Biophysical Properties and Slow Voltage-dependent Inactivation of a Sustained Sodium Current in Entorhinal Cortex Layer-II Principal Neurons

A Whole-Cell and Single-Channel Study

Jacopo Magistretti*† and Angel Alonso*

From the *Department of Neurology and Neurosurgery, McGill University and Montreal Neurological Institute, Montréal, Québec, H3A 2B4 Canada; and †Dipartimento di Neurofisiologia Sperimentale, Istituto Nazionale Neurologico “Carlo Besta”, 20133 Milano, Italy

abstract The functional and biophysical properties of a sustained, or “persistent,” Na⁺ current (I_{NaP}) responsible for the generation of subthreshold oscillatory activity in entorhinal cortex layer-II principal neurons (the “stellate cells”) were investigated with whole-cell, patch-clamp experiments. Both acutely dissociated cells and slices derived from adult rat entorhinal cortex were used. I_{NaP}, activated by either slow voltage ramps or long-lasting depolarizing pulses, was prominent in both isolated and, especially, in situ neurons. The analysis of the gating properties of the transient Na⁺ current (I_{NaT}) in the same neurons revealed that the resulting time-independent “window” current (I_{NaTW}) had both amplitude and voltage dependence not compatible with those of the observed I_{NaP}, thus implying the existence of an alternative mechanism of persistent Na⁺-current generation. The tetrodotoxin-sensitive Na⁺ currents evoked by slow voltage ramps decreased in amplitude with decreasing ramp slopes, thus suggesting that a time-dependent inactivation was taking place during ramp depolarizations. When ramps were preceded by increasingly positive, long-lasting voltage prepulses, I_{NaP} was progressively, and eventually completely, inactivated. The V_{1/2} of I_{NaP} steady state inactivation was approximately −49 mV. The time dependence of the development of the inactivation was also studied by varying the duration of the inactivating prepulse: time constants ranging from ~6.8 to ~2.6 s, depending on the voltage level, were revealed. Moreover, the activation and inactivation properties of I_{NaP} were such as to generate, within a relatively broad membrane-voltage range, a really persistent window current (I_{NaPW}). Significantly, I_{NaPW} was maximal at about the same voltage level at which subthreshold oscillations are expressed by the stellate cells. Indeed, at −50 mV, the I_{NaPW} was shown to contribute to >80% of the persistent Na⁺ current that sustains the subthreshold oscillations, whereas only the remaining part can be attributed to a classical Hodgkin-Huxley I_{NaTW}. Finally, the single-channel bases of I_{NaP} slow inactivation and I_{NaPW} generation were investigated in cell-attached experiments. Both phenomena were found to be underlain by repetitive, relatively prolonged late channel openings that appeared to undergo inactivation in a nearly irreversible manner at high depolarization levels (−10 mV), but not at more negative potentials (−40 mV).

Keywords: persistent Na⁺ current • window current • stellate cells • oscillations • patch clamp

INTRODUCTION

The so-called persistent sodium current (I_{NaP}) is known to be expressed, together with the classical, transient sodium current (I_{NaT}), by numerous mammalian neuronal types. Its basic features include persistence during prolonged depolarizations, lower threshold of activation than I_{NaT}, and low amplitude (the underlying conductance normally representing 0.2–2% of the total sodium conductance; French and Gage, 1985; French et al., 1990; for review see Taylor, 1993; Crill, 1996. After the initial descriptions of the actions of sustained Na⁺ currents on neuronal electroresponsiveness (Hotson et al., 1979; Llinàs and Sugimori, 1980; Connors et al., 1982), I_{NaP} was first demonstrated with voltage-clamp studies in neocortical neurons (Stafstrom et al., 1982, 1985), and biophysically characterized in the hippocampus (French and Gage, 1985; French et al., 1990). I_{NaP} has also been observed, directly or indirectly, in an increasing number of neuronal structures including basal ganglia (Cha and Alzheimer, 1995; Cepeda et al., 1995), amygdala (Pape and Driesang, 1998), thalamus (Jahnsen and Llinàs, 1984; Parri and Crunelli, 1998), hypothalamus (Llinàs and Alonso, 1992; Uteshev et al., 1995), cerebellum (Jahnsen, 1986; D’Angelo et al., 1998; Kay et al., 1998), and peripheral ganglia (Baker and Bostock, 1997). Due...
to its voltage-dependent properties, $I_{NaP}$ can contribute to important integrative functions such as amplification of excitatory postsynaptic potentials (Deisz et al., 1991; Stuart and Sakmann, 1995; Schwindt and Crill, 1995; Lipowsky et al., 1996), generation of pacemaker activity (Alonso and Llinás, 1989; Amitai, 1994; Pennartz et al., 1997; Pape and Driesang, 1998), and firing-pattern shaping (Jahnsen and Llinás, 1984; Klink and Alonso, 1993; Franceschetti et al., 1995; Parri and Crunelli, 1998).

Since $I_{NaP}$ can generate membrane bistability and plateau potentials, it has also been implicated in the pathogenesis of some forms of epilepsy (Segal, 1994; for review see Ragsdale and Avoli, 1998). In addition, the ability of $I_{NaP}$ to sustain long-lasting Na$^+$ influxes, and therefore to steadily increase intracellular Na$^+$ concentration, has raised interest as to its possible role in mechanisms of neurodegeneration (Taylor and Meldrum, 1995).

The entorhinal cortex (EC) has proven to be a particularly interesting neuronal system for the study of $I_{NaP}$ functions. In the stellate cells of EC layer II, which give rise to the main cortical projection to the hippocampus (Steward and Scoville, 1976), $I_{NaP}$ has been shown to critically participate in the generation of subthreshold membrane-potential oscillations in the theta-rhythm range (Alonso and Llinás, 1989; Klink and Alonso, 1993). This particular intrinsic subthreshold activity is considered to be a critical determinant for the generation of the population theta oscillations generated by the EC network (Adey et al., 1957, 1960; Holmes and Adey, 1960; Mitchell and Ranck, 1980; Alonso and García-Aust, 1987a,b). The theta rhythm has been shown to contribute to synaptic-plasticity processes (Larson and Lynch, 1986; Larson et al., 1986; Greenstein et al., 1988; Alonso et al., 1990; Huerta and Lisman, 1996; Hölscher et al., 1997), and it is thus believed to play a major role in temporal lobe learning and memory functions (Doyère and Laroche, 1992; Buzsáki, 1996). On the other hand, since the EC is also known to play a crucial role in temporal lobe epileptogenesis (see references in Dickson and Alonso, 1997), it has been hypothesized that the presence of a robust $I_{NaP}$ in EC neurons may contribute to epileptogenic processes (Klink and Alonso, 1993).

For the above considerations, a detailed knowledge of the biophysical properties of $I_{NaP}$ expressed by EC layer II neurons seems of great interest since it would help to understand how this current specifically influences the physiological behavior of these cells. In this study, we have characterized $I_{NaP}$ in both acutely isolated and in situ EC layer II neurons. Our results revealed the existence of some interesting biophysical properties of $I_{NaP}$ that had not been thoroughly investigated yet, including: (a) a slow voltage- and time-dependent inactivation occurring with voltage-dependent time constants in the order of seconds; (b) a full inactivation at sufficiently positive potentials; and (c) a truly persistent, or “window,” current arising from the particular activation and steady state inactivation properties of the corresponding conductance. Moreover, we describe the single-channel events that account for all of the above-mentioned macroscopic phenomena.

**Materials and Methods**

**Slice and Cell Preparation**

Young-adult Long-Evans rats (P25–P35) were killed by decapitation. The brain was quickly removed under hypothermic conditions, blocked on the stage of a vibratome (Pelco), and submerged in an ice-cold cutting solution containing (mmol/liter): 115 NaCl, 5 KCl, 4 MgCl$_2$, 1 CaCl$_2$, 20 PIPES, and 25 d-glucose, pH 7.4 with NaOH, bubbled with pure O$_2$. Osmotic pressure ($\pi$) of the latter solution, as measured with a Micro Osmette 5004 osmometer, was typically 320 mOsm. Horizontal slices of the retro-hippocampal region were cut at 350–400 μm. For in situ recordings, slices were stored at room temperature in the above solution until use. For recordings on isolated neurons, the layer II of medial entorhinal cortex was dissected from each slice (White et al., 1993). Neurons were acutely isolated from the tissue fragments thus obtained following an enzymatic and mechanical dissociation procedure described elsewhere (Magistretti and de Curtis, 1998).

