KNa channels increased to 0.193 (30 mM Na+) from 0.107 (Control) in this cell (Fig. 2A). The average value of the relative Po examined from six different cells was significantly increased to 1.67 ± 0.088 (30 mM Na+) from 1.0 (Control; P < 0.05; Fig. 2D). Next, we examined the effect of further increasing Na+ concentration on KNa-channel activity. When 50 mM Na+ was perfusate, the Po of KNa channels increased to 0.118 (50 mM Na+) from 0.0255 (Control) in this cell (Fig. 2B). The average value of the relative Po examined from six different cells was significantly increased to 5.37 ± 0.98 (50 mM Na+) from 1.0 (Control; P < 0.05; Fig. 2D). By washout of the cell for 5 min with control solution (10 mM NaCl), Po recovered partially to 3.30 ± 1.13 (n = 3).

Effect of substituting Na+ with Li+ in a bath solution on KNa-channel currents. The permeability of voltage-gated Na+ channels for Li+ is nearly equal to that of Na+ (Hille 2001). However, Li+ is a very weak activator of KNa channels (Dryer 1994). Therefore, we examined the effect of substituting Na+ with Li+ in a bath solution on KNa-channel activity. When Na+ was replaced by an equimolar concentration of Li+ (10 mM), the Po of KNa channels decreased to 0.0304 (10 mM Li+) from 0.102 (Control) in this cell (Fig. 2C). The average value of the relative Po examined from six different cells was significantly reduced to 0.249 ± 0.071 (10 mM Li+) from 1.0 (Control; Fig. 2D). By washout of the cell for 5 min with control solution (10 mM NaCl), Po recovered partially to 0.265 ± 0.08 (n = 4).

These results further indicated that KNa-channel activity is regulated by Na+ influx through Na+ channels, although the
Fig. 1. Effect of perfusing bath solution and increasing osmolarity on Na\(^+\)-activated K\(^+\) (K\(_{Na}\)) channel activity. Single-channel currents were recorded from cell-attached patch membranes of isolated Kenyon cells. A: K\(_{Na}\)-channel currents at different membrane voltages from -20 to +60 mV with 20-mV increments. The averaged current-voltage (I–V) relationship for single-K\(_{Na}\)-channel currents at full state from 12 different cells is shown at the bottom. Data points from -60 to +60 mV were fitted by linear regression and yielded a slope conductance of 111 pS. B: K\(_{Na}\)-channel currents before (left trace) and after (right trace) perfusion of 80 mM mannitol to the bath solution. The corresponding all-point amplitude histogram is shown at the bottom. C and O are the closed and opening levels, respectively. C: K\(_{Na}\)-channel currents before (left trace) and after (right trace) perfusion of 80 mM mannitol to the bath solution. Data are from 9 different cells. D: the relative open probability (P\(_o\)) of K\(_{Na}\)-channel currents before and after perfusion of 80 mM mannitol to the bath solution. Data are from 3 different cells.

recovery of P\(_o\) is incomplete, similar to the case with perfusion of high concentration of Na\(^+\) in the external solution.

