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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 K_{Na} channels, without substantial contribution from the transient sodium current or bulk $[Na^+]_i$. 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 excitable effects I_{NaP}; the interrelatedness of I_{NaP} and I_{KNa} suggests new ways neurons can tune their excitability.

Introduction

Sodium-activated potassium channels (K_{Na} channels) were first described over 25 years ago as high conductance potassium channels activated by high concentrations of internal sodium (Kameyama et al., 1984). Despite many reports of their expression throughout the nervous system and other tissues (for review, see Dryer, 1994; Bhattacharjee and Kaczmarek, 2005), it was unclear whether K_{Na} channels were active under normal physiological conditions. Experiments with inside-out patches appeared to show that K_{Na} channel activity requires bulk sodium concentrations in excess of that normally present in bulk cytosol. Such experiments were taken as evidence against the participation of I_{KNa} during normal electrophysiological activity. Rather, it was suggested that K_{Na} channels might represent a type of "reserve conductance" active only under conditions of hypoxia when $[Na^+]_i$ might substantially increase. However, recent studies have shown that K_{Na} channels produce large outward currents in several types of neurons under normal physiological conditions, and appear to be dependent on sodium influx rather than high bulk $[Na^+]_i$ (Budelli et al., 2009; Lu et al., 2010; Nuwer et al., 2010).

I_{KNa} is a tetrodotoxin (TTX)-sensitive delayed outward current that lasts for seconds but the transient sodium current (I_{NaT}) is over within a few milliseconds; I_{NaT} thus seems an unlikely source of Na^+ to activate I_{KNa}. Here we explore the possibility that the TTX-sensitive persistent sodium current (I_{NaP}) is the source of Na^+ activating I_{KNa}. Amplitudes of I_{NaP} are tiny compared with peak amplitudes of I_{NaT}, and even prolonged I_{NaP} activity will not quickly increase bulk $[Na^+]_i$. Therefore a mechanism permitting I_{NaP} to deliver sufficient cytosolic Na^+ to activate I_{KNa} would require that channels underlying both currents be present together in single membrane patches. We show that the activity of I_{KNa} indeed depends on the activity of I_{NaP} by recording single-channel I_{NaP} and I_{KNa} currents in membrane patches isolated from the soma of olfactory bulb mitral cells. Single-channel currents recorded from outside-out patches revealed that treatments that eliminated I_{NaP}, also removed I_{KNa}; conversely, enhancing I_{NaP} resulted in increased I_{KNa} activity. This dependence of I_{KNa} on I_{NaP} was demonstrated under conditions where I_{NaT} was absent, and bulk $[Na^+]_i$ was 0, thus verifying the importance of I_{NaP}. These results focus further attention on I_{NaP} which is increasingly being recognized for its role in controlling many aspects of cell excitability (Crill, 1996; Vervaeke et al., 2006; Huang and Trussell, 2008). Although the action of I_{NaP} is usually thought of as producing an increase in cell excitability (Stafstrom et al., 1984) our results suggest an unanticipated role for I_{NaP} in activating a negative feedback system, I_{KNa}, which provides an opposing inhibitory current.