**Whole-Cell Recordings on Slices**

The recording chamber was mounted on the stage of an upright microscope (see below). Slices were laid onto the bottom of the chamber and perfused with an extracellular solution containing (mmol/liter): 34 NaCl, 26 Na$_2$CO$_3$, 80 tetraethylammonium (TEA)-Cl, 5 KCl, 3 CsCl, 2 CaCl$_2$, 3 MgCl$_2$, 2 BaCl$_2$, 2 CoCl$_2$, 0.4 CdCl$_2$, 4 4-aminopyridine (4-AP), 10 glucose, pH 7.4 when bubbled with 95% O$_2$, 5% CO$_2$. The solution of 0.2 rather than 0.4 mM CdCl$_2$ was found to depress residual inward rectification insensitive to the application of tetrodotoxin (TTX; Sigma-Aldrich Canada Ltd.) more effectively than Cd$^{2+}$ alone in slices (but not in freshly dissociated neurons; see below). Preliminary experiments were performed in situ neurons in the absence of extracellular Co$^{2+}$ and Ba$^{2+}$ and in the presence of 0.2 rather than 0.4 mM Cd$^{2+}$. Both average peak amplitude and current density of ramp (50 mV/s)-evoked, TTX-subtracted $I_{NaP}$ were significantly different ($P = 0.07$ and 0.51, respectively). Hence, possible enhancing effects of nonphysiological extracellular divalent cations on $I_{NaP}$ amplitude (Cummins et al., 1998) did not significantly affect measures in our experimental conditions.

Patch pipettes were fabricated from thick-wall borosilicate glass capillaries by means of a P-97 horizontal puller (Sutter Instruments Co.). The intrapipette solution contained (mmol/liter): 110 CsF, 10 HEPESNa, 11 EGTA, 2 MgCl$_2$, pH 7.25 with CsOH ($\pi$ adjusted to ~290 mOsm with mannitol). When filled with the above solution, the patch pipettes had a resistance of 3–5 MΩ. Slices were observed with an Axioskop microscope (Carl Zeiss, Inc.) equipped with a 40× water-immersion objective lens and differential-contrast optics. A near-infrared charge-coupled device camera (XC-75; Sony Corp.) was also connected to the microscope, and used to improve cell visualization for identification of neuron types and during the approaching and patching procedures. With this equipment, the principal cells of EC layer II were easily distinguished based on their somato-dendritic shape
Whole Cell Recordings on Isolated Neurons

The recording chamber was mounted on the stage of an inverted microscope (see below). After seeding into the chamber, dissociated cells were perfused with a standard HEPES buffer containing (mmol/liter): 140 NaCl, 5 KCl, 10 HEPES (free acid), 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 25 glucose, pH 7.4 with NaOH, bubbled with pure O<sub>2</sub> (<pH = 320 mOsm). After wash-out of cell debris, cell perfusion was switched to a solution suitable for Na<sup>+</sup>-current isolation containing (mmol/liter): 100 NaCl, 40 TEA-Cl, 10 HEPES (free acid), 2 CaCl<sub>2</sub>, 3 MgCl<sub>2</sub>, 0.2 CdCl<sub>2</sub>, 5 4-AP, 25 glucose, pH 7.4 with NaOH, bubbled with pure O<sub>2</sub> (<pH = 318 mOsm). The intrapipette solution was the same as described in the previous paragraph. Cells were observed at 400× with an Axiosvert 100 microscope (Carl Zeiss, Inc.). After tight-seal formation (>100 GΩ) and the establishment of the whole-cell configuration, series resistance (<pH = 12.0 MΩ (n = 54). Cell capacitance was estimated online by canceling the fast component of whole-cell capacitive transients evoked by −10 mV voltage steps with the amplifier compensation section, and reading out the corresponding value. Voltage-clamp recordings were performed at room temperature (~22°C) using an Axopatch 200A amplifier (Axon Instruments). The general holding potential was −80 mV.

Single Channel Recordings

Single-channel, cell-attached experiments were performed in acutely isolated neurons. After seeding into the recording chamber, cells where initially perfused with the same solution as described in the previous paragraph. The pipette solution contained (mmol/liter): 130 NaCl, 35 TEA-Cl, 10 HEPES-Na, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 5 4-AP, pH 7.4 with HCl (<pH = 338 mOsm). Single-channel patch pipettes had resistances ranging from 10 to 35 MΩ when filled with the above solution, and were always coated with Sylgard® (Dow Corning Corp.) from the shoulder to a point as close as possible to the tip so as to minimize stray pipette capacitance. After obtaining the cell-attached configuration, the extracellular perfusion was switched to a high-potassium solution containing: 140 K-acetate, 5 NaCl, 10 HEPES (free acid), 4 MgCl<sub>2</sub>, 0.2 CdCl<sub>2</sub>, 25 glucose, pH 7.4 with KOH (<pH = 320 mOsm) so as to hold the neuron resting membrane potential at or near 0 mV. Recordings were performed at room temperature with an Axopatch 200B amplifier (Axon Instruments). Capacitive transients and linear current leakage were minimized online by acting on the respective built-in compensation sections of the amplifier. Long-duration (20 s) depolarizing voltage steps were delivered one every 40 s from a holding potential of −80 or −100 mV.

Data Acquisition

Voltage protocols were commanded and current signals were acquired with a Pentium PC interfaced to an Axon TL1 interface, using the Clampex program of the pClamp 6.02 software (Axon Instruments). Current signals were filtered online (using the amplifier’s built-in low pass filter) and digitized at different frequencies according to the specific experimental aim. Filtering and acquisition frequencies were 5 and 20 kHz, respectively, for I<sub>NaP</sub> recordings 0.1-1 and 0.67-10 kHz (depending on the protocol duration), respectively, for I<sub>NaP</sub> recordings 1 and 2 kHz, respectively, for single-channel recordings. In all of the voltage protocols applied, cell-membrane potential was kept at the holding level for 15 (in whole-cell experiments) or 20 s (in single-channel experiments) between the end of each sweep and the beginning of the subsequent sweep (or of the conditioning prepulse preceding it, when applied). This avoided the development of cumulative voltage-dependent inactivation of I<sub>NaP</sub> during consecutive acquisition cycles.

Data Analysis

Whole-cell recordings were analyzed by means of the Clampex program (Axon Instruments). Offline leak subtraction was performed on I<sub>NaP</sub>− (but not I<sub>NaP</sub>) protocol traces. Current density was calculated by dividing the peak current amplitude by cell capacitance, estimated as explained above. Conductance values were calculated from Na<sup>+</sup>-current amplitudes by applying the extended Ohm’s law in the form: G<sub>Na</sub> = I<sub>Na</sub>/(V − V<sub>Na</sub>), where V<sub>Na</sub> is the nominal (Nernst) Na<sup>+</sup> reversal potential. Data were fitted with exponential functions, I = A<sub>1</sub> · exp(−t/τ<sub>1</sub>) + C, using Clampfit, or with Boltzmann functions, G = G<sub>max</sub> · (1 + exp[(V − V<sub>1/2</sub>)/k]) using Origin 3.06 (MicroCal Software).

Single-channel recordings were analyzed using Clampfit, Fetchan, and pStat (Axon Instruments). Residual capacitive transients were nullified by offline subtracting fits of average blank traces. Residual leakage currents were carefully measured in every single sweep at trace stretches devoid of any channel openings, and digitally subtracted. Channel dwell times were determined using a standard threshold routine of the Fetchan program. Ensemble-average traces were fitted with single exponential functions, I = A<sub>1</sub> · exp(−t/τ<sub>1</sub>) + A<sub>2</sub> · exp(−t/τ<sub>2</sub>) using pStat.

Average values were expressed as mean ± SD, unless otherwise explicitly stated. Statistical significance was evaluated by means of the two-tail Student’s t test for unpaired data.

Modeling I<sub>NaP</sub>

For a phenomenological description of I<sub>NaP</sub> activation and slow inactivation, a simple Hodgkin-Huxley model was assumed. We applied the basic relationship:

\[ I_{NaP}(V, t) = G_{NaP}(V, t) \cdot (V - V_{Na}), \]  

where

\[ G_{NaP}(V, t) = G_{NaP(max)} \cdot m(V) \cdot h(V, t), \]

and m and h are the probabilities of the activating and inactivating particles, respectively, to be in the permissive position. I<sub>NaP</sub> activation was assumed to be instantaneous, and m(V) was derived directly from the G<sub>NaP</sub> activation curve. h(V) was derived directly from the G<sub>NaP</sub> steady state inactivation curve. The transitions of the inactivating particle, h, were modeled according to the following first-order kinetic scheme:

\[ \frac{\alpha}{1 - h} \Leftrightarrow h \quad \frac{\beta}{} \]

from which it follows:
Plots of the voltage dependence of activation of methods subtracting the traces recorded in 1 μM TTx from control traces (both shown in the insets; calibration bars: 100 pA in B, 200 pA in C). (D) Plots of the voltage dependence of activation of $I_{\text{NaP}}$ in the same two neurons as shown in B and C (D1 and D2, respectively). $I_{\text{NaP}}$ conductances ($G_{\text{NaP}}$) were derived from the early parts of the TTX-subtracted currents by applying the extended Ohm equation (mat erial s and met hods). $G_{\text{NaP}}$ plots were normalized for the maximal values and fitted with single Boltzmann functions (empty lines). Fitting parameters were: $V_{1/2} = -47.6$ mV, $k = -5.5$ mV (D1); $V_{1/2} = -50.8$ mV, $k = -4.5$ mV (D2). The perpendicular, dotted lines indicate the half-maximal activation and the corresponding $V_{1/2}$.

