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Sodium-Activated Potassium Channels Are Functionally Coupled to Persistent Sodium Currents

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We report a novel coupled system of sodium-activated potassium currents (I_{KNa}) and persistent sodium currents (I_{NaP}), the components of which are widely distributed throughout the brain. Its existence and importance has not been previously recognized. Although I_{KNa} was known to exist in many cell types, the source of Na^+ which activates I_{KNa} remained a mystery. We now show in single membrane patches generated from the somas of rat neurons that sodium influx through I_{NaP} is sufficient for activation of KNa channels, without substantial contribution from the transient sodium current or bulk [Na^+]. I_{NaP} was found to be active at cell membrane resting potentials, a finding that may explain why I_{KNa} can be evoked from negative holding potentials. These results show an unanticipated role for I_{NaP} in activating a negative feedback system countering the excitabile effects I_{NaP}; the interrelatedness of I_{NaP} and I_{KNa} suggests new ways neurons can tune their excitability.
inhibit division of glia. Whole-cell and excised patch recordings were made up to 4 and 10 d after plating, respectively.

Voltage-clamp recordings. Tufted/mitrals cells were identified on the basis of their pyramidal shape and large size (Trombley and Westbrook, 1990). Recordings were made with borosilicate glass pipettes with tip resistances of 3–6 MΩ. Outside-out patches were obtained by steadily moving the pipette away from the cell after establishing a whole-cell patch at the soma. Voltage-clamp recordings were made with an Axoclamp 200B, filtered at 2 kHz and digitized at 50 kHz with a Digidata 1440A. Internal pipette solution contained the following (in mM): 140 KCl, 10 HEPES, 5 EGTA, 1 MgCl₂, pH 7.40 with KOH. Bath solutions contained the following (in mM): 150 NaCl, 10 HEPES, 10 dextrose, 5 KCl, 2 MgCl₂, pH 7.30 with NaOH. For recordings of \( I_{\text{Na}} \), the internal solution contained 130 CsCl, 10 NaCl, 1 MgCl₂, 10 HEPES, 5 EGTA, pH 7.40 with KOH. The external solution for recording \( I_{\text{Na}} \) was the same as the standard external recording solution with 10 mM tetraethylammonium (TEA) • Cl, 2 mM 4 aminopyridine (4-AP) • Cl, and 0.2 mM CdCl₂ replacing equimolar NaCl. Zero Na⁺ external solutions replaced all NaCl with choline • Cl or LiCl as indicated. TTX and veratridine were used at 1 and 50 μM concentrations, respectively. Veratridine was stored as a 50 mM stock solution in DMSO. Many of the transient sodium current by holding at \(-50\) mV, TTX-sensitive outward currents and persistent inward currents were largely unchanged. c, d, I–V plots of sustained currents (mean current during final 50 ms of voltage step) from \(-70\) mV and \(-50\) mV holding potentials, respectively. Recordings were made from the soma of dissociated tufted/mitral cells of the olfactory bulb.

**Figure 1.** The TTX-sensitive delayed outward current, \( I_{\text{Na}} \), does not depend on the transient sodium current. a, Whole-cell currents before and after TTX (1 μM) and TTX-sensitive currents evoked by voltage steps (−90 to +40 mV, 10 mV intervals) from a holding potential of −70 mV. Note the large component of inward Na⁺ current present in a (arrows) but absent in b. b, Currents evoked from the same neuron as in a from a holding potential of −50 mV. Note that despite the substantial diminution of the transient sodium current by holding at −50 mV, TTX-sensitive outward currents and persistent inward currents were largely unchanged. c, d, I–V plots of sustained currents (mean current during final 50 ms of voltage step) from −70 mV and −50 mV holding potentials, respectively. Recordings were made from the soma of dissociated tufted/mitral cells of the olfactory bulb.
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Results

