Novel description of ionic currents recorded with the action potential clamp technique: application to excitatory currents in suprachiasmatic nucleus neurons

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Clay JR. Novel description of ionic currents recorded with the action potential clamp technique: application to excitatory currents in suprachiasmatic nucleus neurons. J Neurophysiol 114: 707–716, 2015. First published June 3, 2015; doi:10.1152/jn.00846.2014.—The traditional method of recording ionic currents in neurons has been with voltage-clamp steps. Other waveforms such as action potentials (APs) can be used. The AP clamp method reveals contributions of ionic currents that underlie excitability during an AP (Bean BP. Nat Rev Neurosci 8: 451–465, 2007). A novel usage of the method is described in this report. An experimental recording of an AP from the literature is digitized and applied computationally to models of ionic currents. These results are compared with experimental AP-clamp recordings for model verification or, if need be, alterations to the model. The method is applied to the tetrodotoxin-sensitive sodium ion current, $I_{\text{Na}}$, and the calcium ion current, $I_{\text{Ca}}$, from suprachiasmatic nucleus (SCN) neurons (Jackson AC, Yao GL, Bean BP. J Neurosci 24: 7985–7998, 2004). The latter group reported voltage-step and AP-clamp results for both components. A model of $I_{\text{Na}}$ is constructed from their voltage-step results. The AP clamp computational methodology applied to that model compares favorably with experiment, other than a modest discrepancy close to the peak of the AP that has not yet been resolved. A model of $I_{\text{Ca}}$ was constructed from both voltage-step and AP-clamp results of this component. The model employs the Goldman-Hodgkin-Katz equation for the current-voltage relation rather than the traditional linear dependence of this aspect of the model on the Ca$^{2+}$ driving force. The long-term goal of this work is a mathematical model of the SCN AP. The method is general. It can be applied to any excitable cell.

action potential clamp; suprachiasmatic nucleus neurons; mathematical models; Goldman-Hodgkin-Katz

IN THEIR ORIGINAL WORK, Hodgkin and Huxley (1952) used rectangular voltage-clamp steps to construct models of the ionic currents underlying excitability in squid giant axons. The advantage of this approach is that it allows the investigator to control the primary independent variable of an excitable membrane, the membrane potential $V$, and to make predetermined changes in that variable to activate the primary dependent variable, the net membrane ionic current. Voltage steps also short-circuit the membrane capacitance current, at least ideally. Waveforms other than steps, such as action potentials (APs), can also be used, the so-called AP clamp technique (Bean 2007). In this approach a previously recorded AP is applied to the cell with the amplifier in voltage-clamp mode. The contributions of specific ionic currents during the AP can be determined with the use of ion channel blockers. In contrast, the dynamic clamp technique is an indirect approach in which firing of a cell in current-clamp mode is altered by changes of particular currents with simulations in real time (Sharp et al. 1993).

The primary purpose of this report is a demonstration of a novel application of the AP clamp technique. It is used here as an aid in the construction of models of ion channel conductances. Specifically, AP-clamp results provide a test of models constructed from voltage-step results. Moreover, the two sets of results together provide a richer base from which to construct a model ab initio than rectangular step results alone. The method is illustrated with results from suprachiasmatic nucleus (SCN) neurons (Jackson et al. 2004). This group recorded the tetrodotoxin (TTX)-sensitive Na$^{+}$ current ($I_{\text{Na}}$) and the Ca$^{2+}$ current ($I_{\text{Ca}}$), using voltage steps and the AP clamp for both components. A significant feature of the $I_{\text{Ca}}$ analysis is the use of the Goldman-Hodgkin-Katz (GHK) equation for the fully activated current-voltage relation of this component (Clay 2009; Goldman 1943; Hodgkin and Katz 1949). The descriptions of $I_{\text{Na}}$ and $I_{\text{Ca}}$ given below may ultimately be incorporated in a mathematical model of the AP in the SCN, the long-term goal of this work.

As noted above, the AP clamp computational method can be used to test models of ion current components including models in the literature. This feature of the analysis was applied to models of $I_{\text{Na}}$ and $I_{\text{Ca}}$ in SCN neurons (Diekman et al. 2013; Sim and Forger 2007). The $I_{\text{Ca}}$ model of Diekman et al. (2013) utilized $I_{\text{Ca}} = g_{\text{Ca}}(V - E_{\text{Ca}})$ as the fully activated current-voltage relation for $I_{\text{Ca}}$, which $g_{\text{Ca}}$ is the conductance of this component and $E_{\text{Ca}}$ is the Ca$^{2+}$ reversal potential. This approach has been traditional for models of ion channel currents beginning with Hodgkin and Huxley (1952). The $I_{\text{Ca}}$ model in this report is based on the GHK equation. The comparison of these models given below illustrates the utility of the GHK equation in the analysis of $I_{\text{Ca}}$.