Effect of TTX in a bath solution on K\(_{Na}\)-channel currents. Previously, we showed the presence of two distinct components of TTX-sensitive Na\(^+\) currents, I\(_{NaF}\) and I\(_{NaP}\), in cricket Kenyon cells (Inoue et al. 2014). I\(_{NaF}\) was blocked by 10 nM TTX and less blocked by 50 µM Cd\(^{2+}\), whereas I\(_{NaP}\) was blocked by 1 µM TTX and potently blocked by 50 µM Cd\(^{2+}\) (Inoue et al. 2014). Therefore, TTX and Cd\(^{2+}\) provide us a powerful pharmacological tool for selectively blocking I\(_{NaP}\) and I\(_{NaF}\): if we use 10 nM TTX, we could selectively block I\(_{NaP}\) without a significant effect of on I\(_{NaF}\). Therefore, we next investigated which component of Na\(^+\) currents is responsible for activating K\(_{Na}\) channels by using an appropriate concentration of TTX or Cd\(^{2+}\). When 1 µM TTX, which blocks both I\(_{NaP}\) and I\(_{NaF}\), was applied, the P\(_o\) of K\(_{Na}\) channels decreased to 0.00614 (1 µM TTX) from 0.0775 (Control) in this cell (Fig. 3A). The average value of the relative P\(_o\) examined from five different cells was significantly reduced to 0.246 ± 0.091 (1 µM TTX) from 1.0 (Control; Fig. 3D). Conversely, the application of TTX (0.01 µM), which blocks only I\(_{NaF}\), had little effect on the P\(_o\) of K\(_{Na}\) channels: NP\(_o\) was 0.398 (Control) and 0.297 (0.01 µM TTX) in this cell (Fig. 3B). The average value of the relative P\(_o\) examined from three different cells was as follows: 1.0 (Control) and 0.877 ± 0.15 (10 nM TTX); no significant difference was observed between them (Fig. 3D). These results indicated that I\(_{NaP}\) is a source of Na\(^+\) for the activation of K\(_{Na}\) channels.

Effect of Cd\(^{2+}\) as a specific blocker of persistent Na\(^+\) channels in a bath solution on K\(_{Na}\)-channel currents. To confirm further whether K\(_{Na}\)-channel activity is regulated by I\(_{NaP}\), we examined the effect of Cd\(^{2+}\) at a concentration of 50 µM, which selectively blocks persistent Na\(^+\) currents in cricket Kenyon cells (Inoue et al. 2014; Schafer et al. 1994) on K\(_{Na}\)-channel activity. The P\(_o\) of K\(_{Na}\) channels decreased to 0.00864 (50 µM Cd\(^{2+}\)) from 0.0600 (Control) by this treatment (Fig. 3C). The average value of the relative P\(_o\) examined from three different cells was significantly decreased to 0.241 ± 0.095 (50 µM Cd\(^{2+}\)) from 1.0 (Control;
By washout of the cell for 5 min with control solution, representative unitary KNa-channel currents before (Control) and after (30 mM Na\textsuperscript{+}) application of a bath solution containing 30 mM Na\textsuperscript{+}. The corresponding all-point amplitude histogram is shown at the bottom. B: representative unitary KNa-channel currents before (Control) and after (50 mM Na\textsuperscript{+}) application of a bath solution containing 50 mM Na\textsuperscript{+}. The corresponding all-point amplitude histogram is shown at the bottom. C: unitary KNa-channel currents before (Control) and after (10 mM Li\textsuperscript{+}) replacement of Na\textsuperscript{+} with an equimolar concentration of Li\textsuperscript{+}. The corresponding all-point amplitude histogram is shown at the bottom. D: average relative Po of KNa-channel currents in Control (1.0), 30 mM NaCl (1.67 ± 0.088, n = 6), 50 mM NaCl (5.37 ± 0.98, n = 6), and 10 mM LiCl (0.249 ± 0.071, n = 6). *P < 0.05.

Fig. 2. Effect of altering Na\textsuperscript{+} concentration and replacing Na\textsuperscript{+} with an equimolar concentration of Li\textsuperscript{+} in a bath solution on KNa-channel activity. A: representative unitary KNa-channel currents before (Control) and after (30 mM Na\textsuperscript{+}) application of a bath solution containing 30 mM Na\textsuperscript{+}. The corresponding all-point amplitude histogram is shown at the bottom. B: representative unitary KNa-channel currents before (Control) and after (50 mM Na\textsuperscript{+}) application of a bath solution containing 50 mM Na\textsuperscript{+}. The corresponding all-point amplitude histogram is shown at the bottom. C: unitary KNa-channel currents before (Control) and after (10 mM Li\textsuperscript{+}) replacement of Na\textsuperscript{+} with an equimolar concentration of Li\textsuperscript{+}. The corresponding all-point amplitude histogram is shown at the bottom. D: average relative Po of KNa-channel currents in Control (1.0), 30 mM NaCl (1.67 ± 0.088, n = 6), 50 mM NaCl (5.37 ± 0.98, n = 6), and 10 mM LiCl (0.249 ± 0.071, n = 6). *P < 0.05.

