Kinetic and Stochastic Properties of a Persistent Sodium Current in Mature Guinea Pig Cerebellar Purkinje Cells

ALAN R. KAY, 1 MUTSYUKI SUGIMORI, 2 AND RODOLFO LLINÁS 2
1Department of Biological Sciences and Neuroscience Program, University of Iowa, Iowa City, Iowa 52242; and 2Department of Physiology and Neuroscience, New York University Medical School, New York, New York 10016

Kay, Alan R., Mutsuyuki Sugimori, and Rodolfo Llinás. Kinetic and stochastic properties of a persistent sodium current in mature guinea pig cerebellar Purkinje cells. J. Neurophysiol. 80: 1167–1179, 1998. Whole cell voltage-clamp techniques were employed to characterize the sodium (Na) conductances in acutely dissociated, mature guinea-pig cerebellar Purkinje cells. Three phenome-
nological components were noted: two inactivating and a persistent component (I\textsubscript{Na\_p}). All exhibited similar sensitivities to tetrodotoxin (TTX; IC\textsubscript{50} ~ 3 nM). The inactivating Na current demonstrates two components with different rates of inactivation. The persistent component activates at a more negative membrane potential than the inactivating components and shows little inactivation during a 5-s pulse. The amplitude of the persistent Na conductance had a higher Q\textsubscript{10} than the inactivating Na conductance (2.7 vs. 1.3). I\textsubscript{Na\_p} rapidly activates (~1 ms) and deactivates (<0.2 ms) and like the fast component appears to be exclusively Na permeable. I\textsubscript{Na\_p} is not a “window” current because its range of activation exceeds the small overlap between the steady-state activation and inactivation characteristics of the inactivating current. Anomalous tail currents were observed during voltage pulses above ~40 mV after a prepulse above ~30 mV. The tails rose to a maximum inward current with a time constant of 1.5 ms and decayed to a persistent inward current with a time constant of 20 ms. The tails probably arose as a result of recovery from inactivation through the open state. The noise characteristics of I\textsubscript{Na\_p} were anomalous in that the measured variance was lower at threshold voltages than would be predicted by a binomial model. The form of the variance could be partially ac-
counted for by postulating that the maximum probability of activa-
tion of the persistent current was less than unity. The noise charac-
teristics of I\textsubscript{Na\_p} are such as to minimize noise near spike activation threshold and sharpen the threshold.

INTRODUCTION

The threshold for action potential generation in central neurons is determined by the kinetics and distribution of channels that are activated at subthreshold voltages. A number of channels generating both inward and outward currents are activated at subthreshold voltages that can potentially determine spike threshold. Prominent among these is a per-
sistent sodium current (I\textsubscript{Na\_p}) (for review see Crill 1996) activ-
ated at subthreshold potentials that may effectively lower spike threshold and may serve to boost excitatory postsynap-
tic potentials EPSPs.

A noninactivating Na conductance has been known to occur in mammalian neurons (Llinás and Sugimori 1980; Stafstrom et al. 1982). I\textsubscript{Na\_p} may arise from a channel distinct from the conventional inactivating Na channel (I\textsubscript{Na\_i}) (Masu-
kawa et al. 1991; Sugimori et al. 1994) or it may result from the persistent activation of I\textsubscript{Na\_i}. It was argued that a persistent current could arise through the overlap of the activation and inactivation curves of the inactivating Na current, resulting in a so-called “window” current (Attwell et al. 1979; Gah-wiler and Llano 1989). Single-channel data suggest in some cases that a persistent Na current may arise from the sporadic failure of inactivation in the conventional Na channel (“mode switching”) (Alzheimer et al. 1993; Nilius 1988; Patlak and Ortiz 1986). It was also difficult to exclude its origin in space-clamp problems that are inevitable in intact neurons or to unequivocally demonstrate that the persistent current is not associated with a pump or exchanger. Some of the difficulty in resolving these issues results from the difficulty of imposing a complete voltage clamp in the spatially complex neurons of the CNS and of controlling both the external and internal milieu in the slice preparation.

Despite the rapid advances made in the molecular analysis of Na channels, there are still uncertainties about the number of different types of Na channels (Black et al. 1994) ex-
pressed in the CNS. Indeed there are recurrent questions as to whether the channels expressed in oocytes are the same as those in its natural setting, as different postranslational modifications might be made in a heterologous system. There is therefore still a strong case to be made for measuring channel kinetics in situ.