METHODS

This work requires an experimental recording of an AP from an SCN neuron such as the result in the inset of Fig. 5B of Jackson et al. (2004). SCN neurons fire APs spontaneously, especially during the daytime. The waveform used was taken from a train of spontaneously occurring APs. That waveform was digitized (Fig. 1). The same result is shown with the points connected by straight lines (Fig. 1). This $V_{m}$ vs. $t$, data set ($t$ is time in milliseconds, $V_{m}$ is membrane potential in millivolts) was applied computationally to models determined from voltage steps. The procedure is illustrated by consideration of a generic ion current component, $I_{i}$. We assume that voltage-step
analysis indicates that $I_\text{Na} = g_N q^2(t)(V - E_N)$, where $g_N$ and $E_N$ are the conductance and the reversal potential of this component, respectively. The gating variable $q$ is given by
\[ dq(t)/dt = -[\alpha_q(V) + \beta_q(V)]q(t) + \alpha_q(V) \] where $\alpha_q$ and $\beta_q$ are voltage-dependent functions based on chemical reaction rate theory similar to the $\alpha$ and $\beta$ by Hodgkin and Huxley (1952) for their models of $I_{\text{Na}}$ and $I_K$ in squid axons. Note that $q$ is raised to the second power in Eq. 1, a degree of complexity that allows a good fit by eye to a family of $I_{\text{Na}}$ and $I_K$ in squid axons. They observed that both components exhibited a sigmoidal time-dependent activation following voltage steps—a delay—that they modeled by raising the activation gating variables $n(t_K)$ and $m(t_{\text{Na}})$ to a power $-n^3$ for $I_K$ and $m^3$ for $I_{\text{Na}}$. We assume that raising $q$ to the second power is sufficient for this result for $I_\text{Na}$. We further assume that the values of the expressions for $\alpha_q$ and $\beta_q$ (Eq. 1) were adjusted so that the model provided a good fit by eye to a family of $I_{\text{Na}}$ records, a procedure similar, again, to the approach of Hodgkin and Huxley (1952) in their analysis of $I_{\text{Na}}$ and $I_K$. The $V$ vs. $t$ data set in Fig. 1 can be applied to the $I_\text{Na}$ model in AP clamp, computationally, with software routines such as the ones in Mathematica (Wolfram Research, Champaign, IL). The start point of the AP in Fig. 1 is $t_0 = 0$, $V_0 = -56.7$ mV. If $V_0$ is below the activation range of $I_{\text{Na}}$, the start value of the gating variable $q$ is given by its steady-state value $q = q_0 = \alpha_q(V_0)/(\alpha_q(V_0) + \beta_q(V_0))$. The next iterative value of $q$, $q_1$, is determined from Eq. 1 with NDSolve (Mathematica), using $V(t) = V_0 + (V_i - V_0)(t - t_0)/(t_1 - t_0)$ for $t_0 < t < t_1$ ($V_i = -56.1$ mV; $t_1 = 0.44$ ms). This procedure is continued throughout the $V$ vs. $t$ data set of the AP waveform. The resulting digitized values of $I_\text{Na}$ can be determined according to $I_{\text{Na}} = g_N q^2(V - E_N)$. This result is compared with an experimental AP-clamp recording of $I_{\text{Na}}$ obtained by applying the waveform in Fig. 1 to the cell before and after the addition to the bathing medium of a blocker of $I_{\text{Na}}$, assuming such a blocker exists. The difference between the two results gives the time course and amplitudes of $I_\text{Na}$ during the AP. If the comparison between theory and experiment for $I_\text{Na}$ is satisfactory, AP-clamp analysis would be merely confirmatory of the model obtained from rectangular steps. On the other hand, if significant differences are found, an attempt would be made to modify the model by changing $\alpha_q$ and $\beta_q$ and perhaps also the gating scheme from $q^2$ to, for example, $q^3$, so as to provide a good description of both AP-clamp and voltage-step results.

The other part of the $I_\text{Na}$ model, the fully activated current-voltage relation, $g_N(V - E_N)$, may also require revision. Many ion channel current components have a nonlinear dependence on driving force, which in some instances, $I_{\text{Ca}}$ for example, is well described by the GHK equation (Clay 2009; McCormick and Huguenard 1992). The GHK equation is used in the $I_{\text{Ca}}$ results given below.

**RESULTS**

$I_{\text{Na}}$ component: voltage-step results. The first part of the analysis is the development of a model based on voltage-step recordings, in this case the $I_{\text{Na}}$ results in Fig. 8, B and C, of Jackson et al. (2004). A model widely used throughout neurosciences including the results of Engel and Jonas (2005) on $I_{\text{Na}}$ in hippocampal mossy fiber boutons is that of Hodgkin and Huxley (1952), $I_{\text{Na}} = g_{\text{Na}} m^3 h(V - E_{\text{Na}})$, where $m$ refers to channel activation, $h$ refers to inactivation, and $E_{\text{Na}}$ refers to the reversal potential for Na$^+$ ($E_{\text{Na}} = 45$ mV). The $m$ and $h$ variables are determined by
\[
\frac{dm}{dt} = -[\alpha_m(V) + \beta_m(V)]m + \alpha_m(V);
\frac{dh}{dt} = -[\alpha_h(V) + \beta_h(V)]h + \alpha_h(V)
\] (2)
The $\alpha$ and $\beta$s in Eq. 2 for squid axons do not provide a good description of the $I_{\text{Na}}$, rectangular step results of Jackson et al. (2004). The start point here is the equations of Engel and Jonas (2005):
\[
\alpha_m(V) = -93.8(V - 105)/\{\exp[ -(V - 105)/17.7] - 1\};
\alpha_h(V) = 0.0035\exp(-V/18.7);
\beta_m(V) = 0.17\exp(-V/23.3);
\beta_h(V) = 6.6/\{\exp[-(V + 17.7)/13.3] + 1\}
\] (3)
The $\alpha$s and $\beta$s are in units of inverse milliseconds. The above expressions were modified to give a qualitative description, by eye (Fig. 2A), for the $I_{\text{Na}}$ results in Fig. 8B of Jackson et al. (2004) as well as a description of steady-state inactivation, i.e., the $h_{\text{Na}}(V)$ curve (Huang 1993) and the $m_{\text{Na}}(V)$ curve (Fig. 8C of Jackson et al. 2004), with $h_{\text{Na}}(V) = \alpha_{\text{Na}}(V)/[\alpha_{\text{Na}}(V) + \beta_{\text{Na}}(V)]$ and $m_{\text{Na}}(V) = \alpha_{\text{Na}}(V)/[\alpha_{\text{Na}}(V) + \beta_{\text{Na}}(V)]$. The $h_{\text{Na}}(V)$ and $m_{\text{Na}}(V)$ curves are illustrated in Fig. 2B. The results in Fig. 2A correspond to $I_{\text{Na}} = 120m^3(t)(t)(V - 45)$, with $I_{\text{Na}}$ in picoamperes and voltage steps $V = -53, -48, -43, -38, -33, -28, -38, -28, -18$, $-8$ mV; holding potential $= -78$ mV. B: inactivation, $h_{\text{Na}} = \alpha_{\text{Na}}(V)/[\alpha_{\text{Na}}(V) + \beta_{\text{Na}}(V)]$, and activation, $m_{\text{Na}}(V)$, with $m_{\text{Na}}(V) = \alpha_{\text{Na}}(V)/[\alpha_{\text{Na}}(V) + \beta_{\text{Na}}(V)]$. Data for the inactivation curve are from Fig. 6A of Huang (1993). The activation results are from Fig. 8C of Jackson et al. (2004). The $\alpha$s and $\beta$s are given by Eq. 4.

