Properties of an intermediate-duration inactivation process of the voltage-gated sodium conductance in rat hippocampal CA1 neurons

Christopher R. French,1,2 Zhen Zeng,2 David A. Williams,3 Elisa L. Hill-Yardin,3 and Terence J. O’Brien1,2

1Department of Neurobiology, Royal Melbourne Hospital, Melbourne, Victoria, Australia; 2Department of Medicine, University of Melbourne, Melbourne, Victoria, Australia; and 3Department of Physiology, University of Melbourne, Melbourne, Victoria, Australia

Submitted 15 December 2014; accepted in final form 2 November 2015

French CR, Zeng Z, Williams DA, Hill-Yardin EL, O’Brien TJ. Properties of an intermediate-duration inactivation process of the voltage-gated sodium conductance in rat hippocampal CA1 neurons. J Neurophysiol 115: 790–802, 2016. First published November 4, 2015; doi:10.1152/jn.01000.2014.—Rapid transmembrane flow of sodium ions produces the depolarizing phase of action potentials (APs) in most excitable tissue through voltage-gated sodium channels (NaV). Macroscopic currents display rapid activation followed by fast inactivation (I_f) within milliseconds. Slow inactivation (I_s) has been subsequently observed in several preparations including neuronal tissues. I_s serves important physiological functions, but the kinetic properties are incompletely characterized, especially the operative timescales. Here we present evidence for an “intermediate inactivation” (I_i) process in rat hippocampal CA1 neurons with time constants of the order of 100 ms. The half-inactivation potentials (V_{0.5}) of steady-state inactivation curves were hyperpolarized by increasing conditioning pulse duration from 50 to 500 ms and could be described by a sum of Boltzmann relations. I_s state transitions were observed after opening as well as at subthreshold potentials. Entry into I_i after opening was relatively insensitive to membrane potential, and recovery of I_i became more rapid at hyperpolarized potentials. Removal of fast inactivation with cytoplasmic papaine revealed time constants of I_{0.5} decay corresponding to I_i and I_s with long depolarizations. Dynamic clamp revealed attenuation of trains of APs over the 10^-ms timescale, suggesting a functional role of I_i in repetitive firing accommodation. These experimental findings could be reproduced with a five-state Markov model. It is likely that I_i affects important aspects of hippocampal neuron response and may provide a drug target for sodium channel modulation.

CA1; inactivation; Na_{V}; rat;

ACTIVATION OF voltage-gated sodium (Na_{V}) channels produces the rapid, large-amplitude sodium currents (I_{Na}) that cause the depolarizing phase of action potentials in most excitable tissue, including hippocampal neurons (Sah et al. 1988). A variety of neurological disorders result from genetic mutations of Na_{V} channels (Eijkelkamp et al. 2012), and identification and quantification of the dynamic behavior of these channels are essential for understanding both normal as well as pathophysiological states.

The original studies of this conductance in squid axon identified a rapid sigmoidal activation phase followed by a slower monoeponential fall in amplitude termed inactivation (Hodgkin and Huxley 1952). This decrement in current became faster with larger depolarizations and was considered voltage sensitive, although it has been subsequently demonstrated that this “fast” inactivation derives voltage sensitivity largely from the activation process (Bezanilla 2008b). Fast inactivation (I_f) plays an important role in neural dynamical behavior as it contributes to the repolarization of the action potential as well as paradoxical hyperexcitability with hyperpolarization (“anode break excitation”). Additionally, the rate of recovery from I_f will affect firing rates and might be expected to influence firing probability close to threshold by determining the fraction of activatable channels.

A “slow” inactivation process, here termed I_s, identified in squid axons (Rudy 1978; Ulbricht 2005), is kinetically and structurally distinct from I_f. I_s has also been identified in central neurons, with time constants varying from hundreds (Colbert et al. 1997; Jung et al. 1997) to thousands (Kuo and Bean 1994b; Migliore 1996) of milliseconds, the latter best corresponding to the I_s in squid axon. Additionally, a form of fast-onset long-term open-state block of sodium channels in hippocampal neurons has been identified (Venkatesan et al. 2014).

A similar wide range of time constants of I_s has been observed in CA1 neurons, implying that there may be substantial variability in the time constants of I_s, or alternatively there may be more than one component such as “intermediate” inactivation, which has been identified in rat striated and cardiac muscle Na_{V} channels (Benitah et al. 1999; Kambouris et al. 1998). In the present study we describe an “intermediate” form of inactivation (I_i) with time constants between those of I_f and I_s previously identified in these neurons.