Fig. 3D). These results further confirmed that the major determinant for the activation of KNa channels is $I_{NAP}$.

Effect of riluzole in a bath solution on KNa-channel currents. Previously, we showed that riluzole (20–100 μM) nonselectively blocked both $I_{NAP}$ and $I_{Na}$ (Inoue et al. 2014). When riluzole (100 μM) was applied, the Po of KNa channels decreased to 0.005 (100 μM riluzole) from 0.055 (Control) in this cell (Fig. 4A). The Po after reintroduction of control solution is 0.0344 in this cell. The average value of the relative Po examined from five different cells was significantly reduced to 0.276 ± 0.821 (100 μM riluzole) from 1.0 (Control; Fig. 4B). By washout of the cell for 5 min with control solution, Po recovered partially to 0.383 ± 0.187 (n = 3).

Effect of inorganic Ca\textsuperscript{2+} channel blockers in a bath solution on KNa-channel currents. Na\textsuperscript{+} ions pass through voltage-dependent Ca\textsuperscript{2+} channels on the removal of extracellular divalent cations (Carbone et al. 1997; Zakharov et al. 1999). Therefore, we tested the possibility that Na\textsuperscript{+} influx is via Ca\textsuperscript{2+} channels by investigating the effect of Ca\textsuperscript{2+}-channel blockers on the activity of KNa channels. We utilized Co\textsuperscript{2+}, Ni\textsuperscript{2+}, and Cd\textsuperscript{2+} at high concentrations (500 μM) as nonselective inorganic Ca\textsuperscript{2+}-channel blockers. When we applied 500 μM Co\textsuperscript{2+}, the activity of KNa channels was unaffected: Po of the KNa channels was 0.826 (Control) and 0.884 (500 μM Co\textsuperscript{2+}) in this cell (Fig. 5A). The average value of the relative Po examined from five different cells was as follows: 1.0 (Control) and 1.06 ± 0.105 (500 μM Co\textsuperscript{2+}); no significant difference was observed between them (Fig. 5D). Next, we examined the effects of 500 μM Ni\textsuperscript{2+} (Fig. 5B). Similar to the action of Co\textsuperscript{2+}, the Po of the KNa channels was unaffected by Ni\textsuperscript{2+}: 0.5 (Control) and 0.448 (500 μM Ni\textsuperscript{2+}). The average value of the relative Po examined from three different cells was 1.0 (Control) and 0.979 ± 0.126 (500 μM Ni\textsuperscript{2+}). Contrary to the action of Ni\textsuperscript{2+} and Co\textsuperscript{2+}, the application of Cd\textsuperscript{2+} (500 μM) remarkably decreased the Po of KNa channels to 0.0869 (500 μM Cd\textsuperscript{2+}) from 0.243 (Control) in this cell (Fig. 5C). The average value of the relative Po examined from six different cells was significantly decreased to 0.241 ± 0.095 (500 μM Cd\textsuperscript{2+}) from 1.0 (Control; Fig. 5D). These results indicated that the source of Na\textsuperscript{+} ions for the activation of KNa channels is not via voltage-dependent Ca\textsuperscript{2+} channels.