The Purkinje cells of the cerebellum are endowed with an intricate electroresponsiveness that results from the interplay between an elaborate neuronal architecture and a complex palette of channels. A single climbing fiber input evokes a burst of action potentials termed a complex spike. This complex spike is generated by a large unitary synaptic current resulting from the activation of a single axodendritic distrib-
uted synapse (200–300 active zones) between the climbing fiber axon and its target Purkinje cell (Eccles et al. 1966). This large unitary synaptic potential in turn activates voltage-gated calcium currents mostly on dendrites and a slowly inactivating Na conductance mostly of somatic origin (Lli-
nás and Sugimori 1980) that was shown more recently to be of the persistent variety by experiments employing a Na-
sensitive fluorescent dye (Callaway and Ross 1997).

In this paper we addressed the issue of the origins and influences of the subthreshold noninactivating Na current in cerebellar Purkinje cells. We used acutely dissociated Pur-
kine cells that lost most of their dendritic tree, minimizing space-clamp problems, in whole cell recordings to explore the kinetics of I\textsubscript{Na\_p}. In addition we considered the possible influence of the stochastic properties of this channel on the approach to threshold.
METHODS

Acutely dissociated Purkinje cells

Acutely dissociated Purkinje cells were prepared from guinea pig cerebellum by a modification of the method of Kay and Wong (1986). All experiments were carried out in accordance with the American Physiological Society guidelines. All chemicals were from Sigma unless otherwise stated. Guinea pigs (150–400 g) were killed by decapitation, and the cerebellum was rapidly removed and placed in ice-cold, oxygenated Pipps saline containing (in mM): 115 NaCl, 5 KCl, 1 CaCl2, 4 MgCl2, 20 piperazine-N,N′,N′-bis(2-ethanesulfonic acid) (PIPES), and 25 d-glucose 250, pH 7.0. The cerebellum was hand sliced with a No. 22 scalpel blade. The caudal ~5 mm was blocked off, and the cut surface was placed on a damp paper towel. Slices of ~0.4–0.6 mm were cut by a rapid downward motion of the blade and trimmed into sections of ~1 x 1 mm, excising the white matter. The slices were placed in a 25-ml spinner flaks (Belco) containing Pipps saline to which 1.5 mg/ml of trypsin (Sigma Type XI) was added. The temperature of the flask was raised to 30°C for 1 h, the saline was then replaced with fresh Pipps saline, and the flask was allowed to come to room temperature. Neurons were liberated by triturating the slices with fire-polished Pasteur pipettes in ~0.5 ml of fresh Pipps saline.

Electrophysiological recording

Recordings were performed in the whole cell mode of voltage clamping with the use of electrodes (WPI, TW150) with a resistance of 0.7–2 MΩ, fabricated on a two-stage puller (Narishige, PP-83) and a List EPC7 or an Axopatch 200 (Axon Instruments) amplifier with typically 70% of the series resistance compensated. Voltage-clamp protocols were generated by the pClamp program (Axon Instruments) through a TL-1 A/D (Axon Instruments) and stored on an IBM computer for analysis. Current records were low-pass filtered by a Bessel filter (Frequency Devices). All recordings were performed at a temperature of 20–22°C unless otherwise stated. In some cases a temperature-controlled stage was used (Medical Systems). All current traces are of tetrodotoxin (TTX)-sensitive currents, derived by subtracting current traces in the presence of 2 nM TTX from control traces unless otherwise stated. Voltages were not corrected for junction potentials.

Intracellular and extracellular media were such as to minimize the labile persistent Ca currents would run down rapidly. Furthermore the extracellular medium was not supplemented with Mg-ATP, ensuring that the balance persistent Ca currents would run down rapidly. The intracellular medium was maintained with Mg-ATP, ensuring that the balance persistent Ca currents would run down rapidly. The intracellular medium was not supplemented with Mg-ATP, ensuring that the balance persistent Ca currents would run down rapidly. Solutions with reduced Na were produced by mixing the 1.0 Na solution with the 1.0 TEA solution, e.g., one-fourth Na = 25% 1.0 Na solution + 75% 1.0 TEA solution.

INTRACELLULAR SOLUTIONS. The concentrations for the CsF solution consisted of the following (in mM): 120 CsF, 15 NaCl, 11 ethylene glycol-bis(β-aminoethy1 ether)-N,N,N′,N′-tetraacetic acid (EGTA), and 10 HEPES at pH 7.25. For Tris MeSO4, the solution consisted of the following (in mM): 140 Tris MeSO4, 15 NaCl, 10 EGTA, and 10 HEPES at pH 7.25. For KF the solution contained (in mM) 120 KF, 15 NaCl, 11 EGTA, and 10 HEPES at pH 7.25.