In the present study, previous observations on I_f and I_s in CA1 neurons were confirmed and extended (Kuo and Bean 1994a; Sah et al. 1988), and initial evidence for a component with time constants “intermediate” to conventional I_f and I_s was identified. The equilibrium (h_{Na}) relation was characterized, as well as transition rates through the open-state and closed-state transitions negative to the activation threshold. Elimination of I_f with cytoplasmic papaine allowed direct observation of I_i onset through the time constants of decay with prolonged depolarization. Voltage sensitivity and relative proportionality of I_i were also measured. Dynamic clamp experiments revealed accommodation of action potentials over a timescale consistent with a role for I_i. A relatively simple Markov model of I_i was derived that replicates experimental findings.
MATERIALS AND METHODS

All procedures were carried out in accordance with protocols approved by the University of Melbourne Animal Ethics Committee. Two- to six-week-old Wistar rats (male and female) were deeply anesthetized with pentobarbital and decapitated, and 350- to 400-μm hippocampal slices were prepared in a cutting solution [saturated with a 95% O2-5% CO2 gas mixture (carbogen)] composed of (in mmol/l) 87 NaCl, 2.5 KCl, 25 NaHCO3, 1.25 NaH2PO4, 0.5 CaCl2, 7 MgCl2, 25 D-glucose, and 75 sucrose at ~4°C. The slices were then transferred to a holding chamber containing bicarbonate-buffered saline (in mmol/l: 115 NaCl, 5 KCl, 20 PIPES, 1 CaCl2, 4 MgCl2, and 25 D-glucose, pH 7.4 with NaOH) for use 1–4 h afterwards. Individual neurons were released from the tissue by gentle trituration with gradiens-orifice Pasteur pipettes.

Low-resistance patch short-shanked pipettes (0.8–2 MΩ) fabricated with a Sutter P-1000 electrode puller were used to minimize series resistance artifacts. With these pipettes the neurons generally assumed a spherical profile, facilitating voltage clamp control—retained long axonal segments were almost always associated with either breakthrough spikes or suboptimal voltage control of current-voltage (I-V) or steady-state inactivation curves (Sah et al. 1988). In most experiments, sodium current amplitude was reduced (typically 1–5 nA) to enhance voltage clamp stability by the use of a low-sodium (35 mM) extracellular solution with equimolar substitution of NaCl with Tris chloride (in mmol/l: 35 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 10 D-glucose, 4 4-AP, 55 Tris-HCl, and 0.1 CdCl2, pH 7.4 with Tris base), with TEA (40 mM) added, and 0.1 mmol/l CdCl2 to block calcium conductances. The patch pipette solution consisted of (in mmol/l) 85 CsF, 35 NaCl, 1 CaCl2, 1 MgCl2, 10 EGTA, 10 HEPES, and 20 TEA, pH 7.2 with CsOH.

Currents were recorded with a Molecular Devices Axon 700B or AM Systems model 2400 patch-clamp amplifier with ~90% predictive and compensatory series resistance compensation. Signals were digitized at between 100 and 200 kHz with Axon 1320 or National Instruments (NI USB-6259) devices and filtered at 10 kHz with a four-pole low-pass Bessel filter. pCLAMP 10 (Molecular Devices) or Strathclyde WCP version 4.1.7 software was used to generate suitable pulse protocols and record digitized current and voltage signals that were analyzed with Molecular Devices Clampfit 10, Prism (GraphPad), and Microsoft Excel (Microsoft) software packages. The JSim (University of Washington) package was used for sodium conductance modeling and simulations. The StdpC dynamic clamp package (Kemenes et al. 2011) was used in conjunction with a second computer connected to a National Instruments analog-to-digital converter (PCI-6221) to provide a simulated noninactivating potassium conductance for current clamp.

Experiments were performed at 22°C in a temperature-controlled room with routine verification of chamber temperature. A series of control experiments found no significant time-dependent shifts in steady-state inactivation. Typically, maximal shifts of ~1 mV were noted over periods longer than 15 min, so no correction was necessary. Drugs were applied via a custom-made gravity-flow manifold system, changing from control to test solutions within ~2 s, with a 100-μm-diameter outflow tube positioned ~500 μm from the recorded cell. Electrode/bath potential was zeroed at the beginning and rechecked at the end of recording for significant changes; predicted liquid junction potentials (Barry 1994) were calculated to be +9.6 mV, and observed deviation of reversal from the predicted Nernst potential was close to this value (10–15 mV), so all data points and figures have been adjusted by +10 mV. Statistical errors are presented as SE unless otherwise stated. Rates of onset and offset of inactivation were measured with variable time interval pulse protocols time fitted with exponential functions, as well as the time to 75% onset or recovery (τ1/3, τ75). With double-pulse protocols, the second pulse was normalized to the amplitude of the first pulse; in practice, there was little variability in first pulse amplitudes during individual pulse sequences. Nonlinear least-squares fits to parameters were performed with the Marquardt-Levenberg algorithm as implemented in Clampfit and Prism and a quadratic minimization algorithm in the Microsoft Excel Solver package. Comparisons between single- and double-exponential data fits were analyzed for significance with F statistics as implemented in Prism and pCLAMP.

RESULTS

Activation and Inactivation of I Na

I Na was activated by step depolarizations from a holding potential of ~90 mV with threshold at ~50 mV, reaching maximal conductance at ~0 mV with Boltzmann parameters of half-activation potential ~22.3 ± 1.4 mV and slope 7.1 ± 0.7 mV (Fig. 1). Current reversal occurred about +10 mV positive to the Nernst potential. This was most likely due to the liquid...
junction potential resulting from the dissimilar solutions used in the pipette and bath and is close to a predicted value of +9.6 mV (JPCalc, Barry 1994), and so measured potentials have been adjusted by +10 mV. The rapid activation phase was followed by inactivation with a biexponential decay as previously reported by Sah et al. (1988), leaving a small “persistent” component (French et al. 1990). The biexponential rates of macroscopic inactivation increased with depolarization, with values of 1.9 ± 0.2 ms, 9.4 ± 0.8 ms and 0.8 ± 0.1 ms, 7.7 ± 0.3 ms (n = 7 cells) for the fast and slow components at −20 and +10 mV, respectively, with the fast component dominating (~90% total amplitude) at large depolarizations at up to +40 mV.