![Figure 1](http://jn.physiology.org/doi/10.1152/jn.00846.2014/1)
and $-8\text{ mV}$; holding potential $= -78\text{ mV}$. The $\alpha$s and $\beta$s obtained from this analysis, modified from Eq. 3, were

$$\alpha_m(V) = -14(V - 88)/\{\exp[-(V - 88)/17.7] - 1\};$$
$$\beta_m(V) = 0.026\exp[-(V + 12)/8];$$
$$\alpha_h(V) = 1.2 \times 10^{-4}\exp[-(V + 7)/8];$$
$$\beta_h(V) = 2.3/\{\exp[-(V + 24.7)/13.3] + 1\} \ (4)$$

The expressions in Eq. 4 were used for the voltage-step results (Fig. 2) and the AP-clamp results that follow.

$I_{Na}$ component: AP-clamp results. Jackson et al. (2004) applied the AP in Fig. 1 (shown again in Fig. 3, top) to an SCN neuron in voltage clamp before and after the addition of TTX to the bathing medium. The difference current is shown in Fig. 3, bottom left (dashed line in Fig. 3, bottom) obtained with the procedure described above (METHODS) provides a good description of experiment, other than a slight discrepancy near the peak of the AP (arrow in Fig. 3, bottom). This result was obtained without modifications in the model constructed from voltage-step results (Eqs. 2 and 4).

The AP-clamp result described above illustrates the behavior of inactivation, $h(t)$, and activation, $m(t)$, during repolarization of the AP (Fig. 4A). Inactivation rapidly goes to completion ($h = 0$) close to the peak of the AP [Fig. 4A, middle, $h(t)$ at bottom left]. Before this occurs, $h$ and $m$ (which is effectively 1 at this point of the AP) have an overlap as indicated by the small shaded area (Fig. 4A, middle) resulting in $I_{Na}$ in Fig. 4A, bottom. If $h(t)$ went to 0 even more rapidly during this phase of the AP, the discrepancy between theory and experiment indicated above (Fig. 3) would be reduced, or perhaps eliminated. Attempts to accomplish this result without modifying other aspects of the results were unsuccessful. Activation, $m(t)$, rapidly changes from 1 to 0 during the midportion of repolarization (Fig. 4A) just before inactivation begins to recover from 0. Consequently, no additional overlap of $h(t)$ and $m(t)$ occurs throughout the remainder of the AP (Fig. 4A).

The above results are compared with a similar analysis of the Hodgkin and Huxley (1952) model with their original expressions for the $\alpha$s and $\beta$s for $I_{Na}$ (Fig. 4B legend). Inactivation in their model does not go to completion during the AP. Moreover, $m(t)$ does not return to 0 as early in the AP as is the case in the SCN $I_{Na}$ analysis. For both reasons, $h(t)$ and $m(t)$ overlap considerably during repolarization, which leads to a significant $I_{Na}$ especially since the driving force for Na$^+$ increases as the membrane potential travels down the repolarization phase of the AP. Indeed, peak $I_{Na}$ during the AP is twice as large as the peak $I_{Na}$ during the upstroke phase (Fig. 4B, bottom). The analysis in Fig. 4B appears to be valid for APs in squid giant axons (Clay 2013). As a number of groups have noted, squid giant axon APs are not energetically efficient since $I_K$ must overcome $I_{Na}$ during the AP to return the membrane potential to rest (Alle et al. 2009; Carter and Bean 2009; Crotty et al. 2006; Sengupta et al. 2010). This inefficiency does not appear to have adverse consequences for squid since the giant axon triggers the rapid escape response of the animal, a relatively infrequent occurrence that is triggered by one or at most two or three APs (Otis and Gilly 1990). APs occur much more frequently in mammalian neurons, a result that requires significant separation in time of the $I_{Na}$ and $I_K$ components during an AP for energetically efficient signaling. This result is accomplished when the $I_{Na}$ component flows primarily during the upstroke phase of the AP, as has been shown for hippocampal mossy fiber boutons (Alle et al. 2009), cortical pyramidal neurons (Carter and Bean 2009), and SCN neurons (Jackson et al. 2004).

Alternative model for $I_{Na}$ in SCN neurons. Sim and Forger (2007) have described a model of the AP from SCN neurons. The equations for $I_{Na}$ in their work were based on the voltage-step results of Jackson et al. (2004), as was the case for the model of $I_{Na}$ described above. They used $I_{Na} = 229 m^3 h^2 (V - 45)$, with $dm/dt = (m_{\infty} - m)/\tau_{m}$, $dh/dt = (h_{\infty} - h)/\tau_{h}$, and $m_{\infty} = \{1 + \exp[-(V - 35)/2.7]\}^{-1}$, $\tau_{m} = \exp[-(V + 560)/160], h_{\infty} = \{1 + \exp[(V + 62)/5.5]\}^{-1}$, and $\tau_{h} = 0.51 + \exp[-(V - 26.6)/7.1]$. AP-clamp analysis was applied to these equations (Fig. 5). A discrepancy between theory and experiment is apparent during the latter part of the declining phase of $I_{Na}$ (arrow b in Fig. 5), similar to the results described above above.

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**Fig. 3.** AP-clamp analysis of the $I_{Na}$ model (Eqs. 2 and 4). The AP at top is the same as in Fig. 1. The experimental record at bottom is a digitized version of the $I_{Na}$ record taken from the middle panel of Fig. 5B of Jackson et al. (2004). The experimental and theoretical $I_{Na}$ results are shown superimposed below the AP. The arrow indicates a discrepancy between theory and experiment described in the text.