Figure 1. Basic features of $I_{\text{NaP}}$ in EC layer II principal neurons. (A) $I_{\text{NaP}}$ evoked in a representative acutely isolated neuron (cell E6M15) by 500-ms depolarizing square pulses at -65 to -35 mV. All the traces shown are TTX subtracted (control minus 1 μM TTx). The holding potential was -80 mV. Scale bars: 100 pA, 100 ms. (Inset) Average plot of normalized $G_{\text{NaP}}$ derived, as explained in material s and met hods, from step protocols ($I_{\text{NaP}}$ amplitude was measured by averaging the data points between 400 and 500 ms from the pulse onset; $n = 5$). The best Boltzmann fitting to data points is also shown. Fitting parameters were: $V_{1/2} = -44.4$ mV, $k = -5.2$ mV. (B and C) $I_{\text{NaP}}$ evoked in a representative acutely isolated neuron (B; cell A6M15) and a representative in situ neuron (C; cell D7904) by slow voltage ramps (50 mV/s, from -80 to +20 mV; the voltage protocol is schematized above the insets). The currents shown in the main panels have been obtained by digitally subtracting the traces recorded in 1 μM TTx from control traces (both shown in the insets; calibration bars: 100 pA in B, 200 pA in C).

General Properties of $I_{\text{NaP}}$ in Acutely Dissociated and In Situ Neurons

In acutely dissociated EC layer II principal neurons, a prominent $I_{\text{NaP}}$ could be evoked by delivering long-lasting depolarizing pulses. Fig. 1 A illustrates current traces from a representative neuron. $I_{\text{NaP}}$ was blocked by 1 μM TTX, and was therefore routinely isolated via TTX subtraction. $I_{\text{NaP}}$ threshold of activation was at about -65 mV, and peak at -30 mV. Average $I_{\text{NaP}}$ amplitude (derived by averaging the data points between 400 and 500 ms from the pulse onset) and current density (calculated as explained in materia l s and met hods) were $-96.5 \pm 61.5$ pA and $-12.0 \pm 7.0$ pA/PF, respectively, at the peak of the current-voltage (I-V) relationship ($n = 5$). The average $I_{\text{NaP}}$ I-V relationship showed a linear region from -30 to -5 mV, the linear best fitting of which returned a zero-current level at +63.0 mV (not shown). This value compares favorably with the theoretical Nernst Na$^+$ reversal potential calculated for our ionic conditions ($V_{\text{Na}} = +61.0$ mV).

The voltage dependence of the conductance underlying $I_{\text{NaP}}$ ($G_{\text{NaP}}$ calculated as explained in the material s and met hods) is shown in the average plot of Fig. 1 A, inset. Boltzmann fitting to data points returned a half-activation voltage, $V_{1/2}$, of -44.4 mV, and a slope factor, $k$, of -5.2 mV. The ratio between the peak $G_{\text{NaP}}$ value

$$h(V, t) = h_\alpha(V) - [h_\alpha(V) - h_\beta] \cdot \exp(-t/\tau_h),$$

(3)

where

$$\tau_h(V) = 1/[(\alpha(V) + \beta(V)];$$

(4)

and

$$h_\alpha(V) = \alpha(V)/[(\alpha(V) + \beta(V)].$$

(5)

Numerical values for the rate constants, $\alpha$ and $\beta$, were derived from the experimental values of time constants of inactivation and recovery from inactivation ($\tau_h$) and from the $h_\alpha$ curve by applying Eqs. 4 and 5. After obtaining the analytical functions describing the voltage dependence of the rate constants (see results), the time course of $I_{\text{NaP}}$ activated in response to various voltage protocols was numerically reconstructed on the basis of the Eqs. 1 and 2, and Eq. 3 in its differential form. The simulation programs were compiled using QuickBASIC 4.5 (Microsoft Corp.).
found in each cell and the maximal value of the conductance underlying the transient Na\(^+\) current (I\(_{\text{NaP}}\)) expressed by the same cell was also calculated, and averaged 0.0187 ± 0.0097 (n = 5).

To quickly explore the whole voltage range of I\(_{\text{NaP}}\) activation, ramp protocols were then used. Slow ramps at 50 mV/s were initially selected since they allowed full inactivation of fast-decaying Na\(^+\)-current component(s). Fig. 1 B shows the currents evoked by such a protocol in a representative acutely dissociated neuron, both in control conditions and in the presence of 1-μM TTX (inset). Offline digital subtraction returned the TTX-sensitive I\(_{\text{NaP}}\) in isolation (Fig. 1 B). The continuous I–V relationship thus obtained showed a threshold at -70/ -60 mV and a peak at -40/ -30 mV. Noteworthy in both pulse and ramp protocols, I\(_{\text{NaP}}\) activation was accompanied by an evident increase in current noise, especially at voltage levels close to the peak of the I–V relationship (Fig. 1, A and B), consistent with the relatively high conductance (-20 pS) characterizing the channels responsible for I\(_{\text{NaP}}\) generation in EC layer II neurons (Magistretti et al., 1999).

Ramp protocols were also used in experiments performed in in situ neurons. In this situation, TTX subtraction always returned prominent I\(_{\text{NaPs}}\) in isolation, whose I–V relationship closely resembled that of I\(_{\text{NaPs}}\) in acutely dissociated neurons (Fig. 1 C). I\(_{\text{NaP}}\) amplitude, measured at the peak of the I–V relationship, was significantly higher in in situ than in isolated neurons (-179.3 ± 120.7 pA, n = 54, vs. -65.6 ± 37.9 pA, n = 38; P < 5 x 10\(^{-7}\)), whereas the current density did not significantly differ in the two situations (-17.7 ± 29.1 pA/μF, n = 54, vs. -16.5 ± 8.6 pA/μF, n = 38; P = 0.8). These findings strongly suggest that the channels responsible for I\(_{\text{NaP}}\) are located not only on the soma, but also on neuronal processes severed by the dissociation procedure. Activation curves of I\(_{\text{NaPs}}\) recorded in both in situ and isolated neurons were also constructed. Conductance values were derived from I\(_{\text{NaPs}}\) by applying the extended Ohm's law (see materials and methods), and the resulting activation curves were fitted with single Boltzmann functions (Fig. 1 D). Average half-activation potentials and slope factors were very similar in in situ neurons (V\(_{1/2}\) = -51.3 ± 3.9 mV, k = -4.0 ± 0.7 mV, n = 39) and isolated neurons (V\(_{1/2}\) = -48.7 ± 4.7 mV, k = -4.4 ± 0.9 mV, n = 19). These values compare favorably with those obtained from step protocols (see above), and are also in good agreement with the activation parameters previously reported for I\(_{\text{NaPs}}\) expressed in other neuronal systems (French et al., 1990; Brown et al., 1994; Baker and Bostock, 1997).

Given the effectiveness of TTX subtraction in isolating I\(_{\text{NaPs}}\)s in both isolated and in situ neurons, this procedure was routinely used in our study. All of the data presented from this point on are from TTX-subtracted currents.

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**I\(_{\text{NaP}}\) Is Not a Window Current Generated by Transient Na\(^+\) Channels**