In whole-cell recordings of olfactory bulb tufted/mitral cells (T/M cells) (Fig. 1) or striatal medium spiny neurons (Budelli et al., 2009), blockade of voltage-gated sodium (Na_v) channels with TTX (1 μM) reduces the amplitude of sustained outward currents. This portion of TTX-sensitive outward current removed represents a sodium-activated potassium current (I_{NaP}) (Budelli et al., 2009). As shown in Figure 1, the kinetic properties of the TTX-sensitive outward current (I_{NaT}) do not appear to depend on I_{NaP}; I_{NaP} is a long-duration delayed outward current showing very little inactivation, while I_{NaT} is over in a few milliseconds. Since a key feature of I_{NaP} is its sensitivity to TTX, an alternative to its activation by the TTX-sensitive I_{NaT} component could be its activation by the TTX-sensitive I_{NaP} which is known to accompany I_{NaT} in most neurons (Crill, 1996). Supporting this hypothesis are our observations showing that I_{KNa} persists even when I_{NaT} is eliminated or greatly reduced by a depolarizing holding potential of −50 mV (Fig. 1b).

I_{KNa} in detached outside-out patches

Notably, the whole-cell experiments shown in Fig. 1 were undertaken with the internal bulk concentration of sodium ion reduced to virtually 0 mM. This means that the activation of I_{KNa} by I_{NaP} has to be accomplished by sodium ion crossing the membrane into the cell. This also implies that the local internal concentration of Na^+ in the vicinity of I_{KNa} must be raised to a sufficiently high level without any contribution from the bulk internal [Na^+]. Because of the low conductance of I_{NaP} such a scenario might seem unlikely, but might be possible if channels carrying I_{KNa} and I_{NaP} were closely associated. A rigorous test of the hypothesis of the close association and functional coupling of these two channel types would be demonstrating the dependency of I_{KNa} on the activity of I_{NaP} in an isolated membrane patch.

To test this hypothesis, we recorded I_{KNa} currents in outside-out patches excised from the soma of T/M cells and showed their dependence on I_{NaP} as we had in whole-cell configuration. Figure 2 shows an outside-out macro patch containing a prominent potassium current resembling those recorded in the whole-cell configuration, but lacking any indication of I_{NaT} (Fig. 2a). As we showed for whole-cell currents, outward currents were substantially reduced when external sodium was removed (Fig. 2b) but returned when [Na^+]_o was restored (Fig. 2c). In these experiments, no sodium was included in the internal pipette solution so that activation of K_{Na} currents would be minimally dependent on bulk sodium and primarily determined by sodium influx. The component of K^+ current dependent on Na^+ influx was revealed by subtraction, and closely resembles that seen in whole-cell recordings (Fig. 2d) (amplitude of sustained outward current in external choline was reduced to 63.5 ± 3.01% of control values p < 0.001, n = 5; upon restoration of external Na^+, amplitude of sustained outward current returned to 88.4 ± 7.39% of control values, p < 0.05 compared with choline, mean ± SEM, n = 5, V_m = +60 mV). We also applied TTX to macropatches and, as in whole-cell recordings, saw a significant reduction in the amplitude of total delayed outward current, revealing a large component of I_{KNa} ampltude of sustained outward current in TTX was reduced to 47.3 ± 12.4% of control values p < 0.05, mean ± SEM, n = 4, V_m = +60 mV). As a control for a direct effect of TTX on K_{Na} channels, we measured whole-cell currents from a line of human embryonic kidney cells stably transfected with the Slack gene which is known to encode K_{Na} channels, and found no change in I_{KNa} current amplitude following application of 1 μM TTX (amplitude of sustained outward current in TTX was 101% ± 2.62% of control value. n = 3, measured at +60 mV p = 0.6). Furthermore, replacing external sodium with the Na_v-channel-permeant cation, lithium, also resulted in a significant decrease in the amplitude of sustained outward currents in excised patches (amplitude of sustained outward current in external Li^+ was reduced to 56.2 ± 3.67% of control values p < 0.001, n = 4; upon restoration of external Na^+, amplitude of sustained outward current returned to 78.8 ± 3.01% of control values, p < 0.01 compared with Li^+ mean ± SEM, n = 4, V_m = +60 mV).
Single-channel $K_{Na}$ currents coupled to Na$^+$ influx