Materials and Methods

Primary neuronal culture preparations. Primary cultures of dissociated olfactory bulbs were prepared from either sex of neonatal Sprague Dawley rats (postnatal day 0). Dissected olfactory bulbs were incubated in 0.25% trypsin in PBS for 30 min at 37°C. For the last 5 min, trypsin solution was supplemented with 0.05% DNase. Enzyme digested tissue was then triturated in PBS containing soy bean trypsin inhibitor using Pasteur pipettes of decreasing diameter. Cells were plated on poly-lysine-coated glass coverslips in Dulbecco's Modified Eagle Medium/F-12 medium (Invitrogen) with 10% FBS and 2% B-27 supplement (Invitrogen). Three days after plating, medium was replaced with Neurobasal (Invitrogen) + 2% B-27 supplement and 5 μM arabinofuranosyl cytidine to
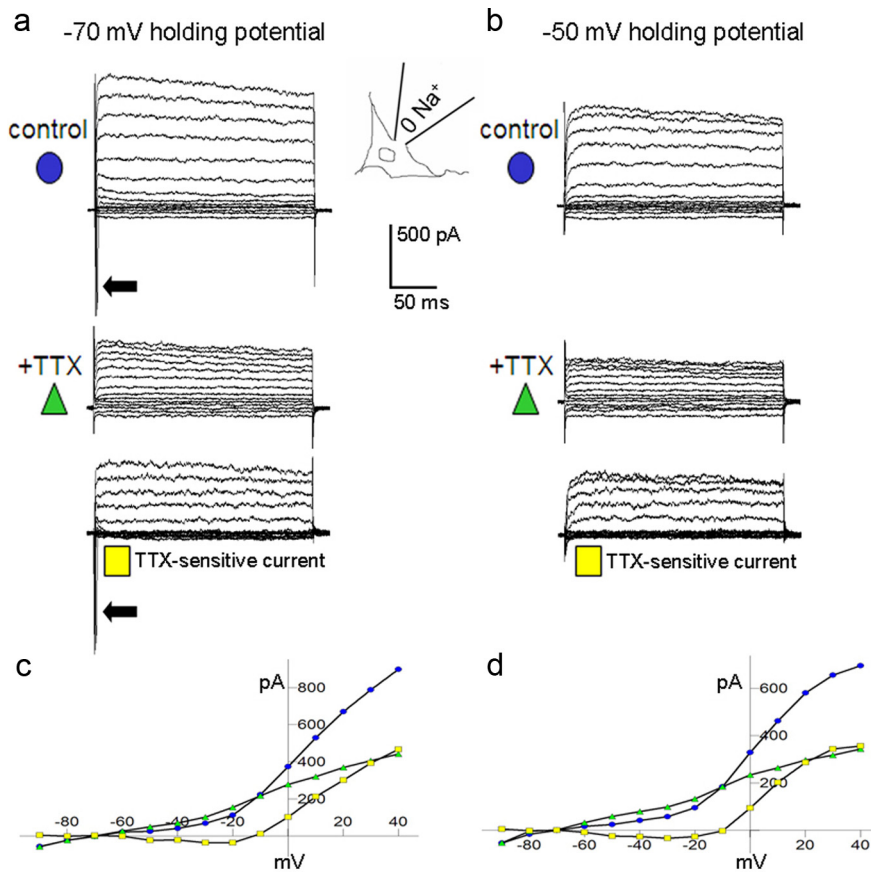
Figure 1. The TTX-sensitive delayed outward current, I_{KNa}, does not depend on the transient sodium current. **a**, Whole-cell currents before and after TTX (1 μM) and TTX-sensitive currents evoked by voltage steps (-90 to $+40$ mV; 10 mV intervals) from a holding potential of -70 mV. Note the large 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.

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/mitral 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 MgCl$_2$, pH 7.40 with KOH. Bath solutions contained the following (in mM): 150 NaCl, 10 HEPES, 10 dextrose, 5 KCl, 2 MgCl$_2$, pH 7.30 with NaOH. For recordings of I_{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_{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 CdCl$_2$ 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 frozen in DMSO. Experiments using veratridine included 0.1% DMSO in control external solution. No effect of this concentration of DMSO on channel activity was noted. In control experiments examining the effect of TTX on *Slack*-transected human embryonic kidney cells, 10 mM NaCl replaced equimolar KCl in the internal solution.

Single-channel analysis. Data were acquired and analyzed with pClamp 10.0 and Microsoft Excel. Single K_{Na} channel activity was analyzed and P$_o$

calculated using the Single Channel Search tool in pClamp 10.0. K_{Na} channel activity was measured during repeated 3 s sweeps to $+60$ mV from a holding potential of -70 mV, using a 10 s intersweep interval. Under differing experimental conditions, no changes in the mean amplitude of detected events or distribution of peaks in histograms of event amplitude were found. The number of channels in a patch was estimated by the maximum number of simultaneously active channels (additive steps) observed over the course an experiment. The minimum estimate of channel number provided by this method was used to determine P$_o$. In the majority of experiments, patches analyzed were estimated to contain 4–6 channels. Single I_{NaP} channel activity was measured using Threshold Search tool in pClamp 10.0. Many of the 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, and the Threshold Search tool as a means of quantification rather than channel open probability. Threshold levels were determined by measuring unitary current levels from selected "box-like" open channel events. The brevity of many events prevented use of an all points histogram to determine unitary current level. Identical threshold searches were performed for control sweeps and sweeps evoked in TTX. A small number of events crossed threshold in TTX and those values were subtracted from corresponding control values to generate the plot in Figure 5B (see Results).