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**Fig. 4.** A: analysis of $m(t)$ and $h(t)$ during repolarization of the SCN AP. Inactivation is almost—but not quite—complete near the initial part of this phase of the AP, resulting in an overlap of $m(t)$ and $h(t)$ as indicated by the small shaded area, middle. The $I_{Na}$ component corresponding to this overlap is shown at bottom. B: similar analysis of the Hodgkin and Huxley (1952) model, which is given by $C dV/dt = -120m(t)h(t)(V - 55) - 36m(t)^3(V + 72) - 0.3(V + 49)$, with $C = 1 \mu F/cm^2$, $\alpha_{Na}(V) = -0.1(V + 35)/[\exp(0.1(V + 35)) - 1]$, $\beta_{Na}(V) = 4\exp[-(V + 60)/18]$, $\alpha_{h}(V) = 0.07\exp[-(V + 60)/20]$, $\beta_{h}(V) = 1/[\exp(-0.1(V + 30)] + 1]$, and $I_{Na}(V) = 0.125\exp[-(V + 60)/20]$. An AP was elicited from the model by a 1-ms-duration 10 $\mu A/cm^2$ amplitude pulse (not shown). Results at middle and bottom are described in the text.
Fig. 5. AP-clamp analysis of the Sim and Forger (2007) model of $I_{\text{Na}}$(SF). The arrows labeled $a$ and $b$ indicate discrepancies between their model and the experimental recording as described in the text.

(3), suggesting that this portion of the AP-clamp recording is a challenge for models of $I_{\text{Na}}$. More significantly, the Sim and Forger (2007) model does not provide a successful description for $I_{\text{Na}}$ just prior to and during the initial upstroke phase of the AP (arrow a in Fig. 5), in contrast to the model of $I_{\text{Na}}$ in this report (Fig. 3). The $I_{\text{Na}}$ component just prior to upstroke of the AP is significant for near-threshold excitability.

$I_{\text{Ca}}$ component. Jackson et al. (2004) reported voltage-step and AP-clamp recordings of $I_{\text{Ca}}$ from SCN neurons by applying steps and APs in control followed by application of the same waveforms in voltage clamp after replacement of Ca$^{2+}$ in the bathing medium by Mg$^{2+}$, a nonpermeant divalent cation. They also reported measurements of $I_{\text{Ca}}$ before and after the addition to the bath of nimodipine, which blocks a portion of $I_{\text{Ca}}$ in SCN neurons (Pennartz et al. 2002). The model of $I_{\text{Ca}}$ contains two intracellular compartments for Ca$^{2+}$ (Diekman et al. 2013; Yamada et al. 1998). One compartment corresponds to a thin spherical shell 0.1 μm in width in near the membrane surface (McCormick and Huguenard 1992). The Ca$^{2+}$ concentration in this compartment is denoted by $C_{\text{a}}$. The other compartment corresponds to the cytosol. The Ca$^{2+}$ parameter is given by

$$\frac{dC_{\text{a}}}{dt} = -K_1 I_{\text{Ca}} - K_2 C_{\text{a}} + c_s$$

where $K_1 = 3 \times 10^{-5}$ M/sec, $K_2 = 0.04$ msec$^{-1}$ (Purvis and Butera 2005), and $c_s = 2$ mM/sec, so that $C_{\text{a}} = 50$ mW when $I_{\text{Ca}} = 0$ (McCormick and Huguenard 1992).

As noted above, the fully activated current-voltage relation for $I_{\text{Ca}}$ is given by the GHK equation (McCormick and Huguenard 1992),

$$I_{\text{Ca}} = -z^2 A P_{\text{Ca}} (F^2 V/RT) \left(\frac{[Ca^{2+}]}{[exp(zFV/RT) - C_{\text{a}}^{2+}]} \right) / \left[exp(zFV/RT) - 1\right]$$

where $z$ is the ionic valence for Ca$^{2+}$ ($z = 2$), $P_{\text{Ca}}$ is the membrane’s permeability to Ca$^{2+}$, $A$ is the membrane surface area, $F$ is the Faraday constant, T is absolute temperature (RT/F = 25 mV at room temperature), and $[Ca^{2+}]$ is the extracellular Ca$^{2+}$ concentration ($[Ca^{2+}] = 1.2$ mM) (Jackson et al. 2004). In this analysis $Ca^{2+}$ is represented by $C_{\text{a}}$, as noted above. Since $Ca^{2+}$ is significantly less than $C_{\text{a}}^{2+}$, Eq. 6 is considerably simplified using $Ca^{2+} = 0$ so that $I_{\text{Ca}} = -a \text{GHK}(V)$, where $a$ is a constant in picamperes and $\text{GHK}(V) = (zFV/RT)/(exp(zFV/RT) - 1) = (V/12.5)/(exp(V/12.5) - 1)$. Note that $\text{GHK}(V = 0) = 1$. As shown below, the assumption of $Ca^{2+} = 0$ for the GHK equation does not significantly alter the results (DISCUSSION).

Voltage-step recordings of the nimodipine-blocked or nimodipine-sensitive $I_{\text{Ca}}$ component—bottom panel of Fig. 9A of Jackson et al. (2004)—appear to be consistent with an activation gating model consisting of a single gate (no delay in onset of the current following a step). In contrast, the recordings of the nimodipine-sensitive component—middle panel of Fig. 9A of Jackson et al. (2004)—do exhibit a slight delay. Those results are modeled by $r_1(t)$ with $dr_1/dt = -[\alpha_1(V) + \beta_1(V)]r_1 + \alpha_1(V)$, where $\alpha_1(V)$ and $\beta_1(V)$ are voltage-dependent functions similar to those used for $I_{\text{Na}}$. Since the kinetics of the nimodipine-blocked $I_{\text{Ca}}$ component do not exhibit a delay, they are modeled by $r_2(t)$ with $dr_2/dt = -[\alpha_2(V) + \beta_2(V)]r_2 + \alpha_2(V)$.

Inactivation of $I_{\text{Ca}}$ in the model was ascribed to a calcium-dependent process (Fox et al. 2002; Kay 1991; Luo and Rudy 1994; Standen and Stanfield 1982). This parameter, $f(t)$, is given by

$$df(t)/dt = [f_1(Ca^{2+}) - f(t)]/\tau_{tCa}$$

with $f_1(Ca^{2+}) = 1/[1 + (Ca^{2+}/K_a)^3]$ and $K_a = 0.18$ μM; $\tau_{tCa} = 30$ ms (Fox et al. 2002). The full model for $I_{\text{Ca}}$ is

$$I_{\text{Ca}}(V,t) = -\text{GHK}(V)f(t)\left[a_1t^2(V,t) + a_2f_2(V,t)\right]$$

with $a_1 = 305$ pA and $a_2 = 31$ pA.