EGTA was obtained from Fluka, CdCl2, CsF, CsOH, and HMeSO4 were obtained from Aldrich, and CaCl2 was obtained from BDH (Gallard Schlesinger).

The morphology of isolated cells was recorded with the aid of a CCD camera (Panasonic) linked to an Apple Macintosh by a Data Translation Quick Capture board. Cell shapes reproduced on all figures are hand-drawn tracings of computer hard copy.

Data analysis

POWER SPECTRA. Analysis programs were written in MathCAD (Mathsoft). Linear DC trend was removed by fitting a line to the data and subtracting it. Eight to 12 overlapping segments were used to compute the average power spectrum. Frequency averaging was then performed on the power spectrum in blocks of 16, averaging 2p points, where n is the number of the block and starts at 0 at low frequencies. All displayed power spectra had a similarly treated power spectrum of the background noise measured in the presence of 2 μM TTX, subtracted.

ANALYSIS OF TIME DOMAIN DATA. Current transients were decomposed into a sum-of-exponential process by the simplex minimization scheme, employing the sum of squares as an optimization criterion. The duration of the sampling interval was at least five times the lowest decay constant; under these conditions it was not necessary to apply differential weighting to the points. All other fitting of equations to data and data display were done in Origin (MicroCal).

Q MATRIX METHOD. Analytic manipulation of the Q-matrices (Colquhoun and Hawkes 1995) was performed in the Maple programming environment (Waterloo Maple Software). Stochastic simulations of channel gating were implemented with the use of an exact method devised by Gillespie (1977). All data are presented as mean ± SD unless otherwise stated.

RESULTS

Three components of the sodium current

Precise control of membrane voltage is difficult in the spatially complex Purkinje cells; we therefore used acutely dissociated cells that were stripped of most of their dendritic arborizations (Fig. 3) The Na current elicited with normal external Na concentrations (125 mM) proved to be too large to control, despite the use of the low-resistance electrodes. To bring the current under control the external Na concentration was diminished, and under these conditions the current elicited exhibited signs of being under control. No latency jitter was observed; the descending limb of the current-voltage (I-V) curve declined continuously and showed no discontinuous breaks (data not shown). This result generally held for Na concentrations below half-normal (i.e., 62 mM); moreover, there was no change in the kinetics of the current at the Na concentration
Note that the only component fully deinactivated at the normal rest potential of $-65 \text{ mV}$ is the slow component. Under normal conditions quite a bit of reserve capacity probably remains in the fast and persistent components that can be restored by deep and extended hyperpolarizations.

There was no obvious difference in the TTX sensitivity of the three components of the Na current when using TTX concentrations of 0.03, 0.3, 3, and 30 nM ($IC_{50} = 3.0 \pm 1.1 \text{ nM}$, mean $\pm SE$, $n = 4$). No TTX-insensitive, Zn-sensitive Na current was observed, such as was found in the rat entorhinal cortex (White et al. 1993).

**FIG. 1.** Three phenomenological components in the Purkinje cell Na current. A: biexponential inactivation of the Na current in response to a current step to $-15 \text{ mV}$ from $-80 \text{ mV}$. The steady-state ($-44.7 \text{ pA}$) value was subtracted from the response, and the log of the current was plotted as a function of time. The continuous line is the best fitting sum of 2 exponentials. *Inset:* current response to step to $-35$, $-30$, $-25$, $-20$, and $-15 \text{ mV}$ from $-80 \text{ mV}$. Sampled at 16.6 kHz and filtered at 5 kHz ($\mu \text{NaCl}$).

B: current responses to a slow voltage ramp (10 mV/s) in the absence and presence of tetrodotoxin (TTX; 2 $\mu \text{M}$). The difference between the control current and that in the presence of TTX represents $I_{P\text{Na}}$. Dotted line is the best-fitting product of the Boltzmann function and $(V - E_{Na})(\delta = 3.8, V_{0.5} = -41.6 \text{ mV}, g_{P\text{Na}} = 2.9 \text{nS})(1.0 \text{Na})$.

The inactivation of the macroscopic Na current elicited by depolarizing pulses could be accounted for by an expression of the form (Fig. 1A)

$$I_{Na}(t) = A_{f}e^{-\delta t / \tau_{f}} + A_{s}e^{-\delta t / \tau_{s}} + A_{p}$$

where $A_{f}$, $A_{s}$, and $A_{p}$ are the amplitudes of the components that inactivate rapidly slowly and the persistent current, respectively. The three phenomenological components of inactivation will be referred to as the fast (f), slow (s), and persistent (p) components. The persistent current can be visualized most clearly by subjecting the cell to a slowly rising voltage ramp (10 mV/s) and subtracting the response in the presence of TTX (Fig. 1B).