Estimation of $[Na^+]_i$ increase. The volume of pipette solution was estimated to be 7.26 μl using the inner diameter of the recording pipettes (0.68 mm) and an approximation of internal solution fill height starting at the tapering of the pipette (20 mm). The number of Na$^+$ molecules entering the patch was estimated based on a high approximation of average I_{Na} throughout the experiment: -50 pA. Using the Faraday constant, this corresponds to 5.18×10^{-16} mol/s. Given this rate of Na$^+$ influx and the volume of pipette solution, the concentration of sodium would increase by 0.257 μM over the course of an hour-long experiment.

This is likely an overestimate, the fill height was usually >20 mm, and there is additional volume in the tapered portion of the pipette not considered here. In most excised patches, the peak amplitude of I_{Na} was much smaller than -50 pA and even smaller at the holding potential that patches were clamped at for the majority of the experiment.

Statistical analysis. We performed statistical analyses using T.W. Kirkman's Statistics to Use (http://www.physics.csbsju.edu/stats/) software. For comparison between two groups, we used paired Student's *t* tests for the same procedures before and after applied treatments.

Animal welfare. It is the policy of Washington University Medical School that all research involving animals be conducted under humane conditions, with appropriate regard for animal welfare. Washington University Medical School is a registered research facility with the United States Department of Agriculture (USDA) and is committed to complying with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services), the provisions of the Animal Welfare Act (USDA), and all applicable federal and state laws and regulations. At Washington University Medical School an Animal Care Committee has been established to insure compliance with all applicable federal and state regulations for the purchase, transportation, housing and research use of animals. Washington
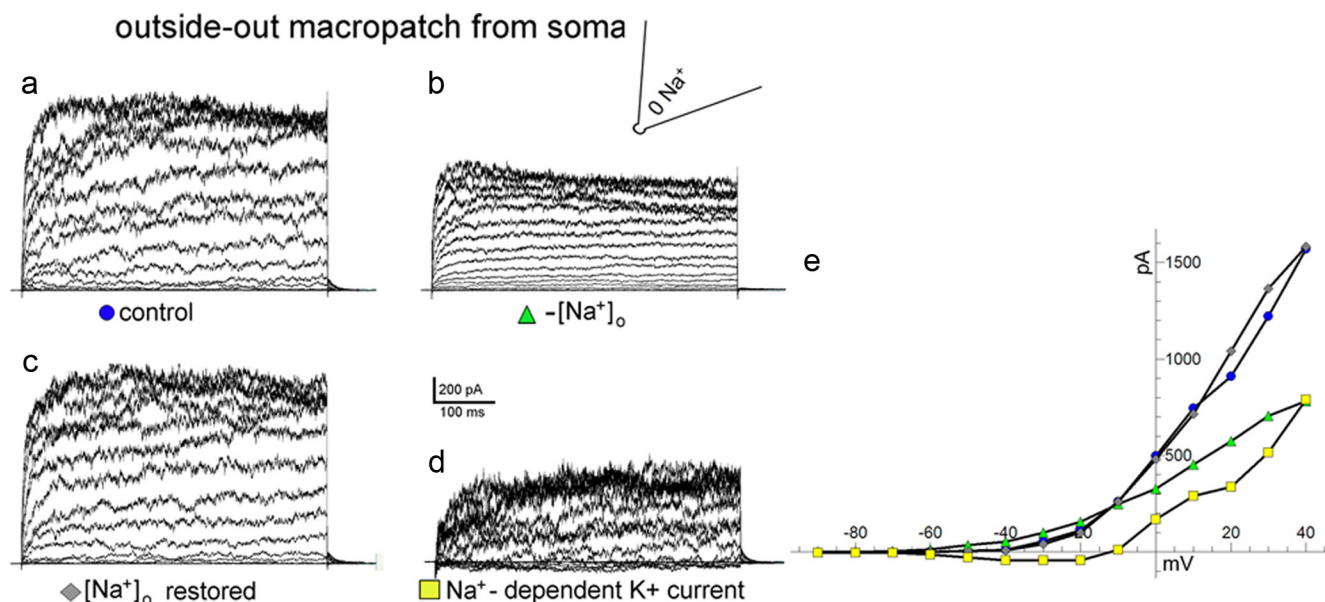
outside-out macropatch from soma