It is well known that a noninactivating, "window" current (I\(_{\text{NaTW}}\)) can be generated by the gating properties of fast, transient Na\(^+\) channels (Hodgkin and Huxley, 1952). To address the issue of whether the I\(_{\text{NaP}}\) expressed by EC layer II neurons can be accounted for by a classical window conductance, we analyzed the voltage-dependence properties of I\(_{\text{NaP}}\) in acutely dissociated neurons. Fig. 2 A shows Na\(^+\)-current traces recorded in a representative neuron in response to an activation--inactivation pulse protocol. Peak-current amplitudes were measured and used for deriving conductance values (G\(_{\text{NaT}}\)); normalized activation and steady state inactivation plots were then constructed (Fig. 2 B), and fitted with Boltzmann functions. The optimal approximation to data points returned by single Boltzmann functions suggested the existence of functionally homogeneous, transient Na\(^+\) channels in the neuronal preparation under examination. In eight cells, average V\(_{1/2}\) and k were -32.5 ± 6.5 mV and -3.6 ± 0.9 mV, respectively, for the activation function, and -59.8 ± 5.2 mV and 4.5 ± 0.9 mV, respectively, for the steady state inactivation function. These values are similar to those reported in a number of other studies on neuronal I\(_{\text{Na}}\) voltage dependence (e.g., Sah et al., 1988; Huguenard et al., 1988; Cummins et al., 1994). The product of the activation and steady state inactivation functions was then calculated in individual cells to derive the theoretically predicted voltage dependence of the window conductance (G\(_{\text{NaTW}}\)) arising from transient Na\(^+\) channels (Fig. 2 B, dotted line). Fig. 2 C illustrates the I\(_{\text{NaP}}\) evoked by a standard ramp protocol, and isolated via TTX subtraction, in the same cell as in A and B. The conductance underlying I\(_{\text{NaP}}\) (G\(_{\text{NaP}}\)) was calculated and compared with the predicted G\(_{\text{NaTW}}\) (Fig. 2 D); an evident discrepancy in the voltage dependence of the two conductances could be observed at potentials positive to about -30 mV, where G\(_{\text{NaTW}}\) rapidly fell towards zero, whereas G\(_{\text{NaP}}\) maintained relatively high values, though it also showed a characteristic decline from its maximum (see below). The same discrepancy between G\(_{\text{NaTW}}\) and G\(_{\text{NaP}}\) was observed in four other acutely dissociated neurons, in which both well-clamped I\(_{\text{NaTs}}\) and sizable I\(_{\text{NaPs}}\) could be recorded. In a broader cell population, the amplitudes of the reconstructed G\(_{\text{NaTW}}\) and of G\(_{\text{NaP}}\) were measured both at the peak [G\(_{\text{max}}\) and at a voltage point positive by 20 mV to that of the peak [G\(_{\text{max}}\)] at and at a voltage point positive by 20 mV to that of the peak [G\(_{\text{max}}\)]. The average ratios G\(_{\text{NaTW}}\)(+20)/G\(_{\text{NaTW, max}}\) and G\(_{\text{NaP}}\)(+20)/G\(_{\text{NaP, max}}\) were 0.076 ± 0.057 (n = 8) and 0.715 ± 0.102 (n = 12), respectively (P < 5 x 10\(^{-12}\)). In addition, in those neurons in which both
$I_{NaP}$ and $I_{NaT}$ were quantified, the size of the predicted $I_{NaTW}$ was much smaller than that of the observed $I_{NaP}$ (see Table I). These data clearly indicate that by far most of the $I_{NaP}$ expressed by EC layer II neurons is not a classical $I_{NaTW}$ similar to what has been previously observed in hippocampal neurons (French et al., 1990).

$I_{NaP}$ Inactivates in a Time- and Voltage-dependent Manner

Whereas a depolarization-activated conductance, once maximally recruited, would be expected to maintain a steady value at more positive voltage levels, $G_{NaP}$, as mentioned above, consistently showed some degree of decline from its maximum. A possible explanation for this observation is the existence of a time-dependent inactivation of $G_{NaP}$ acting during the ramp (Fleidervish and Gutnick, 1996). Under this hypothesis, $G_{NaP}$ should inactivate more when elicited with increasingly slow ramps. To address this issue, we performed a series of experiments in which the amplitude of the $I_{NaP}$ evoked by voltage ramps was analyzed as a function of the ramp slope. Fig. 3 A shows the protocols applied and the TTX-subtracted currents thereby obtained in a representative in situ neuron. $I_{NaP}$ amplitude appeared to markedly depend on the depolarization rate. The average, normalized $I_{NaP}$ amplitude measured at the peak of the $I$–$V$ function was then plotted as a function of the inverse of the ramp slope, a quantity directly related to ramp duration (Fig. 3 B) ($n = 12$). The resulting plot demonstrated a biexponential decay, with a fast “slope constant” and an ~15-fold slower one. These data strongly suggest that at least two kinetic components exist in $I_{NaP}$, each characterized by a different inactivation rate. The ratio between the two slope constants and that between their relative amplitude coefficients were such that, with 50-mV/s ramps, >96% of the ensuing $I_{NaP}$ peak amplitude was accounted for by the slow component. Since we were interested in the slowest Na$^+$-current components, which more closely approach the notion of “persistent” Na$^+$ current, we decided to employ 50-mV/ s ramps in the rest of our study so as to maintain the peak value of “true” $I_{NaP}$ relatively unaffected by time-dependent inactivation, while ruling out most of the “intermediate” kinetic components. Moreover, our observations confirmed the validity of applying 50-mV/ s ramps for obtaining data on $I_{NaP}$ voltage dependence of activation (see previous paragraphs).

The above data clearly pointed to the existence of a time- and voltage-dependent inactivation of $I_{NaP}$. However, inactivation properties of voltage-dependent channels have been shown to be possibly affected by the composition of intracellular milieu, and in particular by intracellular nonphysiological halogenic anions (Chandler and Meves, 1970; Arispe et al., 1984; Kay et al.,
dependence as compared with \( F^- \)-evoked, TTx-subtracted NaPs were --155.4 ± 58.9 pA and --9.8 ± 3.6 pA/pF in \( SO_4^{2-} \) or MeSO\(_3^-\) experiments (data pooled together), not significantly different from the control values reported above (\( P = 0.66 \) and 0.55, respectively). The ratio between the amplitudes of \( I_{NaP} \) evoked by 6.25- vs. 50-mV/s ramps was 0.497 ± 0.134 in \( SO_4^{2-} \) or MeSO\(_3^-\) experiments, again not significantly different from that found when using \( F^- (0.51 \pm 0.08, n = 12, P = 0.79) \). These observations indicate that the above-described phenomena are indeed of physiological relevance.

We then investigated the issue of \( I_{NaP} \) inactivation in further detail by analyzing the effects of variable prepulse potentials on ramp-activated \( I_{NaP} \). The protocol employed is illustrated in Fig. 4 A1. 50-mV/s voltage ramps were preceded by very-long-lasting (15 s) conditioning prepulses at various voltage levels (\( V_{cond} \)). When \( V_{cond} \) of --90 to --50 mV were used, the ramp started from the same voltage level as \( V_{cond} \), rather than from a fixed, negative voltage level; in this way, the possible occurrence of recovery from inactivation during the initial part of the ramp was avoided. At more positive \( V_{cond} \), the ramp started from --50 mV, so as to preserve the voltage region of \( I_{NaP} \) peak. Currents recorded with the above protocol in a representative in situ neuron are shown in Fig. 4 A2. \( I_{NaP} \) peak amplitude turned out to markedly depend on the conditioning potential. Average, normalized current traces obtained from seven in situ neurons are depicted in Fig. 4 B1. It can be observed that voltage-dependent steady state inactivation of \( I_{NaP} \) was nearly complete at about --20 mV. The average plot of \( I_{NaP} \)’s voltage dependence of inactivation (Fig. 4 B2) could be fitted by a single Boltzmann function, with \( V_{1/2} \) at about --49 mV and a slope factor, \( k \), of ~10 mV. In addition, we also constructed an activation plot from the average \( I_{NaP} \) derived from the same neuron pool, and fitted it with a single Boltzmann function (Fig. 4 B2). Note that, importantly, \( G_{NaPW} \) activation and steady state inactivation functions overlapped over a wide voltage range. Due to this phenomenon, a significant window conductance (\( G_{NaPW} \)) is expected to arise from \( G_{NaP} \). The predicted voltage dependence of \( G_{NaPW} \) is depicted in Fig. 4 B2 (dotted line), and will be compared with relevant experimental data later on in the paper.

**Figure 3.** \( I_{NaP} \) looks different, depending on the slope of the ramp applied to elicit it. (A) Voltage ramps exploring the --80/ +20-mV voltage range with different depolarization speeds (100, 66.7, 50, 25, 12.5, and 6.25 mV/s) were tested (A1), and the TTx-sensitive currents recorded in an exemplary in situ neuron (cell B7902) are depicted in A2, all over the same time scale. (B) Average, normalized \( I_{NaP} \) peak amplitude as a function of the inverse of ramp slope (n = 12; same ramp protocols as illustrated in A1). The plot could be fitted by a double exponential function [\( I = A_1 \cdot \exp(-s/s) + A_2 \cdot \exp(-s/s) + C \), where \( s \) is the ramp slope and \( s_1 \) are slope constants] with \( A_1 = 0.295, s_1 = 0.317 \) V/s, \( A_2 = 0.382, s_2 = 20.2 \) mV/s (continuous line).