**Recovery from Fast Inactivation**

Recovery from inactivation was measured with double-pulse protocols (Fig. 2A). After 20-ms conditioning pulses to −10 mV producing almost complete inactivation of the current, recovery occurred with relatively short time constants with a bell-shaped distribution with a maximum of 18 ms at −70 mV, becoming faster at more negative potentials (Fig. 2, B and C).

Additionally, even after a 20-ms depolarization, a second slower component of recovery was evident, as can be seen from the incomplete recovery of $I_{Na}$ over 100 ms (Fig. 2A, arrow), which was subsequently investigated in detail.

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![Fig. 2. Recovery from fast inactivation ($I_f$). A, left: $I_f$ recovery protocol. A prepulse to −10 mV for 20 ms is followed by a recovery period varying from 0 to 100 ms at potentials ($V_{recovery}$) from −110 to −60 mV, followed by 5-ms test pulse at −10 mV, 5-s interpulse interval. Right: $I_f$ recovery current trace (note incomplete recovery over 100 ms, arrow and dashed line). B: normalized peak sodium current plotted against the recovery period at recovery potentials from −110 to −60 mV. C: recovery curves at different potentials in B were each fitted with a single-exponential function, and the resultant time constants are plotted against the corresponding recovery potentials (n = 5 cells in each group, mean ± SE). D–F: extended recovery period shows fast and intermediate components. D: modified double-pulse protocol similar to that shown in A was used with an extended recovery period (Δt) to 400 ms at −60, −70, and −90 mV to identify slower components. E: peak sodium amplitude normalized and plotted against recovery period at potentials −60, −70, and −90 mV. F: the resultant time constants of recovery from intermediate inactivation ($I_i$) and $I_f$ are plotted against the corresponding recovery potentials (left axis, circles and squares). Right axis: % of $I_i$ component amplitudes are plotted against recovery potentials (triangles) (n = 6 for −70 mV, n = 4 for −80 mV, and n = 7 for −100 mV; mean ± SE), and 75% recovery ($R_{75}$) is plotted against recovery potential (inverted triangles); 5-s interpulse intervals for all protocols. Test pulses were normalized to first pulse amplitude.](http://jn.physiology.org/doi/10.1152/jn.01000.2014/ www.jn.org)
Steady-State Inactivation of $I_F$

The steady-state inactivation relation ($h_{ss}$) for $I_F$ was measured with a 50-ms conditioning pulse (i.e., sufficiently long to reach equilibrium), followed by short depolarizations to $-10$ mV. This was parameterized by a Boltzmann distribution of the form

$$h_{ss} = \frac{A}{1 + e^{-\frac{V-V_s}{k}}}$$

where $V$ is the command potential (mV), $V_s$ is the half-inactivation potential, and $k$ is the slope. A half-inactivation potential ($V_{0.5}$) of $-54.8 \pm 1.1$ mV and a $k$ of $9.9 \pm 0.3$ mV were observed ($n = 5$ cells; Fig. 3B).

Slow Inactivation

Steady-state inactivation with long conditioning pulses. Depolarizations to $-10$ mV of 10-s duration followed by a test pulse to $-10$ mV after repolarization for 100 ms to $-90$ mV revealed considerable ($\sim 80\%$) attenuation of $I_{Na}$, indicating the presence of “slow” inactivation (Fig. 4 and Fig. 8).

An $h_{ss}$ relation was measured with 10-s conditioning pulses to assess the equilibrium distribution for channels affected by $I_S$. Boltzmann fits revealed $V_{0.5}$ of $-66.7 \pm 0.7$ mV and $k$ of $6.1 \pm 0.3$ mV ($n = 5$ cells), showing a significant hyperpolarizing shift compared with the 50-ms $I_F$ relation (Fig. 3B). Further analysis revealed that the 10-s $h_{ss}$ relation was better parameterized by a sum of two Boltzmann functions of the form

$$h_{ss} = \frac{A}{1 + e^{-\frac{V-V_s}{k}}} + \frac{1-A}{1 + e^{-\frac{V-V_s}{k}}}$$

consistent with more than one inactivation process. The double Boltzmann form was preferred over the conventional single form by testing $F$ statistical significance, setting $P = 0.05$, $F = 6.6$. $A$ is the proportion of the larger component of the normalized conductance, $V_{0.5} = -77.3 \pm 2.2$ mV and $V_{0.5}' = -63.1 \pm 1.7$ mV are the two half-inactivation potentials, and slope factor $k = 4.7 \pm 0.8$ mV.