To examine the functional association of $K_{Na}$ channels with $I_{NaP}$ at higher resolution, we used smaller outside-out patches where we could distinguish the activity of individual $K_{Na}$ channels (Fig. 3a). These channels resemble $K_{Na}$ channels in inside-out patches from T/M cells as previously reported to be abundantly expressed in this cell type (Egan et al., 1992). $K_{Na}$ channels from these native cells also resembled sodium-activated SLO2 channels expressed in heterologous systems (Bhattacharjee et al., 2003; Yuan et al., 2003; Santi et al., 2006). The excised patch in Figure 3a contains at least 8 active high conductance channels, and even though single-channel openings are easily discerned, the evoked current as a whole resembles that seen in macropatches as in Figure 2. When TTX (1 μM) was perfused on the outside of this patch and others like it, there was a substantial decline in channel activity (Fig. 3a) ($P_o$ decreased to 57.3 ± 3.00% of control value after application of 1 μM TTX, $p < 0.001$ n = 5, measured at +60 mV). These results suggest that, in addition to the high conductance $K_{Na}$ channels in the patch which are obvious, the patch also contains TTX-sensitive $Na_p$ channels which are not. We will show in a following section that single-channel $I_{NaP}$ can be revealed when measures are taken to block K$^+$ channels.

Like macroscopic $I_{NaP}$ seen in whole-cell experiments and in macropatches, TTX-sensitive single $K_{Na}$ channel activity was observed without the addition of Na$^+$ to our pipette solution (Fig. 3a). Thus, even at the single-channel level it appears that TTX-sensitive sodium influx and not bulk internal Na$^+$ was activating $I_{NaP}$. This seems especially persuasive in these experiments with excised outside-out patches because the volume of solution inside the pipette is enormous relative to the miniscule fluid volume in contact with the inner surface of the membrane patch. In these experiments, bulk [Na$^+$], is not likely to be significantly altered (see calculations in Materials and Methods). Yet, these experiments clearly show the Na$^+$ dependence of single $K_{Na}$ channels. Such activity implies that local [Na$^+$]$_i$ near $K_{Na}$ channels at the intracellular membrane surface was being raised to a higher level than bulk internal Na$^+$ by persistent TTX-sensitive Na$^+$ influx. As previously reported in whole-cell experiments (Budelli et al., 2009), replacement of external Na$^+$ with Li$^+$, decreased observed single $K_{Na}$ channel activity in outside-out patches (Fig. 3b). ($P_o$ decreased to 66.0 ± 4.16% of control value after replacement of Na$^+$ with Li$^+$, $p < 0.001$, mean ± SEM n = 3, measured at +60 mV).

External veratridine increases local [Na$^+$]

After demonstrating that removing external Na$^+$ or blocking Na$^+$ influx decreases $K_{Na}$ channel activity in isolated patches, we hypothesized that increasing Na$^+$ influx would increase $K_{Na}$...
channel activity. We applied veratridine (50 μM), which prolongs the open state of the sodium channel, to outside-out patches containing active KNa channels, and observed a significant increase in KNa channel activity (Fig. 4). The activating effect of veratridine on single KNa channel activity could be abolished by the removal of external Na⁺ after application of veratridine. (KNa channel Po increased to 171% ± 9.44% of control value after application of 50 μM veratridine, p < 0.001, n = 10, and decreased to 55.7 ± 12.7% value following subsequent removal of external Na⁺, p < 0.001, mean ± SEM, n = 10, measured at +60 mV). Thus, our interpretation of these results is that veratridine increases KNa channel activity by increasing sustained Na⁺ influx via NaV channels. We emphasize that the effect of veratridine must be via increasing the local [Na⁺] in the vicinity of KNa channels and cannot be due to changing the internal bulk [Na⁺].