Effect of the substitution of external Na\textsuperscript{+} with choline\textsuperscript{+} on net outward currents. To examine whole cell macroscopic KNa currents in cricket Kenyon cells, we performed perforated whole cell recordings using β-escin. In particular, we validated whether the macroscopic KNa currents are Na\textsuperscript{+}-influx-dependent. Figure 6A shows the net currents obtained by step
depolarization to +50 mV from a holding potential of −60 mV with 10-mV increments. By substituting all extracellular NaCl (140 mM) with an equimolar concentration of choline chloride, both the inward and outward currents were decreased (Fig. 6A). The inward current that remains in the Na+-free condition is Ca2+-current carried by Ca2+ channels as has been reported in the previous paper (Inoue et al. 2014). The I–V relationship for the inward and outward currents indicated that both currents were reduced between −30 and +50 mV (Fig. 6, B1–B3). In an Na+-free solution, the average values of relative outward currents at the early and late part decreased significantly by 62.88 ± 3.71% and 63.73 ± 3.4% (both n = 23) of control, respectively (Fig. 6, E2 and E3). To confirm further the Na+ dependence of the outward currents, we examined the effect of lowering external Na+ to a concentration of 70 mM. Figure 6C shows the control records. After replacing the extracellular NaCl (140 mM) with 70 mM NaCl plus 70 mM choline chloride, both the inward and outward currents decreased (Fig. 6C). The I–V relationship for both the inward and outward currents indicated that both currents were reduced between −30 and +50 mV (Fig. 6, D1–D3). In a low-Na+ (70 mM) solution, the average value of the relative outward currents at the early and late part was decreased significantly by 79.99 ± 2.68% and 81.14 ± 2.56% (both n = 8) of control, respectively (Fig. 6, E2 and E3). These results indicate that outward currents exist that are activated by Na+ entry through Na+ channels.

To confirm further the presence of Na+ entry-dependent outward currents in cricket Kenyon cells at the level of whole cell currents, the current trace was reconstructed by subtracting the net current in an Na+-free or 70 mM Na+ solution from the net currents in the control solution. The resultant current was found to be composed of rapid inward and subsequent rapid and steady outward currents (Fig. 6, A3 and C3). As shown in Fig. 6, B3 and D3, the average I–V curve for the subtracted currents further indicated the presence of the component of K+ outward current dependent on Na+ entry. These whole cell experiments further confirm the functional coupling of KNa channels and Na+ channels.

**DISCUSSION**

This study showed that KNa channels are functionally coupled with both TTX- and Cd2+-sensitive voltage-dependent persistent Na+ channels in Kenyon cells isolated from the MB of the cricket *G. bimaculatus* brain.

**Source of Na+ entry for the activation of KNa channels.** The hypothesis that Na+ entry through persistent Na+ channels is
increasing Na
tions strongly support the hypothesis that Na
outward currents as well as inward currents. These observa-

tions are based on the following evidence: 1) the $P_o$ of $K_{Na}$ channels was increased with increasing Na$^+$ concentration in a bath solution; 2) the $P_o$ of $K_{Na}$ channels was reduced by the replacement of Na$^+$ with an equimolar concentration of Li$^+$ in a bath solution; 3) the $P_o$ of $K_{Na}$ channels was reduced in the presence of 1 μM TTX, which blocks both $I_{Na}$ and $I_{Na}$, 4) the $P_o$ of $K_{Na}$ channels was unaffected in the presence of 10 nM TTX, which selectively blocks $I_{Na}$; 5) the $P_o$ of $K_{Na}$ channels was reduced in the presence of Cd$^{2+}$ (50 μM), which selectively blocks $I_{Na}$; 6) the $P_o$ of $K_{Na}$ channels was reduced in the presence of riluzole (100 μM), which blocks both $I_{Na}$ and $I_{Na}$; 7) the $P_o$ of $K_{Na}$ channels was unaffected by the blockade of voltage-dependent Ca$^{2+}$ channels using the nonspecific Ca$^{2+}$-channel blockers Co$^{2+}$ and Ni$^{2+}$; and 8) lowering extracellular Na$^+$ by replacing Na$^+$ with choline$^+$, Na$^+$, reduced the open state of Na$^+$ channels and Na$^+$ by replacing Na$^+$ with choline$^+$, caused a reduction of whole cell outward currents as well as inward currents. These observations strongly support the hypothesis that Na$^+$ entry via voltage-dependent Na$^+$ channels participates in the activation of $K_{Na}$ channels. In this study, we especially suggest that both TTX- and Cd$^{2+}$-sensitive $I_{Na}$ are obviously related to the activation of $K_{Na}$ channels based on the experiments using 10 nM TTX or 50 μM Cd$^{2+}$. Thus, we conclude that both TTX- and Cd$^{2+}$-sensitive $I_{Na}$ may participate largely in elevating intracellular Na$^+$ concentration ([Na$^+$]$_i$) and accordingly activate $K_{Na}$ channels. These results are consistent with previous studies demonstrating that TTX-sensitive $I_{Na}$ are the source of Na$^+$ for the activation of $K_{Na}$ channels in tufted/mitral cells of the olfactory bulbs (Budelli et al. 2009; Hage and Salkoff 2012).