Figure 2. I_{KNa} depends on sodium influx into an outside-out macropatch. ***a***, Currents were evoked from a holding potential of −70 mV by steps from −90 to +80 mV. ***b***, Removal of external sodium dramatically reduced magnitude of outward currents. ***c***, Currents returned to previous levels upon restoration of external sodium. ***d***, Delayed outward K_{Na} currents, revealed by subtracting 0 [Na$^+$]$_o$ currents from control currents, resemble those recorded in whole-cell configuration. ***e***, *I–V* plot of currents in ***a–d*** (mean current of final 50 ms of voltage step). Note that sodium ion was absent from the internal bulk solution.

University Medical School has filed appropriate assurance of compliance with the Office for the Protection of Research Risks of the National Institutes of Health.

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_{KNa}) (Budelli et al., 2009). As shown in Figure 1, the kinetic properties of the TTX-sensitive outward current (I_{KNa}) do not appear to depend on I_{NaT}; I_{KNa} 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_{KNa} 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 depolarized 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} (amplitude 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).
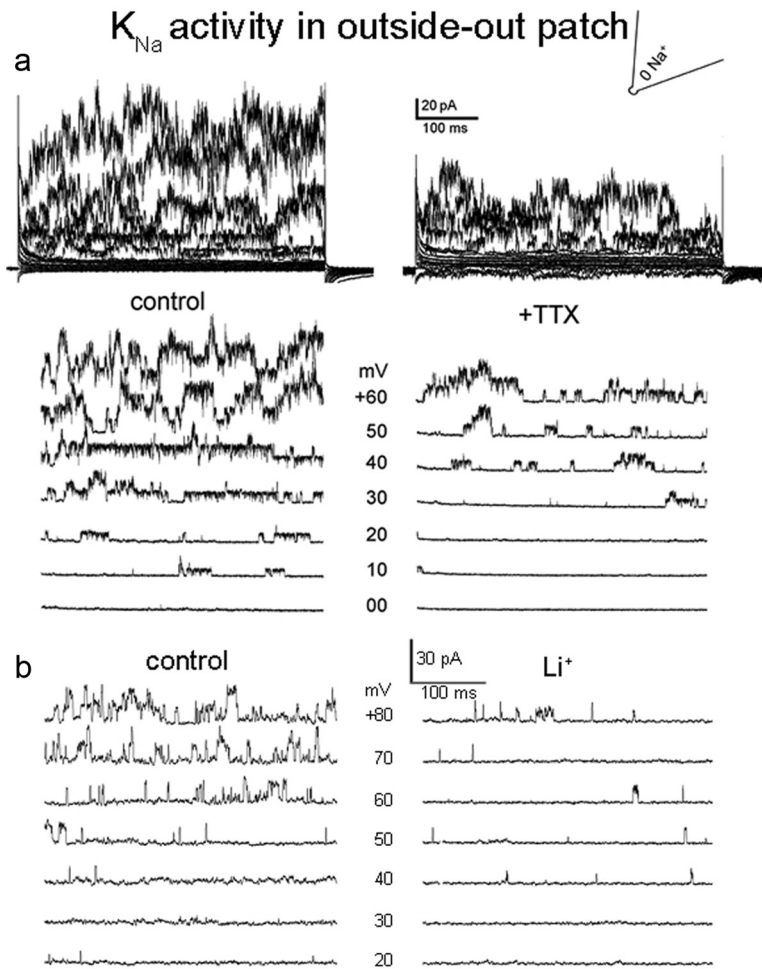
Figure 3. Single-channel K$_{Na}$ activity depends on TTX-sensitive persistent sodium influx into an outside-out patch. **a**, Currents evoked by a family of voltage steps in control conditions and in the presence of TTX (1 μM). K$_{Na}$ channel activation was stimulated by TTX-sensitive entry of sodium ion from the external medium; sodium ion was absent from the internal bulk solution. Upper traces show currents evoked by step pulses from −90 to +60 mV in 10 mV intervals from a holding potential of −70 mV. Lower traces display distributed traces at indicated voltages to allow resolution of individual K$_{Na}$ channel events. **b**, Currents evoked by steps to indicated voltages from a holding potential of −70 mV in control conditions and after complete replacement of external Na$^+$ with Li$^+$.