Persistent Na⁺ currents in patch recordings
While many of the recordings described above had some indication of macroscopic and single-channel I_{NaP} currents, accurate recording of I_{NaP} currents in outside-out patches required blockade of K⁺ channels. This is due to the small single-channel conductance of NaV-channels, simultaneous activity of Na⁺ and K⁺ currents and prominent subconductance states of KNa channels. We found that both transient and persistent TTX-sensitive sodium currents were present in outside-out patches from the soma (Fig. 5a,b). We observed that I_{NaP} at a single-channel level consists of brief openings occurring over a wider voltage range than I_{NaT} and displaying no significant inactivation over the course of 1 s voltage steps. Many of these persistent TTX-sensitive openings were so brief that we could not reliably measure a mean channel open time, even when digitizing at high rates. Thus, we used the number of detectable events as a means of quantification rather than channel open probability (Fig. 5b). Because of the brevity of openings it is likely that we failed to detect many of them. Although TTX-sensitive openings of I_{NaP} occurred less frequently at hyperpolarized voltages, they were clearly present even at −90 mV (Fig. 5b). I_{NaP} with similar voltage-sensitivity was identified in whole-cell recordings using voltage steps and slow voltage ramps (Fig. 5c,d). These results suggest I_{NaP} is a determinant of
during voltage steps and a prominent, slowly decaying tail current upon repolarization (Fig. 6a,b) consistent with previous reports of its action on $I_{K\text{Na}}$ (Barnes and Hille, 1988).

**Discussion**

Upon initial identification in cardiac myocytes, the suggested role for $K\text{Na}$ channels was that of providing a reserve conductance that would protect cells during hypoxia when an elevation in $[\text{Na}^+]_i$ occurred as a result of blocking the Na$^+$/K$^+$ ATPase (Kameyama et al., 1984). This idea arose as a result of observations of $K\text{Na}$ channels in inside-out patches which showed that $K\text{Na}$ channel activation required a much higher $[\text{Na}^+]_i$ than that normally found in bulk cytoplasm. However, we have uncovered an unexpected phenomenon by which Na$^+$ entering the cell via a small but persistent sodium current is surprisingly effective in activating $I_{K\text{Na}}$ even in the total absence of bulk internal Na$^+$. The seeming implausibility of such a mechanism may be partially responsible for keeping this major and widespread component of delayed outward conductance unappreciated by electrophysiologists for many years. These observations suggest a mechanism by which $I_{K\text{Na}}$ can commonly participate in providing a large outward conductance in many neuronal types. $K\text{Na}$ channels are expressed throughout the brain (Bhattarchjee et al., 2002, 2005). The Allen Brain Atlas shows that one of two genes which encode $K\text{Na}$ channels (KCNT1, also known as Slack and Slo2.2) is as widespread and abundantly expressed as KCNB1, the gene encoding the major voltage-dependent delayed rectifier (http://mouse.brain-map.org/).

The functional coupling of $I_{K\text{Na}}$ to $I_{\text{NaP}}$ that we observe at the single-channel level in isolated outside-out patches suggests a highly specialized relationship between $K\text{Na}$ and $\text{NaP}$ channels perhaps similar to the colocalization between calcium channels and the Slo1 calcium-activated potassium channel (BK) (Marrión and Tavalin, 1998). Although the coupling of $K\text{Na}$ to Na channels may be analogous, the two orders of magnitude difference in the required concentrations of Na$^+$ and Ca$^{2+}$ needed to activate the respective K$^+$-channels suggests some differences. Hence, we are unlikely to observe a correlation between the opening of single Na$^+$ and $K\text{Na}$ channels as is observed between calcium channels and single BK channels. Nevertheless there are parallels between the two systems. In both systems the channels are activated by two major factors, an intracellular ion ligand, and voltage (albeit $K\text{Na}$ channels have lower voltage sensitivity). In both systems, the effect of increasing the concentration of the intracellular ion ligand is to shift the conductance-voltage relation leftward to more hyperpolarized voltages. We previously demonstrated this for $I_{K\text{Na}}$ by loading cells with higher concentrations of Na$^+$ and noting a leftward shift in the conductance-voltage relation of $I_{K\text{Na}}$ (Budelli et al., 2009). Signif-