It has been shown that application of veratridine, which prolongs the open state of the sodium channel, increases $K_{Na}$-channel activity (Hage and Salkoff 2012). The use of this and other toxins that prolong Na$^+$-channel inactivation such as BgII and BgIII (the toxins isolated from the sea anemone Bunodosoma granulifera; Bosmans et al. 2002; Salceda et al. 2002) and CgNa (the toxin isolated from the sea anemone Condylactis gigantea; Billen et al. 2010; Ständker et al. 2006) would further support the functional coupling between $K_{Na}$ channels and voltage-dependent persistent Na$^+$ current in cricket Kenyon cells. Hage and Salkoff (2012) suggested that $I_{Na}$ is not responsible for the activation of $K_{Na}$ channels because $I_{KNa}$ persists even when $I_{Na}$ is eliminated or reduced by a depolarizing holding potential of −50 mV in tufted/mitral cells. The present result using cricket Kenyon cells has shown that the Na$^+$-dependent net current reconstructed by the subtraction of the current in Na$^+$-free (or 70 mM Na$^+$) bath solution from the control current (Fig. 6, A, B, and C) showed the rapid transient component of the outward current. Therefore, it seems that transient $I_{Na}$ ($I_{Na}$) contributes to $K_{Na}$-channel activity. In our experiment, we used perforated patch-clamp technique with β-escin and relatively high pipette resistances of approximately 6–10 MΩ. Because of this high access resistance, it is extremely difficult to clamp transient $I_{Na}$ properly. Therefore, it remains uncertain whether the activation of $K_{Na}$ channels involves transient $I_{Na}$.

For selective recordings of $K_{Na}$-channel currents and membrane potential control, we used a bath solution containing a high concentration of K$^+$ (140 mM) and 10 mM NaCl follow-
Incomplete recovery of \( I_{KNa} \) after Na\(^+\) replacement and blockade of Na\(^+\) channel. The present results indicated that the recovery of \( I_{KNa} \) after Na\(^+\) replacement and blockade of Na\(^+\) channel is not quite complete. In almost all washout experiments, the activity of \( K_{Na} \)-channel currents recovered partially.

These results are not consistent with the previous results demonstrating that the amplitude of sustained outward current returned to \( \sim 80\% \) of control values on restoration of external Na\(^+\) in tufted/mitral cells in olfactory bulb (Hage and Salkoff 2012). They recorded the activity of single-\( K_{Na} \)-channel currents from the macropatch membranes that contained both Na\(^+\) and \( K_{Na} \) channels using outside-out patch configuration. However, in cricket Kenyon cells, we recorded the activity of single \( K_{Na} \) channels from the cell-attached patch membranes, and the Na\(^+\) channels that supply Na\(^+\) to activate \( K_{Na} \) channels are separated by at least the size of the tip of a hard glass rim. In this study, we used borosilicate glass patch pipettes with electrode resistance of about 6–10 MΩ, which may have an open-tip diameter of \(<1\) μm. According to a study on the tip geometry of a thick-walled, 10-MΩ, hard glass pipette by scanning electron micrograph of the tip-on view, the diameter of the tip opening was measured as 0.98 μm, and the width of the glass rim was measured as 0.4 μm (Sakmann and Neher 1983). The distance from a \( K_{Na} \) channel to a voltage-dependent Na\(^+\) channel under our cell-attached patch configuration is unknown, but we estimate that they are separated by at least 0.4 μm given the size of the tip of a hard glass rim. Therefore, we consider that the differences in the geometry of Na\(^+\) channels and \( K_{Na} \) channels may be one possible

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**Figure 5.** Effect of inorganic Ca\(^{2+}\)-channel blockers on \( K_{Na} \)-channel activity. 