Transition rates and voltage sensitivity of slow inactivation at depolarized potentials. The time course of onset of $I_S$ was measured by performing 10-s depolarizations from $-90$ mV to test potentials between $-30$ and $+10$ mV with 5-ms test pulses to $-10$ mV at 1-s intervals to measure the proportion of channels in the slow inactivated state as a function of time and voltage (Fig. 4A). The test pulses were preceded by 100-ms repolarizations to $-90$ mV to allow $I_F$ recovery. Two exponential components were identified, with a shorter “intermediate” component of several hundred milliseconds and a slow component of the order of seconds. The slow component was not voltage sensitive over this range, with values of $5.7 \pm 0.5$ s and $5.9 \pm 0.7$ s at $-30$ and $+10$ mV, respectively. The faster components were $0.6 \pm 0.1$ s at both $-30$ and $+10$ mV, with the slower component relative amplitudes of $\sim 53\%$ and $71\%$ at the same potentials (Fig. 4C). Later experiments used shorter initial intervals to better characterize the faster component corresponding to the “intermediate” process of interest.

Recovery from $I_S$. Recovery from $I_S$ was measured after 10-s depolarizing pulses to $-10$ mV. Five-millisecond test depolarization to $-10$ mV at 1-s intervals tracked the time course of recovery at a series of potentials from $-60$ to $-110$ mV (Fig. 4, D–F). The time to 75% recovery ($R_{75}$) was shorter at hyperpolarized potentials with values of $264 \pm 90$ ms at $-110$ mV and $1,170 \pm 247$ ms at $-60$ mV ($n = 4$ or 5). In most instances, the recovery curve could be fitted with two exponential components. It should be noted that the sampling intervals in these initial experiments were not optimized for quantifying the faster component.

Identification of an Intermediate-Duration Inactivation Process “$I_I$”

The presence of a slower time constant in the $I_F$ recovery experiments as well as a faster component of both onset and recovery from $I_S$ (Fig. 4, C, E, F) suggested a separate intermediate-duration process, so this was examined with more specific protocols.

The recovery from 20-ms depolarizations was repeated with longer recovery intervals ranging from 400 to 700 ms (Fig. 2, D–F). Forty-two incremented pulse durations were used, with shorter intervals in the initial recovery period to resolve faster time constants. Sodium channel availability was then measured by a 5-ms test pulse to $-10$ mV. A rapid recovery phase corresponding to fast inactivation was observed (Fig. 2, E and
F), as noted above, but a longer component was clearly evident with time constants of 162 ± 25 and 93 ± 10 ms at −90 and −60 mV, respectively, comprising ~20% of the total amplitude. $R_{55}$ became faster with hyperpolarization, and the relative proportion of fast and intermediate components varied with recovery potential (Fig. 2F).

To investigate whether a similar intermediate component was evident at depolarized potentials after channel opening, such as would occur during prolonged repetitive firing, a multipulse protocol with either 5- or 10-ms depolarizations to −10 mV with 25-ms interpulse interval was employed (Fig. 5A). With sequential pulses, the peak current was observed to decay with a time constant of duration comparable to those observed in the recovery experiments above [218.4 ± 28.3 ms at −30 mV ($n = 5$ cells), 232.0 ± 49.8 ms at 10 mV ($n = 3$ cells)], comprising ~22% of the total amplitude of current decay at both potentials (Fig. 5B). It should be noted that the decay rates derived from the multipulse protocols may not reflect the exact time constants of the intermediate process, as they are a composite of inactivation onset and recovery.
Variable-duration transition interval pulses (Fig. 7, A and B) over a 700-ms interval followed by a 5-ms test pulse to −10 mV revealed the rates of transition to inactivated states. Again, an intermediate-duration component was evident, with time constants of 163.3 ± 30.2 ms (n = 3 cells) at −70 mV and 158.1 ± 26.8 ms (n = 5 cells) at −60 mV, comprising ~40% and 53% of the current decay at the same potentials respectively (Fig. 6, B and C). A fast component corresponding to $I_F$ was also present.

Transitions out of closed-inactivated states were examined with a similar protocol, with hyperpolarization to potentials from −60 to −110 mV from a holding potential of −50 mV (Fig. 6D). A slower component of inactivation recovery (183.0 ± 55.0 ms at −60 mV, 214.9 ± 15.0 ms at −110 mV, n = 5 cells) was again identified in addition to fast inactivation recovery, composing approximately 40% and 45% of the total current recovered (Fig. 6, E and F), that is, about double the proportion seen with recovery after depolarization and inactivation through open states.

Comparison of $I_F$ recovery from closed-state inactivation and open-state inactivation. Recovery of $I_F$ and $I_R$ was next measured from two initial potentials, −10 mV, favoring inactivated states reached via the open state, and −60, favoring CSI transitions (Fig. 6G), in the same neuron. $I_R$ time constants were considerably faster at −100 mV from −20 mV compared with −70 mV (148.4 ± 31.0 vs. 702.3 ± 194.7 ms, respectively; Fig. 6H). The effect was distinct in individual cell recordings and not an artifact of variability in recovery time constants.