1986; Nisenbaum et al., 1996). Since the main anion in the intracellular solution used in our experiments was fluoride (\( F^- \)), we performed control experiments in which internal \( F^- \) was substituted with other molecules, namely sulphate (\( SO_4^{2-}; n = 4 \)), and methanesulphonate (MeSO\(_3^-; n = 3 \)). The same ramp protocols as described in the previous paragraph were applied under these ionic conditions. In no case did we observe significant differences in \( I_{NaP} \) amplitude and its ramp-slope dependence as compared with \( F^- \) experiments. In particular, peak amplitude and current density of ramp (50 mV/s)-evoked, TTx-subtracted \( I_{NaP} \)s were --155.4 ± 58.9 pA and --9.8 ± 3.6 pA/pF in \( SO_4^{2-} \) or MeSO\(_3^-\) experiments (data pooled together), not significantly different from the control values reported above (\( P = 0.66 \) and 0.55, respectively). The ratio between the amplitudes of \( I_{NaP} \) evoked by 6.25- vs. 50-mV/s ramps was 0.497 ± 0.134 in \( SO_4^{2-} \) or MeSO\(_3^-\) experiments, again not significantly different from that found when using \( F^- (0.51 \pm 0.08, n = 12, P = 0.79) \). These observations indicate that the above-described phenomena are indeed of physiological relevance.

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**Time Dependence of \( I_{NaP} \) Inactivation and Recovery from Inactivation**

The kinetic properties of \( I_{NaP} \) voltage-dependent inactivation were then further characterized. Time dependence of inactivation was first analyzed by means of prepulse-ramp protocols (Fig. 5 B); the voltage ramp eliciting \( I_{NaP} \) was preceded by a prepulse at various voltage levels (from --60 to --20 mV), which was made to vary in duration from 0 to up to 20 s. Currents recorded in response to such a protocol in a representative in situ neuron are shown in Fig. 5 A. Average, normalized peak-current amplitudes were used for constructing plots of
time dependence of inactivation, each one referring to a specific conditioning potential (Fig. 5 C). These plots could be best fitted with single exponential functions, the time constants were slow and ranged from ~6.8 to ~2.6 s, depending on the conditioning potential.

The time course of $I_{NaP}$ recovery from inactivation was also investigated. The voltage protocols applied (Fig. 6 B) consisted of a first 10-s prepulse at −30 mV that substantially inactivated $I_{NaP}$, followed by a second prepulse at −90 or −80 mV of variable duration (from 0 to 10 s), and by the standard voltage ramp. Currents recorded in response to such a protocol in a representative in situ neuron are shown in Fig. 6 A. Average, normalized peak-current amplitudes were used for constructing plots of time dependence of recovery from inactivation, for both recovery potentials (Fig. 6 C). These plots could be best fitted with single exponential functions, with time constants of ~5.2 (−80 mV), and 4.7 s (−90 mV).

A Major, Persistent Window Current Is Generated as a Consequence of $G_{NaP}$ Gating Properties

As mentioned above and illustrated in Fig. 4 B2, the wide overlapping of $I_{NaP}$ activation and steady state inactivation curves is expected to result in a prominent window current ($I_{NaPW}$) distinct from the classical window current ($I_{NaTW}$) predicted on the basis of the gating properties of the fast, transient Na$^+$ conductance. To test this prediction, voltage protocols consisting of very-long-lasting depolarizing pulses were applied to in situ neurons so as to try to uncover steady current components in $I_{NaP}$. Fig. 7 A1 shows average, TTX-subtracted Na$^+$ currents obtained from five neurons in response to 15-s voltage steps at −60 to −10 mV. After an initial phase displaying fast and intermediate-speed decay components, a slower decaying current component, which was identified as the $I_{NaP}$ under study, became evident. When the last 14 s of the current trace were considered, the decay phase of $I_{NaP}$ could be best fitted by a

![Figure 4](image-url)

**Figure 4.** Voltage dependence of inactivation of $I_{NaP}$. (A) Voltage protocol used for the study of $I_{NaP}$ voltage dependence of inactivation (A1), and TTX-subtracted currents recorded in an exemplary in situ neuron (A2; cell D7808). The conditioning prepulses preceding each ramp varied from −90 to −10 mV in 10-mV steps, and their duration was 15 s. Note that here, as well as in all of the voltage-clamp protocols applied in whole-cell experiments, each conditioning prepulse/ramp cycle was preceded by a 15-s period at −80 mV so as to allow recovery from the inactivation developed during the foregoing cycle (materials and methods). (B) Average currents and steady state inactivation function from seven cells. (B1) Average normalized currents. The currents evoked in each cell as shown in A were divided by the maximal peak-current value observed in the same cell; the traces were then averaged among cells and further normalized to the maximal peak value thus obtained. Standard deviations are also shown at 5-mV intervals in the traces preceded by conditioning prepulses at −90, −50, and −10 mV. (B2) Average steady state inactivation plot (●). The amplitudes of the currents evoked as shown in A were measured at the ramp potential of −40 mV. The values thus obtained were divided by the maximal value observed in each cell, averaged among cells, and plotted as a function of the conditioning prepulse potential. The inactivation plot was best-fitted by a single Boltzmann function, with $V_{1/2} = −48.8$ mV and $k = 10.0$ mV. An average activation plot was also constructed by deriving the normalized conductance (materials and methods) from the average, normalized current obtained from the same cells (B1; prepulse potential = −90 mV), and best-fitted with a single Boltzmann function, with $V_{1/2} = −52.6$ mV and $k = −4.6$ mV. Each plot-fitting pair was further normalized to the value of the fitting-function amplitude coefficient. The dotted line represents the product of the two fitting functions, corresponding to the predicted voltage dependence of the resulting window conductance ($G_{NaPW}$).
single exponential function, with voltage-dependent time constants very similar to those determined for the time-dependent inactivation of $I_{NaP}$ revealed by ramp protocols (see above). In addition to the decaying component, fittings returned a steady (offset) component ($I_{so}$) whose amplitude also displayed a marked voltage dependence. When plotted as a function of test potential (Fig. 7 B), the normalized $I_{so}$ amplitude closely par-
alleled that of the expected $I_{\text{NaPW}}$. Therefore, a steady current component of $I_{\text{NaP}}$ can be directly demonstrated whose voltage-dependent behavior fits that predicted for the time-independent $I_{\text{NaPW}}$.

On the basis of the above data, we then estimated the relative contribution of $I_{\text{NaPW}}$ and $I_{\text{NaTW}}$ to the total, noninactivating Na$^+$ current generated by EC layer II cells in a subthreshold region of membrane voltages, where it is known to sustain theta-like membrane-potential oscillations lasting for indefinitely long periods (Alonso and Llinás, 1989; Alonso and Klink, 1993; Klink and Alonso, 1993). Our measurements indicate that at $-50$ mV, a level close to that at which the maximal amplitude of subthreshold oscillations is observed (Alonso and Klink, 1993), <20% of the total, persistent Na$^+$ current is accounted for by $I_{\text{NaTW}}$, whereas the remaining part must derive from the window current generated by the true $I_{\text{NaP}}$ (Table I).

**Modeling of $I_{\text{NaP}}$: Slow Inactivation**

To further clarify the basis of the experimentally observed decline in $G_{\text{NaP}}$ at positive potentials (Fig. 2 D), a theoretical reconstruction of the biophysical properties of $I_{\text{NaP}}$ inactivation was carried out. A simple Hodgkin-Huxley model, considering a single inactivation gate switching between two energy states, was considered in order to give account for the monoeXponential time course of $I_{\text{NaP}}$ decay and recovery from inactivation. This reconstruction was merely phenomenological and was given no mechanistic meaning since single-channel data clearly indicated different features of the underlying properties.

**Figure 7.** $I_{\text{NaP}}$ inactivation during long-lasting depolarizing voltage steps (A) Averaged, TTX-subtracted currents (A1) from five in situ neurons as obtained in response to 15-s voltage steps to the test potentials indicated on the left. The voltage protocol applied is shown in A2. Standard deviations for some points, single-exponential best fittings of the decay phases (enhanced lines), and time-constant ($\tau$) values are also shown; the starting point of the fitted part of the traces is indicated by the vertical, dashed line. Scale bars: 50 pA, 2.5 s. (B) The amplitude of the nonactivating ("offset") component of average $I_{\text{NaP}}$ ($\bullet$), measured at the different test potentials, is compared with the predicted window current arising from the gating properties of $I_{\text{NaP}}$ ($I_{\text{NaPW}}$, continuous line). The $I_{\text{NaPW}}$ shown has been derived from the $G_{\text{NaPW}}$ depicted in Fig. 4 B2 (dotted line).