These results support the hypothesis that both I_{KNa} and I_{NaP} currents were present in macropatches.

Only trace amounts of I_{NaT} were evoked by step pulses from excised patches held at −70 mV (although patches held at −90 mV exhibited an obvious I_{NaT}). Thus, it is unlikely that I_{NaT} had any substantial role in evoking the Na$^+$-dependent outward current seen in Figure 2d. Since internal Na$^+$ was not included in the pipette solution in these experiments, the results imply that persistent Na$^+$ influx was responsible. However, because of the low amplitude of I_{Nap} it is unlikely that there would be a sufficient influx of Na$^+$ during the first few milliseconds of a depolarizing step pulse to substantially raise the local [Na$^+$]$_i$ near K$_{Na}$ channels to a sufficiently high level to permit I_{KNa} activation. However, as will be shown in a following section, I_{Nap} was observed at all holding potentials and thus, the most likely mechanism is that a small but persistent influx of Na$^+$ at holding or resting potentials maintains Na$^+$ at a higher concentration at the site of K$_{Na}$ channels than in the bulk cell interior. Since K$_{Na}$ channels resemble Ca^{2+}-activated BK channels in having a voltage-dependent component to their activation, current amplitudes increase with depolarizing step pulses, as seen in Figure 2.

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$_V$ 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_{KNa} 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_{KNa}. 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$^+$]$_i$ 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 internal [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$_{Na}$
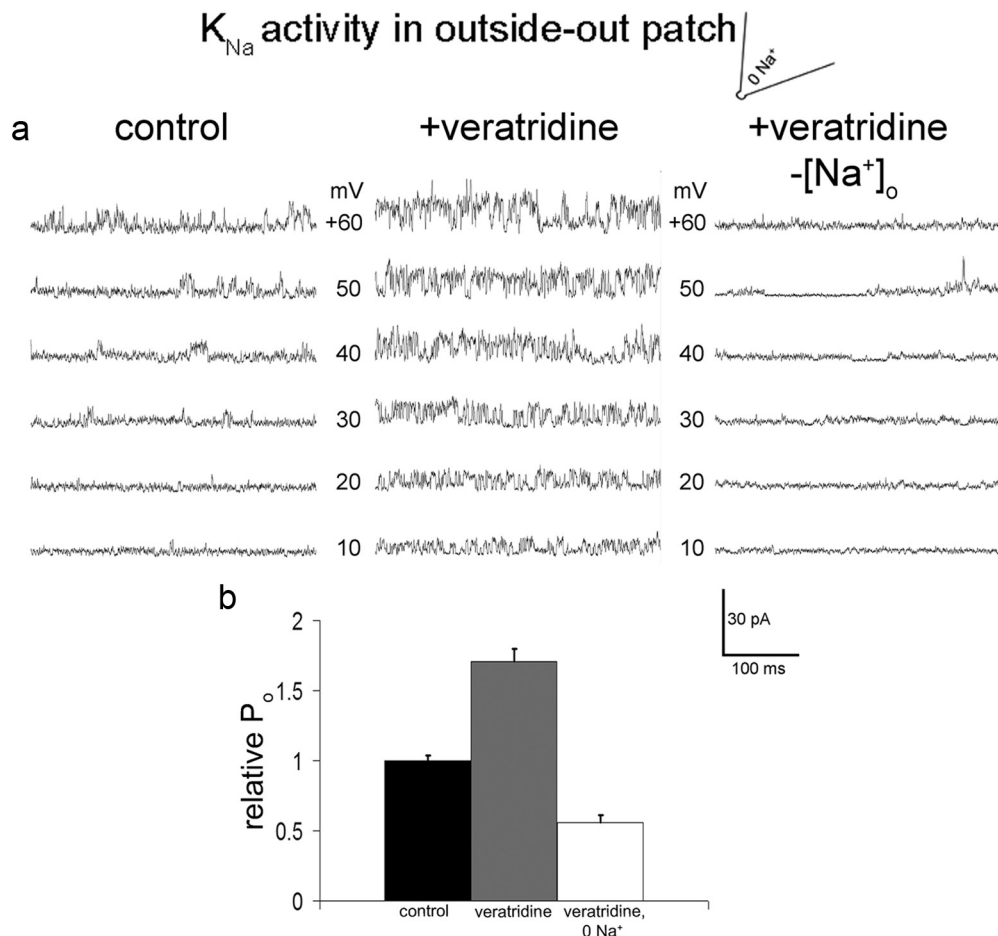
Figure 4. Enhancement of I_{NaP} increases K_{Na} channel activity. *a*, Application of veratridine, which prolongs the open state of the sodium channel, increases K_{Na} channel activity. Single-channel K_{Na} currents were evoked by steps to indicated voltages from a holding potential of -70 mV in control conditions (left), after application of veratridine (50 μM; center), and after removal of external Na$^+$ in the continued presence of veratridine (right). *b*, The effect of veratridine on K_{Na} channel activity in the presence and absence of external Na$^+$ was quantified by calculating the P_o of the channels, as described in Materials and Methods, in each condition ($n = 10$ patches). For each experiment, measured P_o during individual sweeps was normalized against mean P_o in control conditions. Bars represent relative P_o in indicated conditions. Error bars represent SE. $p < 0.001$ comparing control to veratridine and veratridine to 0 Na$^+$.