**Steady-state Inactivation Curve Using Conditioning Pulses with Durations Comparable to Intermediate Inactivation**

As intermediate time constants of transition between inactivated states had been identified, the effect of a longer (500 ms) conditioning pulse of the $h_m$ relation was tested. The $V_{0.5}$ was found to be hyperpolarized by −9 mV ($V_{0.5} = −63.0 ± 0.6$ mV) compared with the 50-ms protocol ($V_{0.5} = −54.8 ± 1.1$ mV), consistent with the longer pulses recruiting channels into a separate prolonged-latency state (Fig. 3B). This change in $V_{0.5}$ was reversed by repeating the original 50-ms protocol. Additionally, the 500-ms $h_m$ relation was better fitted by a double Boltzmann distribution but the 50-ms protocol relation by a single Boltzmann formula. The slope factors were also notably different, 9.9 ± 0.3 and 6.8 ± 0.3 for the 50- and 500-ms protocols, respectively.

Fig. 4. Slow inactivation ($I_F$): at low temporal resolution $I_F$ onset and recovery displayed 2 components. A, left: $I_F$ onset protocol. $V_h = −90$ mV; 10-s-long depolarizations to 0, −10, and −30 mV were punctuated each by a 100-ms repolarization to −90 mV to allow $I_F$ recovery and assess rate of transfer into slower inactivated states. Right: gradual attenuation of sodium current ($I_F$) with successive test pulses with onset of $I_F$. $I_R$: normalized peak $I_R$, plotted against prepulse duration. Prepulse potentials of −30 mV, −10 mV, and +10 mV are shown in black, red, and blue, respectively. C, left: data in A fitted with double-exponential functions and resultant time constants are plotted against prepulse potentials, a slow component order 10^3 ms, and an “intermediate” component order 10^1 ms. The curves were best described by double-exponential functions (using F statistic). Right: relative amplitude as % of intermediate time constant amplitude plotted against prepulse potentials. (n = 7–9 cells at each potential, mean ± SE). D, left: $I_F$ recovery protocol. $V_h = −90$ mV. A 10-s depolarization to −10 mV was followed by recovery at potentials from −110 to −60 mV with 10-ms depolarizations to 0 mV at 1-s intervals to track recovery. Right: inward sodium current in response to the test pulses. E: peak sodium current of test pulses normalized and plotted against total time elapsed from the first prepulse. Recovery potentials of −110, −90, −70, and −60 mV are shown in black, red, blue, and green, respectively. F, left axis: data in E fitted with double-exponential function and the resultant time constants plotted against recovery potentials. Right axis: % amplitude of $I_F$ time constant plotted against recovery potentials. $G$: the $I_F$ protocol with 1-s intervals was augmented with four 250-ms intervals in the first second to better identify the intermediate component. H: entry into inactivated states (onset) from $V_h = −90$ mV to −10 mV over 10 s revealed 2 time constants of 125 and 4,700 ms. $I_F$ recovery from inactivated states (offset) at −90 mV from a 10-s depolarization to −10 mV with similar time interval protocol again revealed dual time constants of 150 and 2,000 ms (n = 7 for onset, n = 5 for offset, mean ± SE). The amplitude ratios of the $I_F$ components are 46.4 ± 4.3% and 22.3 ± 6.8% for onset and offset, respectively.

**Fig. 5.** Trains of depolarizations revealed an “intermediate”-duration component of current attenuation. A, left: trains of 5-ms depolarizations ranging from −30 to +10 mV with 25-ms interpulse interval over a total 1-s period. Right: sodium currents produced by the multipulse protocol showing gradual reduction in amplitude. B, right axis: intermediate time constant over a potential range of −30 to +10 mV. Left axis: $I_f$ component % amplitude (n = 3 for +10 mV, n = 5 for −0 and −30 mV, n = 4 for −10 and −20 mV, mean ± SE).

**Transitions Between Closed States Related to Intermediate Inactivation**

We next examined whether a similar intermediate-duration component of inactivation could be identified with transitions between closed states, that is, at potentials hyperpolarized to channel opening threshold potentials. This may be termed “closed-state inactivation” (CSI; Bahring and Covarrubias 2011) as distinguished from “open-state inactivation” (OSI) resulting after depolarization-activated opening and subsequent inactivation. Double-pulse experiments were performed utilizing variable time interval protocols over several hundred milliseconds. In one set of experiments, transitions into inactivated states from a holding potential of −90 mV to test potentials from −50 to −70 mV were characterized (Fig. 6A).
Fig. 6. Closed-state inactivation (CSI) transitions between closed-inactivated (CI) and closed (C) states at different potentials and comparison between CSI and open state inactivation (OSI) rates. 

**A**. Left: protocol to assess time course of inactivation onset from −90 mV to 3 different potentials (−70, −60, and −50 mV) at intervals up to 700 ms. Right: $I_{Na}$ amplitude attenuation with protocol on left, −90 mV to −60 mV transition. 

**B**. Normalized $I_{Na}$ amplitude vs. interpulse interval. 

**C**. Left axis: current amplitudes in B fitted with biexponential functions, showing both a fast component corresponding to IF as well as an intermediate component at all potentials. Right axis: IF component % amplitude plotted against transition potentials from −70 to −50 mV (n = 3 for −70 mV, n = 5 for −60 and −50 mV, mean ± SE). 

**D**. Left: protocol to assess closed-inactivated state to closed state transitions. Right: inward sodium current trace in response protocol on left (−50 mV to 100 mV). 