An interesting progression occurs in the activation of the three components of the Na current (Fig. 2A). First, the order of activation is persistent, and then slow, and then fast. Second, there is a progressive escalation in the magnitude of the conductance, there being an increase of approximately tenfold at each stage.

All three components separate quite clearly in terms of their steady-state inactivation characteristics (Fig. 2B).

**FIG. 2.** Steady-state activation and inactivation characteristics of inactivating Na current. A: threshold and magnitudes of 3 components of the Na current increase in the order $P > s > f$. The steady-state activation of the components are plotted on a log scale. Lines are the best fit to the Boltzmann equation $(f: \delta = -5.4, V_{0.5} = -5.3 \text{ mV}; s: \delta = -6.3, V_{0.5} = -17.1 \text{ mV}; P: \delta = -7.1, V_{0.5} = -27.4 \text{ mV})$. Inset: current responses to steps to $-30$, $-25$, $-20$, $-15$, and $-10 \text{ mV}$. Holding potential is $-70 \text{ mV}$ (0.5 NaCl).

B: steady-state inactivation of all 3 components of the Na current. The protocol used is represented in the inset (prepulse duration 50 s, interpulse interval 60 s). The amplitudes of the components were estimated by fitting a sum of 2 exponentials plus a persistent component to the postpulse response. Lines are the best fit to the Boltzmann equation $(f: \delta = 6.0, V_{0.5} = -52 \text{ mV}; s: \delta = 4.4, V_{0.5} = -44.4 \text{ mV}; P: \delta = 14.0, V_{0.5} = -46.7 \text{ mV})$. *Inset:* postpulse currents in response to different prepulses ($-35$, $-40$, $-45$, $-50$, and $-55 \text{ mV}$). All traces sampled at 16.6 kHz and filtered at 5 kHz (1.0 Na).
The persistent sodium current, \( I_{Na}^P \)

A sawtooth voltage input with a rise of 10 mV/s was used to obtain the steady-state \( I-V \) relationship of \( I_{Na}^P \) (Fig. 1B). There was little sensitivity of the shape of the \( I-V \) for ramps rising at rates between 20 mV/s and 5 mV/s, and the \( I-V \) s so gathered were similar in form to those measured with the use of voltage steps of 1-s duration. As was found previously, \( I_{Na}^P \) activates at a lower membrane potential than the conventional inactivating Na current \( (I_{Na}) \) (French et al. 1990). TEA and 4AP were included in the external medium to block residual potassium currents, and in control experiments they had no significant effect on \( I_{Na}^P \). The current remaining after the application of TTX probably corresponds to the nonspecific cation current described by Alzheimer (1994). Magnesium (4 mM) was used to increase the stability of the recording and to some rightward shift of the \( I-V \) but did not block \( I_{Na}^P \). Likewise Ba\(^{2+}\) was used externally to increase stability; however, on switching to a solution in which Ca\(^{2+}\) was substituted for Ba\(^{2+}\), a reduction of \( I_{Na}^P \) occurred that could not be reversed on the reintroduction of Ba\(^{2+}\). The origins of this effect remain obscure. Unlike the persistent Na current in rabbit cardiac Purkinje fibers (Carmeliet 1987), \( I_{Na}^P \) was not blocked by Cd\(^{2+}\).

\( I_{Na}^P \) reverses at \(-50 \text{ mV} \) (Fig. 1B), which is close to that predicted for a channel exclusively permeable to Na. The \( I-V \) relationship could be well approximated by an expression of the following form

\[
I_{Na}^P = g_{Na}^P (V - E_{Na})P(V, t) \tag{2}
\]

where \( g_{Na}^P \) is the macroscopic channel conductance, \( E_{Na} \) is the reversal potential, and \( P(V, t) \) the activation variable that ranges from 0 to 1.

Knowing \( E_{Na} \) and the steady-state \( I-V \) relationship of \( I_{Na}^P \), Eq. 2 can be used to calculate \( P(V, t) \), which in the case of the results in Fig. 1B corresponds to the steady-state activation that follows the form of a Boltzmann distribution

\[
P_a(V) = \frac{\phi}{1 + e^{(V - V_{0.5})/\delta}} \tag{3}
\]

where \( V_{0.5} \) is the voltage at which half-maximal activation occurs, \( \phi \) is maximal probability of activation, and \( \delta \) is the slope factor.