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**Figure 5.** Voltage-gated sodium currents present in the soma of T/M cells include a persistent component. Sodium currents were isolated by using Cs$^{+}$ in the patch pipette and TEA and 4-AP in the bath to block potassium channels. a–d, Recordings were made in outside-out patches (a, b) and whole-cell configuration (c, d) with the internal sodium concentration set to 10 mV to obtain better voltage control of inward current and allow measurement of channel reversal potential. a, Single-channel sodium currents evoked by a voltage step from $-90$ to $-40$ mV have both early occurring, high open probability, transient openings, as well as low probability, persistently recurring, brief openings (asterisks). Both components were blocked by TTX (1 μM). b, Plot of the number of channel opening events versus voltage. Channel events were counted from 50 ms after the initiation of a 1 s voltage step to its end. c, Whole-cell, TTX-sensitive persistent sodium currents evoked by 500 ms steps to indicated voltages from a holding potential of $-90$ mV. Mean current is plotted during the last 100 ms of the pulse (arrow). Error bars represent SEM. n = 5 cells. d, Whole-cell, TTX-sensitive sodium currents evoked by a voltage ramp from $-100$ to $+20$ mV, 40 mV/s. In e and d, TTX-sensitive currents were isolated and plotted by subtracting residual currents evoked in TTX (1 μM) from currents evoked before TTX application.
icantly, those experiments showed that cells loaded with higher concentrations of [Na\(^{+}\)], still retained an obvious delayed outward component of \(I_{\text{KNa}}\) due to the influx of Na\(^{+}\). Thus, the TTX-dependent influx of Na\(^{+}\) appears to be additive to the [Na\(^{+}\)], contributed by the bulk solution.

A possible mechanism for the functional coupling of \(K_{\text{Na}}\) and \(I_{\text{NaP}}\) is their presence in a region of limited diffusion near the plasma membrane, sometimes described as a fuzzy space or unstirred layer. Physiological evidence of such a region in cardiac myocytes has been accumulating for many years (for review, see Barry, 2006). Microheterogeneity of sodium concentrations in internal submembrane space has been inferred many times (Barry, 2006), and has even been directly measured (Wendt-Gallitelli et al., 1993). Measurements using electromagnetic pulse analysis shows [Na\(^{+}\)] near the inner membrane surface can be severalfold higher than the bulk [Na\(^{+}\)], and heterogeneous within a cell, ranging from 0 to 80 mM at the inner membrane surface. Thus, it is possible that a small but constant influx of Na\(^{+}\) through \(I_{\text{Na}}\) is responsible for increasing [Na\(^{+}\)] in some submembrane regions where \(K_{\text{Na}}\) channels are located, and that limited diffusion as well as colocalization of \(I_{\text{Na}}\) and \(I_{\text{KNa}}\) both contribute to activation of \(I_{\text{KNa}}\) by \(I_{\text{NaP}}\).

This study focused on \(I_{\text{NaP}}\) as a major source of sodium for the activation of \(K_{\text{Na}}\) channels. While our data suggest \(I_{\text{NaP}}\) is not necessary for \(K_{\text{Na}}\) activation as a delayed outward current, we have not ruled out a contribution of \(I_{\text{NaP}}\) in all circumstances. The \(K_{\text{Na}}\)-channels that carry \(I_{\text{NaP}}\) are likely to also carry \(I_{\text{Na}}\), and it is difficult to imagine a mechanism by which only persistent Na\(^{+}\)-channel activity would activate \(K_{\text{Na}}\) channels. However, our study indicates that the Na\(^{+}\)-channels present in somal membrane may be largely inactivated at resting membrane potentials with regard to their capacity to carry a transient sodium current on depolarization, while maintaining the ability to carry \(I_{\text{NaP}}\) at most voltages. Activation of \(I_{\text{KNa}}\) by \(I_{\text{STAT}}\) has been previously examined in outside-out patches of neurons from chick brainstem (Dryer, 1991). That study found that while TTX blocked \(I_{\text{Na}}\) it had no effect on potassium currents. However, those experiments observed the effects of \(I_{\text{NaP}}\) on outward currents at \(-25\) to \(-10\) mV, near peak \(I_{\text{NaP}}\) amplitude. We point out that, under conditions of low [Na\(^{+}\)], loading, we also do not detect net outward \(I_{\text{KNa}}\) at those voltages, which we ascribe to the voltage-dependent component of \(I_{\text{KNa}}\) activation. Thus, Dryer’s experiments must be repeated at higher voltages before ruling out a role for \(I_{\text{NaP}}\) in \(I_{\text{KNa}}\) activation.