channel activity. We applied veratridine (50 μM), which prolongs the open state of the sodium channel (Barnes and Hille, 1988), to outside-out patches containing active K_{Na} channels, and observed a significant increase in K_{Na} channel activity (Fig. 4). The activating effect of veratridine on single K_{Na} channel activity could be abolished by the removal of external Na$^+$ after application of veratridine. (K_{Na} channel P_o increased to 171% $\pm$ 9.44% of control value after application of 50 μM veratridine, $p < 0.001$, $n = 10$, and decreased to 55.7 $\pm$ 12.7% value following subsequent removal of external Na$^+$, $p < 0.001$, mean $\pm$ SEM, $n = 10$, measured at $+60$ mV). Thus, our interpretation of these results is that veratridine increases K_{Na} channel activity by increasing sustained Na$^+$ influx via Na$_V$ channels. We emphasize that the effect of veratridine must be via increasing the local [Na$^+$] in the vicinity of K_{Na} channels and cannot be due to changing the internal bulk [Na$^+$]. Given the small amplitude of I_{NaP} in excised patches (see below) and the volume of internal solution used in recording pipettes, increases in bulk [Na$^+$]$_i$ are estimated to be well below 1 μM in experiments using outside-out patches (Materials and Methods), which is orders of magnitude below [Na$^+$] necessary for K_{Na} channel activation. Therefore, changes in activity of K_{Na} channels upon enhancement of I_{NaP} by veratridine must be due to local, subcellular changes in [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 Na$_V$-channels, simultaneous activity of Na$^+$ and K$^+$ currents and prominent subconductance states of K_{Na} 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
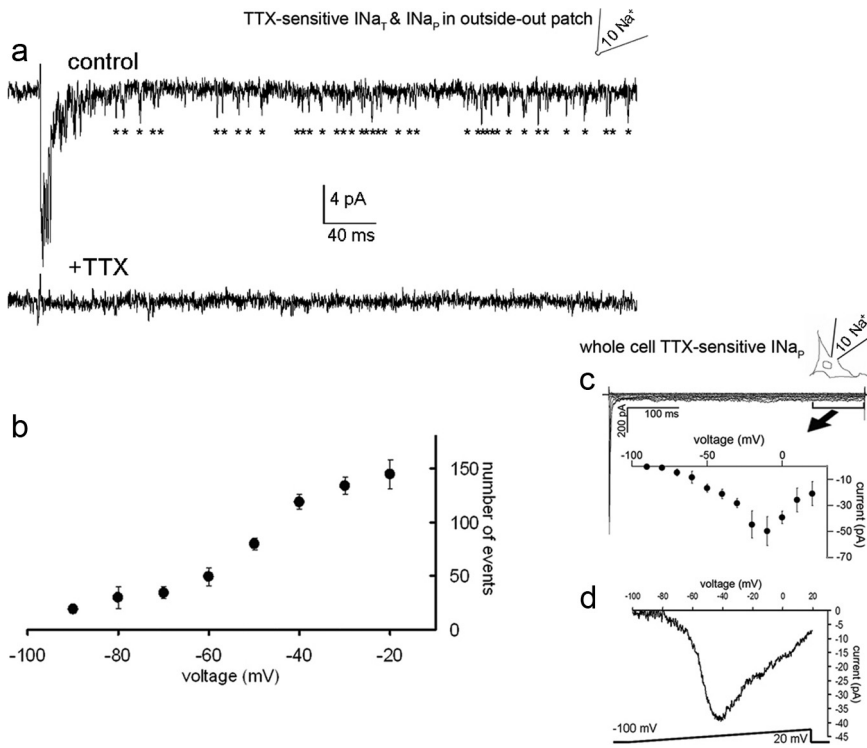
Figure 5. Voltage-gated sodium currents present in the soma of T/M cells include a persistent component. Sodium currents were isolated by using Cs$^+$ in the patch pipette and TEA and 4-AP in the bath to block potassium channels. *a–d*, Recordings were made in outside-out patches (*a*, *b*) and whole-cell configuration (*c*, *d*) with the internal sodium concentration set to 10 mM 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 *c* and *d*, TTX-sensitive currents were isolated and plotted by subtracting residual currents evoked in TTX (1 μM) from currents evoked before TTX application.