For small amplitude voltage steps, before the activation of \( I_{Na} \), it is possible to follow the activation of \( I_{Na}^P \). Its time course is fairly well approximated by a single exponential with a time constant of \(-0.8 \text{ ms} \) (Fig. 3A). The tail current associated with the deactivation of \( I_{Na}^P \) on stepping to voltages below \(-50 \text{ mV} \) behaved conventionally; that is, they relaxed with a monotonic exponential time course (time constant of \(-0.2 \text{ ms} \) at \(-60 \text{ mV} \)). However, for postpulses above \(-30 \text{ mV} \), the tails were “hooked”, rising to an inward peak in \(-5 \text{ ms} \) and decaying to a persistent inward current with a time constant of \(-20 \text{ ms} \) (Fig. 4, inset). The anomalous tail currents reversed at the same potential as \( I_{Na}^P \) and were TTX sensitive, suggesting that they arose from a Na conductance. The \( I-V \) relationship of the anomalous tail is plotted in Fig. 4 and is inconsistent with a classical tail current that would give a linear \( I-V \) relationship. The simplest interpretation of the anomalous tails is that they arise from transitions through the open state on recovery from inactivation and were termed “resurgent” currents by Raman and Bean (1997). Resurgent currents were observed in five out of five Purkinje neurons where the appropriate protocols were deployed.

Unlike the persistent Ca currents (Kameyama et al. 1990), \( I_{Na}^P \) proved to be stable and exhibited no rundown during the course of recording. Moreover, during long pulses very little inactivation of the current was observed (Fig. 3B). Inactivation of the persistent current was \(<10\% \) for a 5-s voltage step.
inactivating Na current and of the pulse the signal variance was measured (V_0.5) of the steady-state activation. Figure 5 shows the steady-state activation and inactivation of the inactivating Na current together with the average current (I) and inactivation of the inactivating component was determined in the same way as that for the inactivating component (V_0.5) of the steady-state activation and inactivation of the inactivating Na current with the use of a protocol similar to that described in Fig. 2B. During this period the mean current exhibited little overlap between the steady-state Na current and E_{Na}. All the data represented in this figure were derived from a single cell (0.5 Na).

I_{pNa} is not a window current

It was suggested that the persistent Na current might be a window current, i.e., a component arising from the overlap of the steady-state activation and inactivation of the inactivating Na current. Figure 5 shows the steady-state activation and inactivation of the inactivating Na current together with the I-V characteristics of the peak and steady-state Na current, all measured in the same cell. It can be seen that the form of the I-V relationship of the persistent current is not well predicted by the product of steady-state activation and inactivation, suggesting that I_{pNa} is not a window current (Fig. 5B). In the case shown in Fig. 5 the measured peak of the I-V relationship of I_{pNa} was 39 pA, whereas the calculated peak window current was 7 pA.

The magnitude of the expected window current was estimated as shown in Fig. 6A. The amplitude of the square of the variance between steady-state activation and inactivation (Win^2) should be proportional to the window current. However, no correlation was found between Win^2 and the fraction of persistent Na current (I_{pNa}/I_{Na}) (Fig. 6B). If I_{pNa} were a pure window current there should be a linear relationship between the two.

The proportion of the Na current carried by the persistent current showed considerable variation over the population of cells examined (Fig. 6C). No correlation was found between the length of the dendritic stub and the magnitude of I_{pNa} per dendritic membrane area, suggesting that the channel is unlikely to be exclusively localized on the dendrites (data not shown). The ratio of I_{pNa} to the inactivating component was not constant, arguing against a fixed mode-switching mechanism (Fig. 6D).

The temperature dependence of the amplitude of both the persistent and inactivating components was measured by slowly changing the bath temperature. The persistent Na current had a Q_{10} of 2.7 ± 0.3 (n = 4) and the inactivating component had a Q_{10} of 1.3 ± 0.2 (n = 3) over the range of 20–30°C. It is unlikely that the sharper temperature dependence of the persistent current arises through metabolic modulation of the conductance (e.g., phosphorylation) as all recordings of the temperature dependence of I_{pNa} were performed with fluoride, which inhibits many enzymes (Baker 1984).

Anomalous properties of I_{pNa} channel noise

Analysis of the macroscopic noise resulting from the gating of channels may be used to estimate the properties of the underlying single channels (DeFelice 1981). The persistent Na current is ideal for noise analysis as it opens in a sustained fashion, making the resulting noise stationary, facilitating the analysis. For a channel with two states, open (O) and closed (C), coupled in the following kinetic scheme

\[ a \rightarrow C \rightarrow O \rightarrow b \]

the average current (I) is given by

\[ I = PNi \]  

where P [\alpha/(\alpha+\beta)] is the probability of opening, N is the number of channels, and i is the single-channel current, which is given by

\[ i = \gamma(V - E_{Na}) \]

where \gamma is the single-channel conductance, V is the transmembrane voltage, and E_{Na} is the reversal potential.