As noted above, a significant result for developing models of voltage-gated ion channel conductances is the activation curve of channel gating. Hodgkin and Huxley (1952) obtained this result for $I_{\text{Na}}$ in squid giant axons by dividing peak $I_{\text{Na}}$ during a voltage step by the driving force ($V - E_{\text{Na}}$). Jackson et al. (2004) used a similar approach to obtain the $I_{\text{Na}}$ activation curve for SCN neurons (Fig. 2B). This method is appropriate for $I_{\text{Na}}$, since the fully activated current-voltage relation of this component is directly related to the driving force. In contrast, $I_{\text{Ca}}$ has a nonlinear dependence on the driving force for Ca$^{2+}$, a result well described by the GHK equation. Consequently, peak $I_{\text{Ca}}$ results during voltage steps should be divided by GHK(V) rather than by ($V - E_{\text{Na}}$). The latter approach with $E_{\text{Ca}} = 57$ mV was used by Jackson et al. (2004) to obtain the data points in their Fig. 9B. Those results were multiplied by ($V - 57$) and then divided by GHK(V) = ($V/12.5$)/[exp($V/12.5$) - 1] to give the activation curves shown here in Fig. 6, bottom. These results are described by $r_{\text{Ca}}(V) = [\alpha_2(V)/([\alpha_1 + \beta_1(V)])^2 for the nimodipine-insensitive $I_{\text{Ca}}$ component and $r_{\text{Ca}}(V) = [\alpha_2(V)/([\alpha_1 + \beta_1(V)])^2 for the nimodipine-sensitive component with

$$\alpha_1(V) = -0.028(V + 11)/[\exp(-0.1(V + 11)) - 1]; \beta_1(V) = 0.552\exp[-0.015(V + 12)]; \alpha_2(V) = -0.154(V + 27)/[\exp(-0.1(V + 27)) - 1]; \beta_2(V) = 1.92\exp[-0.1(V + 37)]$$

The predictions of Eq. 8 for voltage steps with the $\alpha$s and $\beta$s in Eq. 9 are shown in Fig. 6, top.

$I_{\text{Ca}}$: AP-clamp results. An $I_{\text{Ca}}$ result from the AP-clamp recordings of Jackson et al. (2004)—their Fig. 12—is shown in Fig. 7A, bottom (solid line) in response to the AP in Fig. 7A, top. The computational result (dashed line in Fig. 7A, bottom) was obtained in a manner similar to the $I_{\text{Na}}$ analysis given above. Specifically, the $r_1$, $r_2$, and $f$ variables were iterated throughout the AP (METHODS). The model provides a favorable...
and 0 mV and illustrated in Fig. 8. The maximum value of Cas is 18.5 (Jackson et al. 2004, their Fig. 12). The membrane potential AP. The AP triggers entry of Ca$^{2+}$ (dashed line) superimposed on the experimental record.

The GHK current-voltage relation (Eq. 6) is shown (Fig. 9A), scaled as indicated below, along with the current-voltage trajectory of total $I_{Ca}$ of the model during the AP in Fig. 7A (dashed line in Fig. 9A). The arrows on the trajectory indicate the direction of time (Fig. 9A). The GHK result with $Ca^{2+} = 0$ is shown along with the corresponding result for $Ca^{2+} = Ca = 18.5 \mu M$, the highest value attained by Ca$_{a}$ during an AP (Eq. 5 and Fig. 8). The current-voltage relations for Ca$_{a}$ = 0 and 18.5 $\mu M$ differ only slightly for V > 0 and, for practical purposes, not at all for V < 0. Consequently, Ca$_{a}$ = 0 was used, since this assumption simplifies the analysis as indicated above. During the upstroke of an AP and immediately there-

following an AP is in the −80 to −60 mV range, which lies below the activation range of BK channels even with Ca$_{a}$ as high as 10−20 $\mu M$ (Cui et al. 1997; Shelley et al. 2013). In the model Ca$_{a}$ relaxes back to baseline well before the subsequent AP in a spontaneously firing SCN neuron.

The GHK aspect of the $I_{Ca}$ analysis is illustrated in Fig. 9A. The GHK current-voltage relation (Eq. 6) is shown (Fig. 9A), scaled as indicated below, along with the current-voltage trajectory of total $I_{Ca}$ of the model during the AP in Fig. 7A (dashed line in Fig. 9A). The arrows on the trajectory indicate the direction of time (Fig. 9A). The GHK result with $Ca^{2+} = 0$ is shown along with the corresponding result for $Ca^{2+} = Ca = 18.5 \mu M$, the highest value attained by Ca$_{a}$ during an AP (Eq. 5 and Fig. 8). The current-voltage relations for Ca$_{a}$ = 0 and 18.5 $\mu M$ differ only slightly for V > 0 and, for practical purposes, not at all for V < 0. Consequently, Ca$_{a}$ = 0 was used, since this assumption simplifies the analysis as indicated above. During the upstroke of an AP and immediately there-

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after the gates of both $I_{Ca}$ components are strongly activated. Maximal activation of the gates of the nimodipine-insensitive $I_{Ca}$ ($r_1 = 0.8; r'_1 = 0.64$) occurs at the point labeled $a$ in Fig. 9A. The gates of the nimodipine-sensitive component are fully activated ($r_2 = 1$) throughout the top portion of the AP because of the position of the activation curve of this component on the voltage axis (Fig. 6). The inactivation variable, $f$, is $\sim 0.7$ at this point. These values of $r_1$, $r_2$, and $f$ ($r_1 = 0.8$, $r_2 = 1$, $f = 0.7$) were used in Eq. 8 to determine the GHK current-voltage relation shown in Fig. 9A. Subsequent to point $a$, $r_2$ and $f$ change relatively little, whereas $r_1$ begins to deactivate, i.e., closure of those gates thereby causing the trajectory to move away from the GHK curve. Before the $r_1$ and $r_2$ gates close completely, $I_{Ca}$ significantly increases to point $b$ in Fig. 9A in a relatively brief period of time because of rectification of the GHK current-voltage relation.