The activity of \(K_{\text{Na}}\) channels has been shown to be sensitive to intracellular Cl\(^{-}\) (Bhattacharjee et al., 2003; Yuan et al., 2003). We have found the magnitude of TTX-sensitive outward current in whole-cell recordings to be accordingly affected by [Cl\(^{-}\)]. TTX-sensitive potassium currents in recordings using a physiological [Cl\(^{-}\)], of 20 mM comprise \~30\% of the sustained outward current in mitral cells, compared with 57% in 150 mM [Cl\(^{-}\)], (Budelli et al., 2009; our unpublished data). Our current study used an elevated [Cl\(^{-}\)], to increase the likelihood of observing \(K_{\text{Na}}\) channels and to more precisely measure channel \(P_{\text{Na}}\). \(K_{\text{Na}}\) channels have been shown to be activated by the ubiquitous enzyme cofactor nicotinamide adenine dinucleotide (NAD\(^{+}\)) (Tamsett et al., 2009). The presence of NAD\(^{+}\) may increase the activity of \(K_{\text{Na}}\) channels at physiological concentrations of internal bulk Na\(^{+}\).

The contribution of \(I_{\text{NaP}}\) to the activation of a prominent potassium conductance may seem counterintuitive given the established role of \(I_{\text{NaP}}\) in increasing neuronal excitability. We point out that the \(I_{\text{NaP}}\)-\(I_{\text{KNa}}\) coupled system produces a net inward current in mitral cells between typical resting potentials and action potential thresholds (Figs. 1c,d, 2e). Nevertheless, within those voltages the activation of \(I_{\text{KNa}}\) may mitigate depolarization and while it does not overcome \(I_{\text{NaP}}\), could provide some negative feedback against the excitatory role of \(I_{\text{NaP}}\) in the form of membrane accommodation. It may also act as a repolarizing current that shortens action potential duration. In addition, there is evidence for neuromodulatory regulation of both \(I_{\text{NaP}}\) (for review, see Cantrell and Catteral, 2001) and \(I_{\text{KNa}}\) (Santi et al., 2006; Nuwer et al., 2010). Hence, the discrete or combined regulation of either component of the \(I_{\text{NaP}}\)-\(I_{\text{KNa}}\) coupled system could provide a mechanism for long-lasting changes in excitability properties over a wide range of both subthreshold and depolarized voltages. \(I_{\text{NaP}}\) and the upstroke of action potentials are grossly similar across neurons. In contrast, the behavior of \(I_{\text{NaP}}\) varies and contributes to diverse physiological properties such as amplification of synaptic potentials, repetitive firing of action potentials, spike timing, and even membrane resting potential (Grill, 1996; Verfaeke et al., 2006, Huang and Trussell, 2008). Indeed, \(I_{\text{NaP}}\) may be a significant determinant of some of the most subtle excitable membrane behavior such as electrical resonance generating theta frequency firing (Hu et al., 2002). In all of these electrical phenomena in which \(I_{\text{NaP}}\) Participates, \(I_{\text{KNa}}\) may now be found to be
a coparticipant. Many inherited epilepsies are due to gain- or loss-of-function mutations in voltage-gated sodium channels which alter \( I_{NaP} \). Accordingly, several antiepileptic drugs primarily target \( I_{NaP} \); however, understanding of pathophysiological mechanisms of \( I_{NaP} \) toward epilepsy and other disorders is incomplete (Stafstrom, 2007; Saint, 2008; Waxman, 2008). For all these reasons, \( I_{NaP} \) is a topic of great research interest for clinical and basic neuroscientists. The functional coupling of \( I_{NaP} \) to \( K_{Na} \) channels must now also be considered as the contribution of \( I_{NaP} \) to neuronal physiology continues to be investigated.

References