**A:** representative unitary \( K_{Na} \) currents before (Control) and after (500 μM Co\(^{2+}\)) addition of 500 μM Co\(^{2+}\) to the bath solution. The corresponding all-point amplitude histogram is shown at the bottom. **B:** representative unitary \( K_{Na} \)-channel currents before (Control) and after (500 μM Ni\(^{2+}\)) addition of 500 μM Ni\(^{2+}\) to the bath solution. The corresponding all-point amplitude histogram is shown at the bottom. Recordings were made at a holding potential of +60 mV. \( n \): average relative \( P_o \) of \( K_{Na} \)-channel currents in Control (1.0), 500 μM Cd\(^{2+}\) (0.241 ± 0.095, \( n = 6 \)), 500 μM Co\(^{2+}\) (1.06 ± 0.105, \( n = 5 \)), and 500 μM Ni\(^{2+}\) (0.979 ± 0.126, \( n = 3 \)). *P < 0.05.
reason for the incomplete recovery in cricket Kenyon cells. In many cases during the washout of the cells for 5 min, $K_{Na}$-channel activity became unstable. It may take >5 min to gain full recovery of $K_{Na}$-channel activity after treatment of the cells.

It has been demonstrated clearly that ion-channel subtypes coexist within membrane microdomains. A typical example is shown for ion channels between voltage-dependent $Ca^{2+}$ channels and large-conductance $Ca^{2+}$-activated $K^{+}$ (BK) channels. Marrion and Tavalin (1998) reported the
coassembly of Ca$^{2+}$ channels and BK channels in hippocampal neurons. Neher (1998) and Augustine et al. (2003) proposed Ca$^{2+}$ nanodomains (within 20–50 nm of the Ca$^{2+}$ source) and Ca$^{2+}$ microdomains (at distances between 50 and a few hundred nanometers from the Ca$^{2+}$ source). The functional coupling between Na$^+$-activated K$^+$ and voltage-dependent persistent Na$^+$ channels presented in this study may indicate that they are colocализed and act together for the tight regulation of membrane excitability. Additional evidence that would show the colocализation of KNa channels and voltage-dependent persistent sodium channels by the use of fluorescence resonance energy transfer (FRET; Silvius and Nabi 2006) would provide further evidence of the association of these channels within microdomains.

A recent study suggests that KNa channels may be clustered and that clustered KNa channels may display cooperative gating (Kim et al. 2014). From the all-point amplitude histograms obtained from KNa-channel activity in cricket Kenyon cells, however, we rarely observe multiple peaks: only one opening predominated. Furthermore, in many cases, we often observed openings smaller (4 pA) than full-state openings (6 pA) at +60 mV. The 4-pA currents are approximately two-thirds of the main-state level of 6 pA. Subconductance levels have been suggested to occur in KNa channels. Mistry et al. (1996) showed that the stable subconductance levels being one-third and two-thirds of the main-state level of native KNa channels in cricket Kenyon cells. Further study is necessary to elucidate the mechanisms underlying the occurrence of subconductance states.