**Alternative model for $I_{Ca}$ in SCN neurons.** Diekman et al. (2013) described an $I_{Ca}$ model for SCN neurons in which $I_{Ca} = g_{Ca}(V - E_{Ca})$ for the fully activated current-voltage relation, i.e., the traditional approach. The $I_{Ca}$ components are given by $I_1 = g_1 \tau_1 V^{r_1}$ ($V - E_{Ca}$) and $I_2 = g_2 \tau_2 V^{r_2}$ ($V - E_{Ca}$) with $g_1 = 20$ nS, $g_2 = 6$ nS, $E_{Ca} = 54$ mV, $d\tau_1/dt = (r_1 s - r_1)\tau_1 = 1.2$, with $r_1 = 1 + \exp(-V/21.6)/6.7$, $r_2 = 1 + \exp(-(V + 56)/5.1)^{-1}$, $\tau_1 = \tau_2 = 3.1$ ms, $d\tau_2/dt = (f_{1e} - f_{1i})\tau_2$, with $f_{1e} = 1 + \exp((V + 260)/65)^{-1}$. $r_{Ca}$ is given by $E_{Ca}$ with $K_1 = 1.65 \times 10^{-4}$ M/MC, $K_2 = 10$ ms$^{-1}$, and $c_s = 540$ nM/mS. The AP-clamp computational methodology was applied to this model. The result (Fig. 7B) does not provide an accurate description of the $I_{Ca}$ AP-clamp recording. One problem with this model concerns the kinetics, $\tau_1 = \tau_2 = 3.1$ ms, i.e., a lack of voltage dependence and the same kinetics for both $I_{Ca}$ components. The voltage-step recordings of Jackson et al. (2004) demonstrate that the kinetics of the nimodipine-sensitive component are considerably faster than the kinetics of the nimodipine-insensitive component and that both are voltage dependent. One approach to this problem is to graft the kinetics from the $I_{Ca}$ model described in this report onto the Diekman et al. (2013) model while retaining $I_{Ca} = g_{Ca}(V - E_{Ca})$ as the fully activated current-voltage relation for the model. That is, $\tau_1 = \tau_2 = 3.1$ ms is replaced by $\tau_1 = (\alpha_1 + \beta_1)^{-1}$ and $\tau_2 = (\alpha_2 + \beta_2)^{-1}$ with the $\alpha$ and $\beta$ given by Eq. 9. The AP-clamp analysis of this model (Fig. 9B, inset bottom right) provides an improved description of experiment compared with Fig. 7B, although the peak current of the simulation is considerably less than the peak current of the experimental recording. The $I_{Ca}$ current-voltage trajectory of the model (dashed line in Fig. 9B) is tangent to $I_{Ca} = g_{Ca}(V - E_{Ca})$ at a point where activation of the $r_1$ and $r_2$ channel gates is maximal during the simulation (point $a$ in Fig. 9B). The maximum current of the simulation (point $b$ in Fig. 9B) is 20% larger than the current at point $a$. In contrast, the current at point $b$ in the GHK-based model is 65% larger than the current at point $a$ (Fig. 9A). The GHK-based model has a more robust mechanism for an increase in $I_{Ca}$ during repolarization than either version of the alternative model, a result due to rectification of the GHK current-voltage relation, which is consistent with experiment (Fig. 7A).

**Subthreshold $I_{Na}$ and $I_{Ca}$** Jackson et al. (2004) recorded $I_{Na}$ and $I_{Ca}$ with the AP clamp during the interval between spikes in spontaneously firing SCN neurons (their Fig. 5, A and C). In addition, they also recorded the persistent, subthreshold TTX-sensitive Na$^+$ current (reproduced in Fig. 10) sometimes referred to as $I_{Na\text{p}}$ (Crill 1996), using a slow voltage ramp (20 mV/s). The record in the presence of TTX (Fig. 10) was attributed to the leak current, $I_L = 0.07(V + 10)$ pA, plus the steady-state delayed-rectifier K$^+$ current, $I_{K,DR}$, in SCN neurons (Bouskila and Dudek 1995) as indicated by the line through this recording. The model of the latter (Fig. 10 legend) was taken from Clay (2009). The $I_{Na\text{p}}$ component is given by (Clay 2003; McCormack and Huguenard 1992)

$$I_{Na\text{p}} = 2.8(V/25)\left\{\exp[(V - 45)/25] - 1\right\}/\left\{\exp(V/25) - 1\right\}[1 + \exp(-\{V + 62\}/3.5)] \tag{10}\)$$

This result, together with $I_L$ and $I_{K,DR}$, is represented by the line describing the control result (Fig. 10).

The voltage waveform during the interspike interval of Fig. 5A of Jackson et al. (2004) was digitized as indicated in Fig. 11, top. The corresponding AP-clamp recordings of $I_{Na}$ and $I_{Ca}$ are reproduced in Fig. 11, bottom. Also shown are results for $I_{Na\text{p}}$ from Eq. 10 (filled circles). This component provides a good description of $I_{Na}$ during the latter part of the interspike interval. The nimodipine-sensitive $I_{Ca}$ component is sufficient for the $I_{Ca}$ results in Fig. 11 (open circles). The nimodipine-insensitive $I_{Ca}$ component is activated at potentials that are considerably more positive (Fig. 6) than those used in Fig. 11.

**DISCUSSION**

This report describes a novel method for constructing models of ionic currents in excitable preparations in which AP-clamp recordings are used in conjunction with voltage-step results. The traditional approach relies on voltage-step results alone (Hodgkin and Huxley 1952). Models of the voltage-gated ion current components underlying excitability, $I_{Na}$ and $I_K$ in the case of squid axons, are fitted to voltage-step recordings. The equations for $I_{Na}$ and $I_K$ obtained from this analysis are then used to simulate an AP. A comparison of simulated and experimental APs provides a test of the model. An inter-
mediate step in this process is proposed here in which the model of each individual ionic component obtained from voltage-step analysis, $I_{\text{Na}}$ for example, is further tested by an experimental recording of an AP that is applied computationally to the model and compared to a recording of $I_{\text{Na}}$ obtained in AP clamp for model verification, or if needed, alterations in the model. In the $I_{\text{Na}}$ results above, the model developed from voltage steps was not altered for the AP-clamp result (Fig. 3). That analysis reveals a discrepancy between experiment and theory that has not yet been resolved. Voltage-step and AP-clamp results were used together for construction of the $I_{\text{Ca}}$ component as described in this text.