The variance (\sigma^2) follows from the binomial theorem

\[ \sigma^2 = P(1 - P)Ni^2 = (1 - P)\sigma^2 \]  

Note that for a channel exhibiting P_{x}^y (where x is an integer) kinetics, the variance is given by \sigma^2 = (1 - P_{x}^y)\sigma^2 + (P_{x}^y)\sigma^2 (Conti et al. 1976).

To measure the steady-state noise characteristics of the persistent Na conductance, currents were elicited by applying steps of 1.5-s duration. One second after the onset of the pulse the signal variance was measured \( n = 1,000 \) points). During this period the mean current exhibited little if any decline. The variance as a function of step voltage is shown in Fig. 7. Application of TTX completely eliminated the voltage dependence of the variance, suggesting that the difference between the control variance and that in the presence of TTX can be attributed to the persistent Na current.
FIG. 6. Variability of $I_{Na}$. A: estimation of the maximum window current ($win^2$). Steady-state inactivation (●) and inverse of the peak Na current (○). The steady-state activation (—) was estimated by fitting a Boltzmann function multiplied by $V-E_{Na}$ to the peak current-voltage curve. B: expected peak window current ($win^2$) plotted against the persistent Na current normalized by the peak current ($I_{PNa}/I_{Na}$). Currents were measured at the peak of their respective $I-V$ relationships. C: frequency of normalized persistent Na current amplitudes ($I_{PNa}/I_{Na}$). D: $I_{PNa}/I_{Na}$ vs. $I_{Na}$.

In the same cell the probability of activation ($P$) was estimated from the $I-V$ (assuming a linear $I-V$ relationship) and, from this and the reversal potential, Eq. 6 can be used to estimate the single-channel conductance. However, contrary to conventional expectations of channel behavior, the single-channel conductance varied dramatically over the voltage range −40 to 10 mV, increasing monotonically from a value of 2.5 pS at −45 mV to 240 pS at −25 mV (data not shown).

For a channel exhibiting binomial statistics the expected dependence of the variance on voltage can be found with the use of the measured values of $P$ from Eq. 6. This is plotted on the same graph as that of the measured variance, and there is a large disparity between the two (Fig. 7). The expected variance peaks exactly midway through the activation curve and declines to zero at the point at which $P$ reaches unity. In contrast the measured variance peaked well above the midpoint of the activation curve and declined very gradually with increasing voltage. This form of behavior was noted in all cells ($n = 7$) where the variance was measured in detail.

Conti et al. (1980) found that the apparent single-channel conductance estimated from the spectral analysis of the steady-state component of squid giant axon Na conductance increased with increasing depolarization. The increase of conductance that they observed was less pronounced than that in this study. They proposed that the apparent voltage dependence of the conductance may have been an artifact arising from filtering of the records. Silberberg and Magelby (1993) explored how band limiting the recorded current can affect the estimation of channel properties by simulating a two-state, single-channel model ($O \leftrightarrow C$). Filtering led to a more pronounced underestimation of the variance at high open probabilities than at low. This would have the effect of shifting the voltage-variance curves to the left. Our voltage-variance curves are right-shifted, and thus it seems unlikely that the skewing of the curve is a filtering artifact. Furthermore, we estimated the voltage-variance curve at three different filter cutoffs (1, 2, and 5 kHz) and found that the filtering had no effect on the form of the curve (data not shown), suggesting again that filtering per se is unlikely to give rise to the anomalous variance.

**Possible origins of the anomalous noise**

The form of the variance-voltage plots can be accounted for by having a maximal probability of activation ($\phi$) that
A minimal canonical model that could account for the two Lorentzian components in the power spectrum has the following form

\[
G(f) = 4N(V - E_{nax})^2 P_i(\infty) \gamma^2 \sum_{\ell=2}^6 \frac{\lambda_{\ell}^j a_{i1}}{[1 + (f/\lambda_{\ell})^2]} 
\]

where \( f_c = \lambda_n/2\pi \) and \( a_{i1} \) is the elements of the first row and column of the \( \ell \)th spectral matrix. The spectral matrices are \( k \times k \) matrices, with elements \( a_{ij} \), with the \( r \)th spectral matrix corresponding to the \( r \)th eigenvalue (\( \lambda_r \)) and \( P_i(\infty) \) is the steady-state probability of the open state.