**E**. $I_{Na}$ normalized against interpulse interval at different potentials. 

**F**. Time constants of IF and II components from E. Right axis: % amplitude of I$_{II}$ (n = 5 for each group, mean ± SE). 

**G** and **H**: IF and II components with OSI (red) and CSI (blue) protocols. Fast time constants were not significantly affected, whereas intermediate time constants were prolonged with CSI protocol (n = 8, mean ± SE); 5-s interpulse intervals between test pulses, which were normalized to first pulse amplitude.

**J Neurophysiol** • doi:10.1152/jn.01000.2014 • www.jn.org
Observation of $I_{II}$ and $I_{IS}$ with Augmented Double-Pulse Protocols

Another set of experiments was performed to identify $I_{II}$ and $I_{IS}$ with a double-pulse protocol encompassing previously observed intermediate and slow timescales. The original 10-s-long $I_{IS}$ onset and offset protocols with 1-s test intervals were modified by adding initial test pulses at 250-ms intervals (Fig. 8). This allowed better characterization of the faster component. A clear biexponential onset and offset of inactivation similar to $I_{II}$ and $I_{IS}$ identified previously were observed, with intermediate inactivation time constant ($\tau_{II}$) and slow inactivation time constant ($\tau_{IS}$) of $150 \pm 30$ ms and $4,700 \pm 300$ ms at $-10$ mV (Fig. 4H).

Effects of Intracellular Papaine on Inactivation

Cytoplasmic application of proteolytic enzymes results in loss of $I_{IF}$ of $I_{Na}$ in several preparations (Armstrong et al. 1973; Goni and Hille 1987). This technique was therefore used to attempt directly observe slower inactivation processes revealed by the double-pulse protocols. Seventeen minutes after whole cell configuration was formed with 0.5 mg/ml papaine included in the patch pipette solution, $I_{Na}$ was almost completely suppressed (Fig. 7A), with little current decrement over 20 ms. With depolarizations for periods comparable to the intermediate and slow time constants (2.5–10 s), $I_{Na}$ decayed with two exponential components with time constants typically of 250 ms and 1,200 ms (Fig. 7B). Similarly, the $h_{Na}$ relation was almost completely suppressed with short conditioning pulses but was similar in form to the untreated neurons with 500-ms duration conditioning pulses, consistent with slower inactivation components being preserved with the proteolytic treatment (Fig. 7C).

Fig. 8. Typical pattern of attenuation of sequential action potentials (APs) indicative of a role of the $I_{II}$ process. In a neuron with potassium and calcium currents blocked, a delayed-rectifier current was introduced with a dynamic clamp protocol, reducing the AP duration to $5 \text{ ms}$ from 30–100 ms without dynamic clamp. In the voltage range between $60$ and $70$ mV repetitive firing could be initiated by depolarizing current injection. The second AP (*) is attenuated as expected from enhanced $I_{IF}$ at the depolarized interspike interval identified by the dashed line. Subsequent APs (**) and (***) are further attenuated, despite the interspike membrane potential remaining very close to $70$ mV, which is unexpected if only $I_{IF}$ was active in this voltage range. Subsequent spontaneous hyperpolarizations (solid and dashed line arrows) restore the AP amplitude (# and ##), likely resulting from recovery of $I_{Na}$ from $I_{IF}$ in a steep region of the $h_{Na}$ curve. B: Markov state model of $I_{II}$: 2 closed states (C1, C2) connect to a single open state (O) with second-order “m2” kinetics and integer ratios between states, connected to the fast inactivation state $I_{IF}$. Closed state transitions are permitted between $I_{IF}$ and C1, C2 is connected to C1, O, and $I_{IF}$ with the same voltage-sensitive parameters ($a_{II}$, $b_{II}$), unadjusted for the slower transitions between closed states found experimentally (see Fig. 6).
Dynamic clamp observations of accommodation of action potentials. Action potentials in dissociated pyramidal neurons (n = 4 cells) were recorded in dynamic current-clamp mode with a simulated noninactivating potassium conductance to reduce the duration of the action potentials (typically >100 ms with potassium channel blockade) close to the physiological range and to provide a relatively stable resting membrane potential. The purpose of these experiments was to determine whether an effect on cell firing attributable to I_t could be observed. Low-amplitude hyperpolarizing current was used to...
bring the membrane potential into the physiological range, typically -50 to -70 mV. Repetitive firing could be induced by reducing the holding current slightly or by superimposed step depolarizing currents (0.1–0.5 nA). Action potential amplitude was closely related to the potential at which it was initiated, as expected from the $I_F$ steady-state inactivation curve, and qualitatively varied at time intervals that would be expected from $I_F$ kinetics. With both step depolarizations and spontaneous repetitive activity, sequential action potentials arising from a constant interspike potential were observed to attenuate over time periods commensurate with $I_F$ kinetics derived from the voltage-clamp studies as shown in Fig. 8A, consistent with a physiological role for $I_F$ in such accommodation. A similar pattern was observed in neurons where the dynamic clamp was not activated, although with much longer action potentials (data not shown).

**Model of $I_I$**

As the time constants and $h_w$ relation were distinct from $I_S$, a five-state Markov model was derived with two closed states, one open and separate states corresponding to $I_F$ and $I_I$ (Fig. 8) without $I_S$ incorporated. The simplest topology compatible with the experimental observations was made with $I_I$ connected to all states except $C_2$.