$I_{\text{Na}}$ component. The model of Engel and Jonas (2005) was a significant part of this analysis. Initial attempts to simulate $I_{\text{Na}}$ during an AP from an SCN neuron based on modifications of the Hodgkin and Huxley (1952) $\alpha$s and $\beta$s for $I_{\text{Na}}$ led to a substantial $I_{\text{Na}}$ during repolarization (simulations not shown), a result that is appropriate for squid axons but not for SCN neurons. The AP model of Engel and Jonas (2005) predicts a separation of the $I_{\text{Na}}$ and $I_{\text{Ca}}$ components on the time axis (Clay 2013), consistent with experiment for hippocampal neurons as well as SCN neurons (Alle et al. 2009; Jackson et al. 2004), which makes their model of $I_{\text{Na}}$ gating a more appropriate starting point for building a model of $I_{\text{Na}}$ gating for SCN neurons than the Hodgkin and Huxley (1952) model. The $\alpha$s and $\beta$s for the SCN $I_{\text{Na}}$ (Eq. 4) correspond to results obtained at room temperature (Jackson et al. 2004). They are $\sim 10$ times smaller than the $\alpha$s and $\beta$s for hippocampal mossy fiber $I_{\text{Na}}$ (Eq. 3), results also obtained at room temperature (Engel and Jonas 2005). This comparison is consistent with considerably faster $I_{\text{Na}}$ gating for the latter preparation compared with the SCN. Both results have been described with the $m^3h$ kinetic scheme, a squid-based model (Hodgkin and Huxley 1952). Later work found their model to be incomplete for squid axons based primarily on gating currents (Vandenberg and Bezanilla 1991a and other studies cited therein). Vandenberg and Bezanilla (1991b) proposed an alternative model that describes most if not all $I_{\text{Na}}$ results from squid axons. Their model is, unfortunately, cumbersome—not easy to use—and perhaps not applicable to mammalian preparations. The original $m^3h$ model is relatively simple, and it does describe whole cell currents from squid axons and mammalian preparations (Clay 2013). Therefore, its continued use appears to be appropriate provided the relevant $\alpha$s and $\beta$s are also used.

Subthreshold $I_{\text{NaP}}$. A time-independent TTX-sensitive $I_{\text{NaP}}$ current activated close to, or slightly below, AP threshold and having relatively small amplitudes—$I_{\text{NaP}}$—has been reported in many mammalian neuron preparations (Bean 2007). The molecular basis of $I_{\text{NaP}}$ has not yet been resolved. Some groups have suggested that it is attributable to a set of channels that are distinct from the traditional $I_{\text{Na}}$ channel (Crill 1996). Other investigators have suggested that it is, in fact, attributable to $I_{\text{Na}}$ (Taddesse and Bean 2002). The former approach was used for the results in Figs. 10 and 11 since it is simpler, computationally, than requiring $I_{\text{NaP}}$ to be incorporated in the $m^3h$ kinetic scheme.

$I_{\text{Ca}}$ component. The voltage-step recordings of $I_{\text{Ca}}$ of Jackson et al. (2004) in the absence of similar results with nimodipine (their Fig. 9A) do not clearly indicate the presence of two kinetically distinct $I_{\text{Ca}}$ components. In contrast, AP-clamp recordings of $I_{\text{Ca}}$ (their Fig. 12) are suggestive of this result, in particular the inflection on the rising phase of $I_{\text{Ca}}$ during the initial phase of AP repolarization. The nimodipine-sensitive component is activated at relatively negative potentials with sufficiently rapid kinetics (Fig. 6) so that it is nearly in step with the membrane potential during the upstroke phase of the AP. The nimodipine-insensitive component is activated at a slower rate and at potentials that are depolarized relative to the nimodipine-sensitive component, thereby accounting for the inflection in $I_{\text{Ca}}$ in the AP-clamp recording. These results provide further evidence for the utility of the AP clamp methodology.

In contrast to $I_{\text{NaP}}$, the $I_{\text{Ca}}$ component clearly does contribute during repolarization of the SCN AP. It may help “shape” the AP. In particular, the “surge” in $I_{\text{Ca}}$ during repolarization that in the $I_{\text{Ca}}$ model is attributable to GHK rectification (Fig. 9A) may explain the relatively long half-width of the SCN AP, $\sim 2.5$ ms (Jackson et al. 2004). Moreover, the influx of $\text{Ca}^{2+}$ associated with $I_{\text{Ca}}$ influences the $\text{Ca}^{2+}$-dependent current, $I_{K,\text{Ca}}$, in particular $K^+$ current associated with BK channels (Jackson et al. 2004). A transient rise of $\text{Ca}^{2+}$ in the vicinity of these channels somewhere in the 10–20 $\mu$M range is believed necessary to shift the voltage-dependent BK activation curve to within the range of potentials spanned by an AP (Berkenfeld et al. 2006; Fakler and Adelman 2008). The simulations in Fig. 7A and Fig. 8 predict that $\text{Ca}^{2+}$ briefly reaches the 18.5 $\mu$M level during the latter part of the AP, a result that serendipitously falls within the 10–20 $\mu$M range. Jackson et al. (2004) reported a robust $I_{K,\text{Ca}}$ component obtained with the AP clamp that they attributed to BK. They did not report voltage-step results for $I_{K,\text{Ca}}$, which would be necessary for the analysis described here.

In addition to being permeable to calcium ions, $\text{Ca}^{2+}$ channels are known to have a small permeability to intracellular $K^+$ (Hille 2001), which influences the reversal potential for the channel but has relatively little effect on $\text{Ca}^{2+}$ currents for $V < 25$ mV, the range of potentials investigated in this report.

Other current components in SCN neurons. Analysis of non-$\text{Ca}^{2+}$-activated $K^+$ currents in SCN neurons with the AP clamp technique does not appear straightforward. Jackson et al. (2004) found that addition of 10 mM TEA$^-$ to the bath completely removed net outward current during an AP. In
contrast, 30 mM TEA does not completely block the delayed rectifier $K^+$ current, $I_{K,DR}$, in voltage-step analysis (Bouskila and Dudek 1995). Perhaps modeling of both sets of results may lead to a resolution of this apparent paradox. SCN neurons also have the transient, rapidly inactivating $K^+$ current, $I_A$ (Bouskila and Dudek 1995; Itri et al. 2010) that is completely blocked by 5 mM 4-aminopyridine (4-AP; Huang et al. 1993). Moreover, 4-AP modifies spiking behavior of SCN neurons and the shape of the SCN AP (Itri et al. 2005). This result suggests that AP-clamp analysis of SCN neurons before and after bath application of 4-AP would be of interest.