<table>
<thead>
<tr>
<th>Voltage (mV)</th>
<th>$I_{\text{NaTW}}$ (pA)</th>
<th>$I_{\text{NaP}}$ (pA)</th>
<th>$I_{\text{NaPW}}$ (pA)</th>
<th>$I_{\text{NaTW}}/I_{\text{NaP}}$</th>
<th>$I_{\text{NaTW}}/I_{\text{NaPW}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>-50</td>
<td>-3.4 ± 1.3</td>
<td>-58.1 ± 25.0</td>
<td>-30.8 ± 13.2</td>
<td>0.071 ± 0.051</td>
<td>0.133 ± 0.097</td>
</tr>
<tr>
<td>-40</td>
<td>-5.2 ± 2.0</td>
<td>-80.6 ± 38.4</td>
<td>-38.6 ± 11.2</td>
<td>0.086 ± 0.075</td>
<td>0.293 ± 0.256</td>
</tr>
</tbody>
</table>

Absolute and relative amplitudes of persistent and window Na$^+$ currents. The window currents resulting from the gating properties of both the transient Na$^+$ conductance, $G_{\text{NaT}}$ ($I_{\text{NaTW}}$) and the persistent Na$^+$ conductance, $G_{\text{NaP}}$ ($I_{\text{NaP}}$) were derived according to the following formulas:

$$I_{\text{NaTW}}(V) = G_{\text{NaT}}(V) \cdot (V - V_{\text{Na}}) \cdot m(V) \cdot h(V),$$

where $m(V)$ and $h(V)$ values were taken from the fitting functions of $G_{\text{NaT}}$ activation and steady state inactivation plots determined in each cell; $I_{\text{NaPW}}(V) = I_{\text{NaP}}(V) \cdot h(V)$, where $h(V)$ values were taken from the fitting function of the average $G_{\text{NaP}}$, steady state inactivation curve (see Fig. 4 B2). Measurements are from five acutely dissociated cells in which both well-clamped $I_{\text{NaT}}$s and sizable $I_{\text{NaP}}$s were recorded. Data are mean ± SD.
The time constants of $I_{\text{NaP}}$ inactivation and recovery from inactivation and the data on voltage dependence of $I_{\text{NaP}}$ steady state inactivation were processed to derive numerical values for the rate constants of the inactivation-gate transitions, as explained in materials and methods. Fig. 8 B shows the voltage dependence of the values thus obtained for the two rate constants, $\alpha$ and $\beta$. The plots were then best fitted with the empirical function, $\alpha$ (or $\beta$) = $(a \cdot V_m + b) / \{1 - \exp\{(V_m + b/a) / k\}\}$ where $V_m$ is the membrane voltage. The numerical values returned by the fittings for the $a$, $b$, and $k$ coefficients, in both the $\alpha$ and $\beta$ plots, are indicated in the legend to Fig. 8. The voltage-dependence functions thus obtained for $\alpha$ and $\beta$ were then used to derive the predicted voltage dependence of $I_{\text{NaP}}$ inactivation-gating time constants (see materials and methods). The concordance between the reconstructed, theoretical function and the real data is shown in Fig. 8 A.

The kinetic parameters obtained in the above manner were then used to verify the possible effects of $I_{\text{NaP}}$ slow inactivation on the results of voltage-clamp ramp protocols. Reconstructed $I_{\text{NaP}}$'s evoked by simulated voltage ramps of variable slope are illustrated in Fig. 8 C. It is apparent that progressively reducing the depolarization rate causes a decrease in $I_{\text{NaP}}$ peak amplitude, and the appearance of increasing discrepancies between the true $I_{\text{NaP}}$ voltage dependence and that measured in the late part of the ramp protocol. Fig. 8 E shows the voltage dependence of $G_{\text{NaP}}$ as obtained in response to a simulated 50 mV/s ramp protocol: the reconstructed $G_{\text{NaP}}$ declined at voltages positive to about $-30$ mV very similarly to the experimentally observed $G_{\text{NaP}}$ (see Fig. 2 D), whereas $G_{\text{NaP}}$ voltage dependence

Figure 8. Modeling of $I_{\text{NaP}}$ inactivation and of its consequences on ramp protocols (A) Time constants of $I_{\text{NaP}}$ inactivation (circles) and recovery from inactivation (triangles) as a function of membrane voltage. Time constants of inactivation ($\tau_\alpha$), determined with both ramp (•) and step (○) protocols are shown. The continuous line is the theoretical function describing $\tau_\alpha(V)$, as obtained from the fitting functions of the voltage dependence of rate constants (see below). Crosses indicate time constants of inactivation of ensemble-average traces obtained in single-channel, cell-attached experiments (see text), here included for comparison. (B) Rate constants of the transitions of the inactivating gate, $h$, to $\alpha$ and from $\beta$ the permissive position (see results and materials and methods for details). Values for $\alpha$ (squares) and $\beta$ (diamonds) have been calculated, as explained in materials and methods, on the basis of time constants measured from both ramp (filled symbols) and step (empty symbols) protocols. The continuous lines represent the fitting functions (described in results) for $\alpha(V)$ and $\beta(V)$, with $a = -2.88 \cdot 10^{-3}$ mV$^{-1}$ s$^{-1}$, $b = 4.63$ mV, $k = 4.63$ mV, $k = 4.63$ mV, $k = 4.63$ mV.

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of activation, as measured by considering only the early part of the ramp protocol, was negligibly affected. Moreover, the plot of reconstructed $I_{\text{NaP}}$'s peak amplitude as a function of the inverse of ramp slope (Fig. 8D) could be fitted, with some approximation, with a single exponential function, with a slope constant (<s>) similar to the slow slope constant observed in experimental plots (see Fig. 3B), and no sign of any fast, early component. The latter results further confirm the adequacy of the ramp protocol we routinely used for characterizing the biophysical properties of $I_{\text{NaP}}$ and in particular the slow inactivation of this current.

Finally, a simulated protocol of steady state inactivation revealed that, during a ramp preceded by a prepulse that fully inactivates $I_{\text{NaP}}$, a significant recovery from inactivation can occur provided that the ramp starts at sufficiently negative levels and is sufficiently slow. For instance, during a 25-mV/s ramp starting at $-80 \text{ mV}$, 17% of the total current can recover from inactivation (not shown). By contrast, voltage protocols similar to those we employed for the study of $I_{\text{NaP}}$ voltage-dependent inactivation (see above) determined no appreciable ramp-dependent recovery from inactivation of $I_{\text{NaP}}$.

**Single-Channel Bases of $I_{\text{NaP}}$ Slow Inactivation and $I_{\text{NaPW}}$ Generation**

The single-channel basis of $I_{\text{NaP}}$ in EC layer II neurons has been described elsewhere (Magistretti et al., 1999). In that study, we found that, after membrane step depolarization, $I_{\text{NaP}}$ is generated by early as well as late single Na$^+$-channel openings of much more prolonged duration and of significantly higher conductance than the usual, transient Na$^+$-channel openings responsible for $I_{\text{NaT}}$ generation. Here, we report how the same persistent Na$^+$-channel activity also accounts for some particular biophysical properties described for macroscopic $I_{\text{NaP}}$, namely $I_{\text{NaP}}$ time-dependent inactivation and $I_{\text{NaPW}}$ generation.

Recordings from cell-attached patches in acutely isolated EC layer II neurons frequently revealed the presence of a persistent Na$^+$-dependent channel activity that proved to remain stable even over prolonged periods of time (tens of minutes). When very-long-lasting (20 s) depolarizing steps at $-10 \text{ mV}$ were commanded from a holding potential of $-80 \text{ mV}$, multiple, repetitive single-channel openings were observed that tended to cluster preferentially at the beginning of the test pulse. Typical examples of this channel activity for a series of consecutive 20-s test pulses is shown in Fig. 9A1. A detail of some prolonged, late channel openings is also provided by Fig. 9A1, inset. Ensemble averaging of multiple traces was then carried out for each patch (Fig. 9A2). Due to the very long overall duration of the recording cycle required for every single sweep (40 s), only a limited number of traces could be recorded in each patch (15 on average), what explains the low signal-to-noise ratio of ensemble-average traces. In all cases, however, the averaged currents showed a noticeable trend to decay towards zero, and this decay could be properly fitted by a single exponential function (Fig. 9A2, blank trace). The time constant of average-current decay was $2.66 \pm 0.52 \text{ s}$ in six patches at $-10 \text{ mV}$, a value that compares favorably with those found in whole-cell protocols on macroscopic $I_{\text{NaP}}$ inactivation at the most positive voltage levels tested (see Fig. 8A). Ensemble averaging of all of the available sweeps recorded from the same six patches returned a better signal-to-noise ratio (Fig. 9B). The decay time constant of the resulting average current was 2.46 s, again in good agreement with the data obtained from the analysis of both individual patches and whole-cell recordings.

The open-time distribution of the single-channel activity evoked by 20-s depolarizing steps at $-10 \text{ mV}$ was then investigated. Fig. 10A shows the open-time distribution found in the same patch as illustrated in Fig. 9A. In this case, all plots were best fitted by double-exponential functions, with average time constants of $3.35 \pm 0.86$ and $21.07 \pm 17.74 \text{ ms}$, and an average ratio of the slow vs. fast exponential-component weight ($W = A \cdot \tau$) of 0.118 $\pm$ 0.048 ($n = 6$). It seems important to point out at this time that: (a) even the faster of the two time constants exceeds by more than six times the mean open time found in classical, transient Na$^+$ channel openings at approximately the same test voltage level (Aldrich et al., 1983; Alzheimer et al., 1993b); yet (b) the values of both time constants were much smaller than those of the time constants of inactivation of ensemble-average currents as well as whole-cell $I_{\text{NaP}}$. This specific issue will be further addressed below.