the resting potential in T/M cells. Consistent with this, the resting potential of mitral cells undergoes subthreshold oscillations in membrane voltage known to be mediated by TTX-sensitive I_{Na} (Desmaisons et al., 1999). This finding was consistent with our earlier whole-cell experiments that seemed to indicate that at cell resting potentials K_{Na} channels were maintained in a "primed" state under normal physiological conditions, which, given the small voltage-sensitive component to K_{Na} channel gating, produces a delayed outward current upon sufficient membrane depolarization. Such a "primed" state is likely to be the result of a tiny but persistent replenishment of Na$^+$ in the local vicinity of K_{Na} which maintains local [Na$^+$]$_i$ at a higher level than bulk [Na$^+$]$_i$. I_{NaT} in excised patches displayed an unusually hyperpolarized steady-state inactivation curve (V$_{1/2}$ = −81.7 ± 1.3 mV, peak current measured at −20 mV following 1 s prepulses of −120 to −30 mV, *n* = 6 patches). Consistent with the lack of apparent I_{NaT} in our recordings of K_{Na} channel activity, only ~10% of the sodium channels contribute to I_{NaT} evoked from a holding potential of −70 mV. Atypically hyperpolarized sodium channel inactivation (of the transient component) has recently been reported in other mammalian neurons as well (Grimes et al., 2010; Scott et al., 2010). To confirm that I_{Na} in excised patches was sensitive to veratridine, we applied veratridine to an outside-out patch in which I_{Na} was isolated. Upon application of 50 μM veratridine, we observed substantially increased I_{NaP} activity

during voltage steps and a prominent, slowly decaying tail current upon repolarization (Fig. 6*a,b*) consistent with previous reports of its action on I_{Na} (Barnes and Hille, 1988).

Discussion

Upon initial identification in cardiac myocytes, the suggested role for K_{Na} channels was that of providing a reserve conductance that would protect cells during hypoxia when an elevation in [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_{Na} channels in inside-out patches which showed that K_{Na} channel activation required a much higher [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_{KNa}, 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_{KNa} can commonly participate in providing a large outward conductance in many neuronal types. K_{Na} channels are expressed throughout the brain (Bhattacharjee et al., 2002, 2005). The Allen Brain Atlas shows that one of two genes which encode K_{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_{KNa} to I_{NaP} that we observe at the single-channel level in isolated outside-out patches suggests a highly specialized relationship between K_{Na} and Na$_V$ channels perhaps similar to the colocalization between calcium channels and the Slo1 calcium-activated potassium channel (BK) (Marrion and Tavalin, 1998). Although the coupling of K_{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$_V$ and K_{Na} channels as is observed between calcium channels and single BK channels. Nevertheless there are may parallels between the two systems. In both systems the channels are activated by two major factors, an intracellular ion ligand, and voltage (albeit K_{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_{KNa} by loading cells with higher concentrations of Na$^+$ and noting a leftward shift in the conductance-voltage relation of I_{KNa} (Budelli et al., 2009).Signif-

icantly, those experiments showed that cells loaded with higher concentrations of $[Na^+]_i$ still retained an obvious delayed outward component of I_{KNa} due to the influx of Na^+. Thus, the TTX-dependent influx of Na^+ appears to be additive to the $[Na^+]_i$ contributed by the bulk solution.