**SCN and circadian rhythms: relationship to ionic currents.** Circadian rhythms in mammals are coordinated by the hypothalamic SCN (Brancazio et al. 2013). Spontaneously occurring APs in the SCN exhibit diurnal patterning. During the day SCN neurons are more active than at night, having firing frequencies of 8–10 Hz. At night, activity is suppressed to <2 Hz, on average, with many neurons in the silent state (Green and Gillette 1982; Groos and Hendriks 1982; Inouye and Kawamura 1979; Shibata et al. 1982; Yamazaki et al. 1998). Potential roles of $I_{Ca}$, $I_A$, and $I_{K,Ca}$ have been emphasized in the regulation of day-night differences of SCN firing rate (Kent and Meredith 2008). Pennartz et al. (2002) found that Ca$^{2+}$-free bathing media reversibly suppressed firing of APs in SCN neurons during the day. Moreover, firing of APs during the day was blocked by the addition of 2 $\mu$M nimodipine to the bathing medium. At night $I_{Ca}$ was reduced ~50%, which is consistent with a reduction in firing rate at night (Pennartz et al. 2002). Nimodipine-sensitive $I_{Ca}$ is activated close to AP threshold with relatively fast kinetics, so that blockade of this component is consistent with block of firing during the day even though this portion of $I_{Ca}$ is relatively small (Jackson et al. 2004; Pennartz et al. 2002). Itri et al. (2005) found a day-night difference in the amplitude of $I_A$ in SCN neurons, with a reduction of this component by ~50% at night relative to the day. Moreover, 4-AP, a blocker of $I_A$, reduces firing rate during the day by ~50% (Itri et al. 2005). Finally, Meredith et al. (2006) found that daily expression of $I_{K,Ca}$ BK channels, is controlled by the intrinsic circadian clock. Specifically, BK channel-null mice have increased spontaneous firing rates selectively at night and weak circadian amplitudes in multiple behaviors timed by the SCN. A number of reports including Jackson et al. (2004) have provided indirect evidence for the presence of BK channels and other $I_{K,Ca}$ channel subtypes in SCN neurons from electrophysiological recordings (Cloes and Sather 2003; Pitts et al. 2006; Teshima et al. 2003). A complete description of this component will also require voltage-step results. That work necessitates the use of the excised patch-clamp technique in the inside-out configuration so that Ca$^{2+}$ and membrane potential V can both be controlled during the experiments. This approach has been used for BK currents from mslo channels heterologously expressed in Xenopus oocytes (Cui et al. 1997) and BK splice variants heterologously expressed in HEK cells (Shelley et al. 2013). Similar results from SCN neurons at various points in the day-night cycle will provide information on diurnal changes in $I_{K,Ca}$ in the SCN.

The results of Jackson et al. (2004), the focus of this work, were taken from acutely dissociated single SCN cells. This approach appears necessary to ensure isopotentiality during voltage-step and AP-clamp recordings especially for $I_{Na}$. These cells are physiologically relevant since they exhibit electrical properties similar to those described in SCN brain slices (Pennartz et al. 2002). Nevertheless, contributions to excitability of ion channels in dendritic spines and axons, anatomical features that are not clearly present in dissociated cells, cannot be excluded. Furthermore, SCN neurons are not homogeneous. The preparation used by Jackson et al. (2004) was enriched with neurons stained for arginine vasopressin (AVP). These neurons exhibited rhythmic subthreshold oscillations in the presence of TTX but were still likely a heterogeneous population reflected by a dramatically different ratio of $I_{Na}$ and $I_{Ca}$ between SCN neurons that were firing at the same frequency (Fig. 6, Jackson et al. 2004).

**Dynamic clamp: comparison with AP and voltage clamp step techniques.** The AP clamp technique is similar to the dynamic clamp method, an approach originally used in cardiac electrophysiology that also has been used in neuroscience and in other fields (Goaillard and Marder 2006; Wilders 2006). As the name implies, dynamic clamping involves real-time injection of current into the preparation under investigation during an ongoing experiment, a procedure not required with AP clamp. The AP clamp method does require the preparation under study to be sufficiently stable to permit several steps: 1) recording of an AP, 2) application of the AP waveform to the preparation in voltage-clamp mode in control conditions, 3) voltage-step recordings in control with a particular ionic conductance in mind, $I_{Na}$ for example, and 4) application of the AP waveform and voltage steps in voltage clamp after the addition of TTX to the bath. The results are the differences in membrane currents in control and test conditions both for the AP and for voltage steps. This report describes an extension of the method in which an experimentally recorded AP is digitized and the resulting $V_i$ vs. $t_i$ data set applied to mathematical models of the relevant ionic conductance, such as $I_{Na}$. The result is compared with an experimental AP-clamp recording of that component either for model validation or for modification of model parameters as noted above. A review of the literature did not yield any other reports in which this procedure has been used. A related approach concerning models of $I_{Na}$ has been reported for raphe pacemaker neurons (Milescu et al. 2008). Moreover, waveforms other than rectangular steps have been used previously. For example, Fohlmeister and Adelman (1985) used sinusoids in voltage clamp to measure $I_{Na}$ gating currents in squid giant axons, and they analyzed their results with the Hodgkin and Huxley (1952) $I_{Na}$ model. An AP waveform is, perhaps, of greater interest compared with sine waves or rectangular steps since it has direct physiological relevance.

**Summary.** A novel extension of the AP clamp technique is described involving models of the ionic conductances in the cell from which the AP was recorded. The method is general. It has been applied to SCN neurons with a goal of describing a complete mathematical model of the AP in these cells. The equations for $I_{Na}$ and $I_{Ca}$ given above form one part of that model, a model that may have broad applicability not only for the SCN but also for other mammalian preparations.

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