Long-lasting depolarizing protocols at more negative test-voltage levels were also applied in cell-attached recordings so as to investigate the possible bases of $I_{\text{NaPW}}$ generation. A typical example of the recordings obtained at the test voltage of $-40 \text{ mV}$ is shown in Fig. 11A. Again, multiple, repetitive single-channel openings were observed and these were able to generate a measurable inward current in ensemble-average traces (Fig. 11B). Note, however, that at these more negative voltage levels channel openings were more widely distributed over the entire 20-s sweeps. Consequently, at $-40 \text{ mV}$ the ensemble average-current decayed at a slower rate (with a time constant of $4.33 \text{ ms}$) than at $-10 \text{ mV}$. In addition, this decay was towards a steady value (C in exponential fittings; see materials and methods) that was higher than zero and represented 25.1% of the current's total amplitude coefficient (namely $A + C$). These data are in good agreement with the whole-cell data on $I_{\text{NaP}}$ inactivation and $I_{\text{NaPW}}$ generation illustrated above.
They are also consistent with the idea that the same single-channel events can account for $I_{\text{NaP}}$ as well as a steady, nondecaying Na-$^{+}$-current component (namely $I_{\text{NaPW}}$) generated within a limited voltage window, where it represents a substantial fraction of total $I_{\text{NaP}}$.

Finally, the analysis of open-time distribution at $-40$ mV also revealed the existence of two exponential components (Fig. 10 B), with average time constants of $1.33 \pm 0.14$ and $6.63 \pm 3.47$ ms, and an average $W_{2}/W_{1}$ ratio of $0.069 \pm 0.042$ ($n = 3$). At this potential, therefore, the discrepancy between mean open times and time constants of inactivation of both ensemble-average currents and macroscopic $I_{\text{NaP}}$ was even bigger than at $-10$ mV.

The observation that the channel mean open times are far exceeded by the time constants of ensemble-average current inactivation clearly indicates that the latter do not reflect average channel-opening lifetimes in a simple model considering two open states each undergoing one single closure process (with rate constant $b$) towards an absorbing state, like:

$$C \rightarrow O_{i} \rightarrow I_{i},$$

with the index $i$ being either 1 or 2, and with $a_{i} > b_{i}$.

Rather, alternative models considering late first openings and/or late reopenings must be taken into account. In an extreme situation, late channel openings such those of Fig. 9 A1 might all be first openings of as many distinct channels inactivating towards an absorbing state, in a model qualitatively similar to that depicted in Scheme I, but in which the kinetics of the macroscopic inactivation process would rather reflect the rate constants of the closed-to-open reactions, $a_{i}$.

This interpretation, which resembles the classical Aldrich-Corey-Stevens model of the kinetics of transient Na-$^{+}$ channels (Aldrich et al., 1983), seems very unlikely since, in patches containing most likely only one “persistent” channel, delayed first openings occurred very infrequently, whereas late reopenings were often observed (Magistretti et al., 1999). An alternative possibility is that the channel, once open, can reach multiple nonconductive states, one of which is virtually absorbing at positive potentials, thus causing a true channel inactivation. Under this assumption, a channel behavior such as that in Fig. 9 A1 could be accounted for by repetitive reopenings of a small number of persistent channels. The most economical kinetic scheme of such a behavior is seen in Scheme II.

$$\alpha_{i}$$
$$O_{i} \rightleftharpoons C$$
$$\gamma_{i} \downarrow \beta_{i}$$
$$I_{i}$$

(SCHEME II)
A nonnegligible rate constant ($\delta$) for the reaction $I_1 \rightarrow O_2$ should also be considered for more negative voltages, at which a major $I_{NaPW}$ is produced. The analytical derivation of relaxation time constants from rate constants (see Colquhoun and Hawkes, 1977, 1981) reveals that if $\alpha_1 >> \gamma_1$, and $\gamma_1 \approx \gamma_2$ (or if only one inactivated state exists, communicating with one of the two open states), one slow time constant of ensemble-average current inactivation would be produced, which would faithfully reflect $1/\gamma$. Other possible kinetic schemes consider that an inactivated state is reached from a closed rather than a conducting state. The evaluation of the relationships between transition rate constants in such a scheme and the time constants of ensemble-average current inactivation would require the study of closed-time distribution. This could not be reliably accomplished from our data since in no patches in which slow inactivation was studied could the presence of one single channel be assumed. Despite these limitations in providing precise kinetic schemes, our data clearly demonstrate the importance of late channel (re)openings, rather than of early, very-long-lasting openings, for the generation of slow kinetic components in macroscopic $I_{NaP}$.

**Discussion**

The present study provides a biophysical characterization of the $I_{NaP}$ expressed by rat EC layer II principal neurons. The major issues we addressed deal with the mechanism of generation of $I_{NaP}$, the possible influence of the voltage protocols employed for the study of $I_{NaP}$ biophysical properties, and the real degree of $I_{NaP}$ persistence over time and specific voltage windows.

In general, the hypotheses on generation of persistent Na$^+$ currents consider two main possibilities: (a) $I_{NaP}$ simply derives from an incomplete steady inactivation of transient Na$^+$ channels over a narrow voltage window, due to the partial superimposition of activation and steady state inactivation voltage-dependence curves of the corresponding conductance; (b) $I_{NaP}$ is the result of channel openings that do not functionally behave according to the properties of transient Na$^+$ channels, but derive from a rare and atypical gating modality of the same channels (Alzheimer et al., 1993b), possibly under modulatory control by G proteins (Ma et al., 1997) or other factors, or, alternatively, from a different channel species. To discriminate between these two possibilities, it is necessary to accurately determine the properties of the window current generated by transient Na$^+$ channels and compare them with those of $I_{NaP}$ present in the same cell preparation. The study of acutely dissociated EC neurons allowed us to perform careful measurements on $I_{NaT}$ and, therefore, to make reliable predictions on the resulting $I_{NaTW}$. The comparison between $I_{NaTW}$ and $I_{NaPW}$ was done both on a statistical basis and, in some cases, in the same cells. Our data on the voltage dependence and amplitude of these currents indicate that $I_{NaTW}$ in contrast with what was reported in a previous study (Fan et al., 1994), cannot be a major source of the $I_{NaP}$ expressed by EC principal neurons. An alternative, specialized mechanism must be implied in the generation of the large $I_{NaP}$ found in these neurons. An exciting possibility, already suggested elsewhere on the basis of whole-cell data (French et al., 1990), is that $I_{NaP}$ is generated by a specialized Na$^+$ channel at least biophysically distinct from those generating the fast transient Na$^+$ current (Masukawa et al., 1991). Indeed, single-channel, patch-clamp experiments in EC layer II neuron somata indicate that while $I_{NaT}$ is generated by a typical ~15-pS channel with fast activation and inactivation kinetics, $I_{NaP}$ is due to an ~20-pS channel activity with a 10-mV lower threshold of activation, and a sustained, high open probability during prolonged depolarizations (Magistretti et al., 1999). The issue regarding the molecular diversity of Na$^+$ channels (see Ragsdale and Avoli, 1998, for recent review) expressed by EC layer II neurons is beyond the scope of the present study and will be discussed elsewhere.
activation varies considerably among different experimental works (Alzheimer et al., 1993a; Uteshev et al., 1995; Cepeda et al., 1995; Pennartz et al., 1997; Parri and Crunelli, 1998; Cummins et al., 1998). The experiments we carried out by running voltage ramps of variable slopes clearly indicate that the amplitude of the ensuing $I_{NaP}$ strictly depends on the depolarization rate applied. Our data show how, when ramps of progressively decreasing slopes (from 100 to 6.25 mV/s) are commanded, $I_{NaP}$ amplitude decreases in a roughly biexponential fashion. The existence of a faster exponential component may be due to the presence of Na$^+$-current components kinetically intermediate between classical, “fast” Na$^+$ currents and the persistent Na$^+$ current. These intermediate kinetic components were easily observed in our step-protocol recordings (see Figs. 1 A and 7 A1), and their properties in EC layer II stellate cells will be described in detail elsewhere. The measurements on $I_{NaP}$S evoked with “slow” voltage ramps may therefore be contaminated by the superimposition of such current components, unless the commanded ramp is slow enough.