Relation to Slick and Slack channels. Recently, it has been shown that two genes, Slack (Slo2.2 or K$\text{Ca}_4.1$) and Slick (Slo2.1 or K$\text{Ca}_4.2$), encode KNa channels. Slack and Slick subunits differ in their unitary conductance, kinetic behavior, and pharmacological properties (Wei et al. 2005). The Slick KNa channel is activated rapidly and Slack channels slowly in response to depolarization. Unlike Slack channels, there is an ATP-binding site on the Slick COOH terminus (Bhattacharjee et al. 2003). Bithionol, a bis-phenol compound known for its antiparasitic actions, potently activates Slack currents (Yang et al. 2007). Activation of PKC increases the amplitude of Slack-B currents and decreases the amplitude of Slick currents (Santi et al. 2006). Furthermore, a recent study shows that the Slick and Slack subunits coassemble to form heteromeric channels that differ from the homomers in their unitary conductance (Chen et al. 2009). In the cricket Kenyon cells, native KNa channels have a unitary conductance of 111 pS at full state, and whole cell outward currents activate very rapidly in response to membrane depolarization. These properties are rather similar to those of Slick channels. From the single-channel-current traces in Fig. 2B, it seems that there are two types of single-channel currents having different kinetics, one with very short mean burst times and one with a much longer mean burst duration time (~200 ms). However, at present, it remains uncertain which ones are Slick or Slack channels. It also remains uncertain which type of channel currents show preferential coupling to the persistent Na$^+$ currents. Further studies examining the sensitivity to PKC or ATP are necessary to identify the molecules of native KNa channels. Protein expression level and cellular localization of Slick and Slack subunit in cricket Kenyon cells should be determined by Western blot analysis and immunohistochemistry in the future.

Physiological implications. Inoue et al. (2014) found that 1 μM TTX, replacement of external Na$^+$ with choline, and blockage of Na$^+$-dependent K$^+$ currents with 50 μM quinidine eliminated membrane oscillations. These findings imply that membrane oscillations are mediated primarily by $I_{\text{NaP}}$, cooperation with $I_{\text{KNa}}$: persistent Na$^+$ currents contribute to the depolarizing phase, whereas KNa-channel currents contribute to the subsequent repolarizing phase. Inoue et al. (2014) also suggested that $I_{\text{NaP}}$ and $I_{\text{KNa}}$ participate in the generation of plateau potential. The functional coupling between voltage-dependent Na$^+$ channels and KNa channels presented in this study provides the ionic channel basis for the generation of membrane oscillations and plateau action potentials. Inoue et al. (2014) also indicated that action potential repolarization is mediated by BK-channel currents rather than KNa-channel currents. Therefore, KNa channels may act mainly as a repolarizing current that shortens membrane oscillations or plateau action potentials as a negative feedback mechanism.

In a previous study, the activity of KNa channels in cricket Kenyon cells was found to be regulated potently by a variety of cellular signaling pathways, including cAMP and cGMP. Aoki et al. (2008) showed that the application of octopamine (which mediates reward signals) or cGMP increased KNa-channel activity, whereas dopamine (which mediates punishment signals) or cGMP decreased it. Thus KNa currents seem to be an important target molecule for the formation of olfactory long-term memory. In G. bimaculatus, nitric oxide (NO)cGMP signaling has also been shown to be required to shift short-term memory to long-term memory during multiple rounds of olfactory conditioned stimulation (Matsumoto et al. 2006). Our preliminary observations indicate that NO inhibits KNa-channel activity via the cGMP/PKG signaling pathway and increases persistent Na$^+$ currents (M. Hasebe and M. Yoshino, unpublished observations). Therefore, NO seems to dissociate the functional coupling between KNa and Na$^+$ channels by its opposing action on KNa and Na$^+$ currents. By this uncoupling action of NO, long-lasting membrane depolarization will be induced and thereby membrane excitability of Kenyon cells is increased. Further study is necessary to clarify whether the activity of KNa and Na$^+$ channels is modified in such a manner during multiple rounds of conditioned stimulation.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

M.Y. conception and design of research; I.T. and M.Y. performed experiments; I.T. and M.Y. analyzed data; M.Y. interpreted results of experiments; I.T. and M.Y. prepared figures; I.T. and M.Y. drafted manuscript; M.Y. edited and revised manuscript; M.Y. approved final version of manuscript.
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