The expected power spectrum of this mechanism can be derived with the use of the \( Q \) matrix method of Colquhoun and Hawkes (1995). If states are numbered open (O) = 1, closed (C) = 2, and inactivated (I) = 3, the power spectrum is given by

\[
r_{\text{slow}} = \frac{\text{var}_{\text{slow}}}{\text{var}} = \frac{(1 - h_i)P_e}{1 - h_i P_e} 
\]

(the variance of a Lorentzian distribution is \( \pi^2 A f_c^2 \), where \( A \) is the amplitude of the distribution at zero frequency and \( f_c \) is the corner frequency in Hz). The steady-state activation and inactivation curves can be used to estimate the theoretical \( r_{\text{slow}} \) and is shown in Fig. 10. There is, however, a large mismatch between the estimated and the predicted values, suggesting that the classical model does not account well for the observed variance. The fit was not improved by considering higher powers of \( P_e \).

A minimal canonical model that could account for the two Lorentzian components in the power spectrum has the following form

\[
G(f) = 4N(V - E_{nax})^2 P_i(\infty) \gamma^2 \sum_{\ell=2}^6 \frac{\lambda_{\ell}^j a_{i1}}{[1 + (f/\lambda_{\ell})^2]} 
\]

where \( f_c = \lambda_n/2\pi \) and \( a_{i1} \) is the elements of the first row and column of the \( \ell \)th spectral matrix. The spectral matrices are \( k \times k \) matrices, with elements \( a_{ij} \), with the \( r \)th spectral matrix corresponding to the \( r \)th eigenvalue (\( \lambda_r \)) and \( P_i(\infty) \) is the steady-state probability of the open state.

The expected power spectrum of this mechanism can be derived with the use of the \( Q \) matrix method of Colquhoun and Hawkes (1995). If states are numbered open (O) = 1, closed (C) = 2, and inactivated (I) = 3, the power spectrum is given by

\[
G(f) = 4N(V - E_{nax})^2 P_i(\infty) \gamma^2 \sum_{\ell=2}^6 \frac{\lambda_{\ell}^j a_{i1}}{[1 + (f/\lambda_{\ell})^2]} 
\]

where \( f_c = \lambda_n/2\pi \) and \( a_{i1} \) is the elements of the first row and column of the \( \ell \)th spectral matrix. The spectral matrices are \( k \times k \) matrices, with elements \( a_{ij} \), with the \( r \)th spectral matrix corresponding to the \( r \)th eigenvalue (\( \lambda_r \)) and \( P_i(\infty) \) is the steady-state probability of the open state.

A minimal canonical model that could account for the two Lorentzian components in the power spectrum has the following form

\[
G(f) = 4N(V - E_{nax})^2 P_i(\infty) \gamma^2 \sum_{\ell=2}^6 \frac{\lambda_{\ell}^j a_{i1}}{[1 + (f/\lambda_{\ell})^2]} 
\]

where \( f_c = \lambda_n/2\pi \) and \( a_{i1} \) is the elements of the first row and column of the \( \ell \)th spectral matrix. The spectral matrices are \( k \times k \) matrices, with elements \( a_{ij} \), with the \( r \)th spectral matrix corresponding to the \( r \)th eigenvalue (\( \lambda_r \)) and \( P_i(\infty) \) is the steady-state probability of the open state.

The expected power spectrum of this mechanism can be derived with the use of the \( Q \) matrix method of Colquhoun and Hawkes (1995). If states are numbered open (O) = 1, closed (C) = 2, and inactivated (I) = 3, the power spectrum is given by

\[
G(f) = 4N(V - E_{nax})^2 P_i(\infty) \gamma^2 \sum_{\ell=2}^6 \frac{\lambda_{\ell}^j a_{i1}}{[1 + (f/\lambda_{\ell})^2]} 
\]

where \( f_c = \lambda_n/2\pi \) and \( a_{i1} \) is the elements of the first row and column of the \( \ell \)th spectral matrix. The spectral matrices are \( k \times k \) matrices, with elements \( a_{ij} \), with the \( r \)th spectral matrix corresponding to the \( r \)th eigenvalue (\( \lambda_r \)) and \( P_i(\infty) \) is the steady-state probability of the open state.