With the $h_w$ curve and time constants derived from double-pulse experiments, forward and backward rate constants $\alpha_i$ and $\beta_i$ were determined expressed by the following equations:

\[
\alpha_i = 0.04 \times 1 \left/ \left[1 + \exp\left((-V + 100)/11\right)\right]\right.
\]

\[
\beta_i = 0.0044 \times 1 \left/ \left[1 + \exp\left((-V + 83.7)/13.14\right)\right]\right.
\]

With this model, the negative shift in $h_w$ curve with 500-ms conditioning pulses, intermediate components of entry into and recovery from $I_F$, multipulse inhibition (Fig. 9, A and B) and the intermediate component of the papaine-treated cells could be reproduced (Fig. 9H). Additionally, action potential accommodation in current clamp was reproduced (Fig. 9C). A comparison of this model with and without $I_I$ was made with phase plane plots (Fig. 9, D and F). With $I_I$ present, accommodation occurred so that repetitive firing attenuated with membrane potential, membrane potential trajectories degenerating to fixed points (FP, Fig. 9D), whereas the removal of $I_I$ in the equation manifested the stable membrane potential to limit cycles (LC, Fig. 9F), i.e., nonaccommodating action potential firing.

**DISCUSSION**

In this study a form of sodium channel inactivation with time constants of intermediate duration compared with previously identified $I_F$ and $I_S$ has been identified and termed $I_I$.

$I_F$ and $I_S$ properties were similar to those reported by Kuo and Bean (1994b); although it is reported in that paper that $I_S$ not present at -70 mV, the 50-ms- and 10-s steady-state inactivation curves in the present study continue to diverge up to -90 mV (Fig. 3B), suggesting significant occupancy over a larger range of potentials. $I_I$ was similar in time course to an intermediate component reported by Chen et al. (2000) in rat skeletal muscle as well as Zilberter and Motin (1991) and Benitah et al. (1999) in cardiac sodium channels. Removal of $I_F$ with papaine revealed biexponential inactivation with components corresponding to $I_I$ and $I_S$. The larger question of whether more complex behavior such as fractal or scaled rates is occurring (Marom 2010) is not addressed, but the Markov formulation is likely to be at least a reasonable practical approximation.

It is unlikely that $I_I$ was an artifact resulting from recording from a Na$_v$ subtype, as a similar component has been seen in heterologous expression systems (Rush et al. 2005; Zeng and French 2013).

Changes in the half-inactivation and slope of the steady-state inactivation curves when the conditioning pulse was increased from 50 to 500 ms were also consistent with the recruitment of an additional inactivation process with time constants of the same order as observed by the double-pulse and papaine experiments. The negative shift was not due to time-dependent shifts that are sometimes seen in whole cell recordings of sodium currents (Fozzard et al. 1986), as it was reversible by repeating shorter conditioning pulses. Additionally, separate experiments looking for this effect found no change over the 17-min observation time, generally within the time periods used for experimental observations. This time sensitivity of the steady-state inactivation curve may have implications more generally for studies of sodium channel inactivation, as 500-ms $h_w$ conditioning pulses are often considered to specifically recruit only $I_F$ (e.g., Sheets et al. 2008).

**Comparison with Previous Reports of Slow Inactivation in Sodium Conductances**

Several previous reports (Colbert et al. 1997; Goldfarb 2012; Jung et al. 1997; Katz et al. 2009; Mickus et al. 1999) have identified a slower inactivation process in central neurons. Some of these observations overlap with the intermediate process described here, and others are more consistent with conventional $I_S$ over seconds (Kuo and Bean 1994b). All imply a single $I_S$ process but interestingly have found unusual properties such as paradoxically fast entry into slow inactivated states (Goldfarb et al. 2007; Jung et al. 1997; Mickus et al. 1999) and may coincide with or contribute to $I_I$ described here. In particular, $I_I$ may overlap with the slow recovery process.
identified by Goldfarb et al. (2007) but would seem to be substantially different in regard to rate of onset as well as significant closed-state transitions. Carr et al. (2003) note a 200-ms inactivation time constant in cortical neurons but ascribe this to \( I_F \)—the retention of the intermediate component after enzymatic removal of \( I_F \) suggests this is not the case in hippocampal CA1 neurons. Use of intact neurons in brain slice in several studies may have hindered precise identification of inactivation components, as space clamp and the range of pulse protocols available are limited in this preparation. Park et al. (2013) have described \( I_{NAS} \), a subthreshold component of \( I_{Na} \) that inactivates with time constants similar to the range of \( I_I \) described here in nucleated nuclear patches from rat CA1 neurons, but the range of time constants appears to overlap \( I_S \) and \( I_F \) and have different voltage sensitivity. \( I_I \) is conceptually quite different from \( I_{NAS} \) in that it is an inactivation process rather than a current and that it is active at most potentials rather than just around threshold. It is possible that \( I_{NAS} \) may be a manifestation of the persistent sodium current inactivating through \( I_I \).