A possible mechanism for the functional coupling of K_{Na} and I_{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 $[Na^+]_i$ 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_{NaP} is responsible for increasing $[Na^+]$ in some submembrane regions where K_{Na} channels are located, and that limited diffusion as well as colocalization of I_{NaP} and I_{KNa} both contribute to activation of I_{KNa} by I_{NaP}.

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

The activity of K_{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 $[Cl^-]_i$. TTX-sensitive potassium currents in recordings using a physiological $[Cl^-]_i$ of 20 mM comprise ∼30% of the sustained outward current in mitral cells, compared with 57% in 150 mM $[Cl^-]_i$ (Budelli et al., 2009; our unpublished data). Our current study used an elevated $[Cl^-]_i$ to increase the likelihood of observing
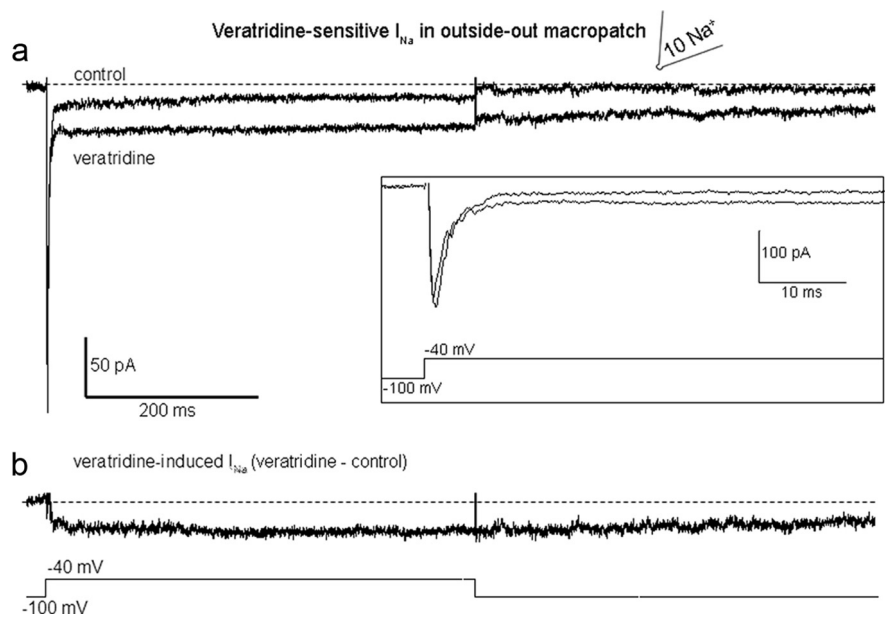

Figure 6. Veratridine enhances I_{NaP} in outside-out excised patches. Sodium currents were isolated by using Cs$^+$ in the internal pipette solution and TEA and 4-AP in the bath solution to block potassium channels. Recordings were made in macroscopic outside-out patches with the internal sodium concentration set to 10 mM. ***a***, Application of 50 μM veratridine to outside-out patches increased I_{NaP} activity during voltage steps, and induced a prominent, very slowly decaying tail current upon repolarization. Inset shows same recordings at higher time resolution. Only a minor difference is seen in the amplitude of I_{NaT} before and after veratridine. Dashed lines represent 0 current level. ***b***, Veratridine-induced I_{Na} was revealed by subtracting control current from current recorded in veratridine.

K_{Na} channels and to more precisely measure channel P_o. K_{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_{Na} channels at physiological concentrations of internal bulk Na^+.

The contribution of I_{NaP} to the activation of a prominent potassium conductance may seem counterintuitive given the established role of I_{NaP} in increasing neuronal excitability. We point out that the I_{NaP}-I_{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_{KNa} may mitigate depolarization and while it does not overcome I_{NaP}, could provide some negative feedback against the excitatory role of I_{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_{NaP} (for review, see Cantrell and Catteral, 2001) and I_{KNa} (Santi et al., 2006; Nuwer et al., 2010). Hence, the discrete or combined regulation of either component of the I_{NaP}-I_{KNa} coupled system could provide a mechanism for long-lasting changes in excitable properties over a wide range of both subthreshold and depolarized voltages.

I_{NaT} and the upstroke of action potentials are grossly similar across neurons. In contrast, the behavior of I_{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 (Crill, 1996; Vervaeke et al., 2006, Huang and Trussell, 2008). Indeed, I_{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_{NaP} participates, I_{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.

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