Moreover, our data show that, importantly, the process of $I_{NaP}$ slow inactivation is a potential source of distortions in the measurements of $I_{NaP}$ biophysical parameters (amplitude, voltage dependence of activation, reversal) when this current is elicited by voltage ramps. All these considerations point to the importance of accurately choosing the voltage protocol applied for the study of $I_{NaP}$ in each specific experimental situation. We propose that some previously reported, atypical biophysical features of $I_{NaP}$, particularly regarding its non-sigmoidal voltage dependence of activation (Brown et al., 1994), are the result of the interaction between multiple Na$^+$-current slow decay components and the ramp protocols employed. In our study, the ramp protocols routinely used were chosen so as to minimize both the possible contribution of intermediate-kinetics Na$^+$ current components and the effects of voltage- and time-dependent inactivation of $I_{NaP}$.

The inactivation properties of what, according to our operative definition, can be considered as $I_{NaP}$ have been characterized in detail in our study. Our experiments indicate that, in our preparation, the steady state inactivation of the conductance underlying $I_{NaP}$ ($G_{NaP}$) (a) has a voltage dependence that extends over a wide voltage window, and (b) reaches a nearly complete level at $-20$ to $-10$ mV. The former of these features, together with the relative position of the $G_{NaP}$ activation curve along the voltage axis, is expected to give rise to a major, time-independent, “window” current over a limited voltage range. This window current ($I_{NaPW}$), clearly different from that arising from the voltage-dependence properties of the transient Na$^+$ conductance ($I_{NaTW}$), could also be directly demonstrated both as nonzero

Figure 11. Single-channel basis of $I_{NaPW}$ generation. (A) Cell-attached recordings at the test potential of $-40$ mV in a representative patch from an acutely isolated EC layer II neuron (patch D8617). The patch was held at $-80$ mV, and 20-s depolarizing pulses were delivered beginning from the time point marked by the arrow. The traces shown are 12 consecutive sweeps (scale bars: 2 pA, 2 s). (Inset) Time expansion of some single-channel open- (o) and shut- (s) and open- (o) channel levels used in dwell-time analysis are remarked by the horizontal dashed lines. (B) Ensemble average of 33 sweeps recorded at the test potential of $-40$ mV, with the same experimental protocol as explained above, from three different patches (scale bars: 0.1 pA, 2 s). The single-exponential best fitting of the average current’s decay phase (blank line) and the corresponding time-constant value are also shown; values for A and C (see materials and methods) were $-51.7$ and $-17.3$ fA, respectively.

Another interesting issue raised by our data relates to the definition itself of $I_{NaP}$. Due to their practicality, ramp protocols are the most widely used means for eliciting and isolating $I_{NaP}$. However, the employment of such protocols presupposes that they can adequately reproduce the voltage dependence of $I_{NaP}$ without significantly recruiting any other kinetically different (i.e., faster-decaying) Na$^+$-current component. This is often implicitly assumed rather than demonstrated. Moreover, the notion of “slow voltage ramp” suitable for $I_{NaP}$...
baselines at the beginning of ramp protocols on voltage dependence of inactivation (see Fig. 4), and as offsets, or pedestals, in exponentially decaying \( I_{\text{NaP}} \) elicited by long-lasting voltage steps (Fig. 7). The peak of the observed \( I_{\text{NaPW}} \) occurred at voltage levels very close to those at which \( I_{\text{NaP}} \)-dependent, theta-like subthreshold membrane-potential oscillations are generated by EC stellate cells (Alonso and Llinás, 1989; Alonso and Klink, 1993; Klink and Alonso, 1993; van der Linden and Lopes da Silva, 1998). Our data indicate that the contribution of \( I_{\text{NaPW}} \) to the total persistent Na\(^{+}\) current over the voltage range of subthreshold-oscillation generation must be substantial, since the peak \( I_{\text{NaPW}} \) amplitude was estimated to exceed that of the predicted \( I_{\text{NaTW}} \) by more than four times. Since the subthreshold oscillations generated by the stellate cells can last indefinitely, our observations also imply that only a fraction of the total \( I_{\text{NaP}} \), namely \( I_{\text{NaPW}} \) itself, is sufficient for sustaining them.

\( I_{\text{NaP}} \) slow inactivation and recovery from inactivation were found to occur with voltage-dependent time constants in the order of a few seconds. We worked out an analytical reconstruction of the kinetics of these processes which, if introduced into suitable neuronal models, should allow us to make predictions on the effects of slow voltage-dependent inactivation on \( I_{\text{NaP}} \) modulation of membrane-voltage events. We demonstrated that \( I_{\text{NaP}} \) inactivation can considerably affect the apparent current maximal amplitude during the delivery of slow depolarizing ramps. Therefore, it is conceivable that the recruitment of \( I_{\text{NaP}} \) and its impact onto membrane-voltage dynamics can be significantly influenced by the speed of membrane depolarization. For instance, the ability of \( I_{\text{NaP}} \) to bring the membrane potential towards threshold for action-potential firing may be expected to be higher in response to a step depolarizing current injection than to slower or sustained depolarizations. This is consistent with the role of \( I_{\text{NaP}} \), demonstrated in various central nervous system neurons (Jahnsen and Llinás, 1984; Franceschetti et al., 1995; Parri and Crunelli, 1998) including EC stellate cells (Alonso and Klink, 1993; Klink and Alonso, 1993) in promoting transient low-threshold spikes and sustaining phasic firing in response to fast depolarizations. On the other hand, \( I_{\text{NaP}} \) inactivation, which we found to be eventually complete above a physiologically interesting range of membrane potentials, may have an important role in limiting detrimental Na\(^{+}\) influx during pathological conditions such as seizures and ischaemia (see also Taylor and Meldrum, 1995).

The question can then be raised whether the biophysical properties we describe here for the \( I_{\text{NaP}} \) expressed by EC stellate cells represent a general feature of this current in various neuronal populations. Slow time-dependent inactivation of \( I_{\text{NaP}} \) has been previously reported in neocortical pyramidal neurons (Fleidervish and Gutnick, 1996), although in that case the voltage dependence of the process was not investigated in detail, whereas a nondecaying “offset” \( I_{\text{NaP}} \) component corresponding to \( \sim 30\% \) of the total was found in experiments on time dependence of inactivation even after inactivating prepules at \( +20 \) mV. This is in contrast with our finding of a virtually complete inactivation at approximately \(-20/-10 \) mV. The discrepancy may imply the existence of interesting functional differences among \( I_{\text{NaP}} \)s expressed by different neuronal populations, which encourages further studies in other cell systems; alternatively, it may be the consequence of the inactivation protocol employed in Fleidervish and Gutnick (1996), in which the depolarizing ramp after the inactivating prepule started from a fixed, negative voltage level (-80 mV): this may allow some degree of recovery from inactivation during the early phase of the ramp, as also suggested by the output of our modeling study (results).

Finally, the present study provides an insight into the fine mechanisms underlying the complex biophysical features displayed by \( I_{\text{NaP}} \). Our previous work has already clarified the nature and elementary properties of the single-channel events responsible for \( I_{\text{NaP}} \) generation in EC principal neurons (Magistretti et al., 1999). The data we report here clearly point to the importance of late Na\(^{+}\)-channel (re)openings, many times longer in duration than those generating classical, transient \( I_{\text{NaT}} \) in central neurons, for determining the behavior of slow kinetic components of macroscopic \( I_{\text{NaP}} \). Late Na\(^{+}\)-channel (re)openings have already been identified as a possible source of \( I_{\text{NaP}} \) in neocortical neurons (Alzheimer et al., 1993b) and ventricular myocytes (Ju et al., 1994), but those we observed were comparatively much longer-lived. The slow inactivation of whole-cell \( I_{\text{NaP}} \) we characterized was paralleled by that of ensemble-average traces from single-channel, cell-attached recordings. In turn, the latter appeared to be the consequence of the slow delivery of channels to an inactivated state. This can be the consequence of either late first openings or, much more probably, low-rate transitions from open states that are more likely to switch to a close, re-recruitable state, or vice versa. The inactivated state was nearly absorbing at \(-10 \) mV, but not at \(-40 \) mV, where the existence of a major window current generated by the voltage-dependent properties of \( G_{\text{NaP}} \) had been predicted and demonstrated (see above). Again, whole-cell \( I_{\text{NaPW}} \) had its single-channel correlate in late, repetitive openings able to generate measurable net inward currents after as long as 20 s of membrane depolarization.

In conclusion, the \( I_{\text{NaP}} \) expressed by EC layer II principal neurons is a prominent current operating in a subthreshold range of membrane potentials, most of which is generated by a process independent of the
classical gating behavior of the transient Na$^+$ conductance. It also displays complex and previously nonrecog-
nized biophysical characteristics that appear to be
tailored to the specific role this current is known to
play in the generation of the sub- and near-threshold
membrane-potential events typical of the same neu-
rons. The concept of “persistent Na$^+$ current” may turn
out to be susceptible to some critical revision also in
other experimental situations, with reference to both
the existence of multiple and heterogeneous func-
tional components, and the expression of kinetic and
voltage-dependence properties that might influence
their impact onto neuronal function in previously un-
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