Another possible reason for diversity of time constants of slower components of inactivation is the possibility of developmental variability. \( I_F \) has been observed to develop slow components in rat neocortical and hippocampal neurons (Fernandes et al. 2001; Huguenard et al. 1998) postnatally, and Carlin et al. (2008) have seen changes in \( I_o \) of sodium currents in motor neurons over 12 days postnatally. It would be useful to more specifically examine the effects of age on the intermediate and slow components described in this study.

**Difference Between Recovery of Open-State Inactivation and Closed-State Inactivation**

The time constants of recovery from \( I_I \) were substantially slower from closed-state inactivated than from open-state inactivated channels. We are not aware of this phenomenon being reported previously in \( NaV \) channels, but recent studies on potassium channels have proposed that different conformational states are involved with OSI and CSI (Bahring and Covarrubias 2011). It is known that OSI results in significant motion of S4 domains (Bezanilla 2008a) and likely interaction with inactivation structures (Kuo and Bean 1994a; Patlak 1991). We suspect that differences in conformational energies between the two states result in different rate constants.

**Model**

The Markov model reproduced the shift in \( h_{a1} \) curves with pulse duration and onset and offset of \( I_F \) as well as the papain effect. It also reproduced the dynamic clamp data showing action potential attenuation resulting from \( I_I \). The differences in rates between OSI and CSI have not been incorporated in his model but could be readily accommodated in the \( I_F \leftrightarrow I_I \) branch rate constants. It could be augmented simply with a conventional slow inactivated state in parallel with the intermediate state.

The dynamic clamp experiment aimed to explore the possibility that \( I_F \) may have effects on action potential firing; it would also be interesting to do a full dynamic clamp simulation with the \( NaV \) Markov model with \( I_I \) incorporated to more thoroughly explore the dynamics of this system and compare it with intact neuronal firing patterns. Because of the rapid kinetics of \( I_{Na} \), software with better capabilities for fast current incorporation, such as RTXI (Bettencourt et al. 2008) or QUB (Miles et al. 2008), are likely to be needed.

The model attempts to minimize kinetic complexity of sodium channel gating, but allowing some flexibility in assignment of the number of states and connectivity. A minimum of two closed states would seem necessary from the study of Baranauskas and Martina (2006) and previous observations in this laboratory (French 2004), and \( I_F \) has been modeled as a single state. All transitions were made voltage dependent for simplicity, although \( I_F \) could almost certainly be made voltage insensitive (Armstrong and Bezanilla 1977; Marom and Abbott 1994). Experimental results suggested connectivity between all states to the intermediate state, with transitions from the open state being rare without inactivation removal. Previous models by Migliore and Fleidervish use an extra variable in the HH equations for the slower inactivation process (Migliore 1996). The model of Migliore is distinctly different from the data from this study, showing time constants of \( \sim 50 \) ms at \( -100 \) mV but dropping to microseconds near 0 mV. Apart from coalescing the timescales of slower inactivation, these models necessarily imply four extra states and impose several constraints on state topology and rate constant ratios (see Hille 1992. p. 488). The Menon et al. (2009) model uses a novel modifiable state topology method to reproduce two components of inactivation, “fast” and a single “delayed” or slow process using a single state for the delayed component, but interestingly incorporates an extra “transition state” with long occupancy that might overlap with \( I_F \). Additionally, there is no connectivity between closed and delayed inactivation states, which is discordant with the present study and that of Vedantham and Cannon (1999). This is a particularly significant difference given the substantial \( I_F \) component in CSI as well as the preservation of the \( h_{a1} \) relationship with papain treatment (Fig. 9C).

**Functional Relevance**

\( I_I \) is likely to have a role in accommodation of action potential firing as suggested by the dynamic clamp experiments, as well as acting to stabilize hyperexcitability states such as epilepsy. It may also contribute to the failure of back-propagation into dendrites (Colbert et al. 1997; Mickus et al. 1999). \( I_I \) could also be a target for drug modulation of sodium channel availability. Khodorov and Shishkova (1976) and Kambaris et al. (1998) have suggested that some local anesthetics act on cardiac \( NaV \) channels through a component of inactivation similar to that presented here. Like these studies with lidocaine, we have observed a shift in the steady-state inactivation curve for \( I_F \), but not \( I_F \), with phenytoin (Zeng and French 2011) suggesting a potential role for this process. Park et al. (2013) have also observed an effect of hexanol and riluzole on a component of decay of hippocampal sodium currents, which, as noted above, may overlap with \( I_F \).

In summary, we have identified an inactivation process of hippocampal sodium currents with time constants of \( 10^2 \) ms that is likely to be associated with important physiological phenomena. This process appears to be separate from conventional fast inactivation and a previously identified “slow” form of inactivation with time constants of seconds but overlaps the time course of inactivation of seen in several previous studies. It will be of interest to perform site-directed mutagenesis of
inactivation-related structures in Na⁺ in order to identify key amino acid residues involved in this process. It will also be of interest to examine possible modulatory effects of drugs, pH, and ionic concentrations on this component of inactivation.

GRANTS

This work was supported by Brain Foundation (Australia), Royal Melbourne Hospital (RMH) Neuroscience Foundation, Bickart Fellowship (C. R. French), and University of Melbourne, Haynes Fellowship (C. R. French).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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