The expected power spectrum of this mechanism can be derived with the use of the \( Q \) matrix method of Colquhoun and Hawkes (1995). If states are numbered open (O) = 1, closed (C) = 2, and inactivated (I) = 3, the power spectrum is given by

\[
G(f) = 4N(V - E_{nax})^2 P_i(\infty) \gamma^2 \sum_{\ell=2}^6 \frac{\lambda_{\ell}^j a_{i1}}{[1 + (f/\lambda_{\ell})^2]} 
\]

where \( f_c = \lambda_n/2\pi \) and \( a_{i1} \) is the elements of the first row and column of the \( \ell \)th spectral matrix. The spectral matrices are \( k \times k \) matrices, with elements \( a_{ij} \), with the \( r \)th spectral matrix corresponding to the \( r \)th eigenvalue (\( \lambda_r \)) and \( P_i(\infty) \) is the steady-state probability of the open state.

The expected power spectrum of this mechanism can be derived with the use of the \( Q \) matrix method of Colquhoun and Hawkes (1995). If states are numbered open (O) = 1, closed (C) = 2, and inactivated (I) = 3, the power spectrum is given by

\[
G(f) = 4N(V - E_{nax})^2 P_i(\infty) \gamma^2 \sum_{\ell=2}^6 \frac{\lambda_{\ell}^j a_{i1}}{[1 + (f/\lambda_{\ell})^2]} 
\]

where \( f_c = \lambda_n/2\pi \) and \( a_{i1} \) is the elements of the first row and column of the \( \ell \)th spectral matrix. The spectral matrices are \( k \times k \) matrices, with elements \( a_{ij} \), with the \( r \)th spectral matrix corresponding to the \( r \)th eigenvalue (\( \lambda_r \)) and \( P_i(\infty) \) is the steady-state probability of the open state.

The expected power spectrum of this mechanism can be derived with the use of the \( Q \) matrix method of Colquhoun and Hawkes (1995). If states are numbered open (O) = 1, closed (C) = 2, and inactivated (I) = 3, the power spectrum is given by

\[
G(f) = 4N(V - E_{nax})^2 P_i(\infty) \gamma^2 \sum_{\ell=2}^6 \frac{\lambda_{\ell}^j a_{i1}}{[1 + (f/\lambda_{\ell})^2]} 
\]

where \( f_c = \lambda_n/2\pi \) and \( a_{i1} \) is the elements of the first row and column of the \( \ell \)th spectral matrix. The spectral matrices are \( k \times k \) matrices, with elements \( a_{ij} \), with the \( r \)th spectral matrix corresponding to the \( r \)th eigenvalue (\( \lambda_r \)) and \( P_i(\infty) \) is the steady-state probability of the open state.
FIG. 9. Spectral density of the persistent Na current. A: spectral power of persistent Na currents elicited by voltage steps to −45 and −25 mV from a holding potential of −80 mV. Inset: current response to voltage step from −80 mV to −25 mV (peak truncated). Rectangle, time period used for the power estimates; solid line, corresponds to the best fitting sum of 2 Lorentzians, with \( f_1 \) and \( f_2 \) (see Eq. 8) = 406, 308 and 6.9, 10 Hz (−45 and −25 mV, respectively). Data were sampled at 5 kHz and filtered at 2 kHz with a Bessel filter and processed as described in METHODS. The power spectrum is result of averaging 9 overlapping segments (total record length is 13,592 points). Voltage dependence of the corner frequency (B) and amplitude (C) of the 2 spectral components of the persistent Na current. D: normalized conductance and variance of \( I_{Na} \) (1.0 Na).

The response of the minimal kinetic model to a voltage step is, if only the closed state (2) is populated at the start

\[
I_i(t) = I(\infty) + N(V - E_{Na}) \gamma P_2(0) \sum_{i=2}^{k} a^{(i)}_{ij} e^{-\alpha_i t}.
\]

where \( P_2(0) \) is the probability of the closed state before the application of the step.

The Maple V symbolic algebra language was used to derive analytically the spectral matrices \((a_{ij}^{(r)})\) in terms of the four rate constants. Maple was then used to calculate the value of rate constants from Eq. 8 with the use of the measured eigenvalues and power spectral amplitudes. At all voltages up to three solution sets were consistent with the measured parameters. The measured probability of opening was used to select the appropriate solution. The estimated time constants are graphed in Fig. 11A. The model predicts that the activation of the persistent Na current is rapid with an inactivating component that decays with a time constant of \(~10\) ms to steady-state persistent components (Fig. 11, B and C). The model suggests, as we found experimentally (Fig. 3), that \( I_{Na} \) has an inactivating component.

DISCUSSION

A clearly discernible persistent current can be activated in acutely dissociated Purkinje somata. The current activates at a lower potential than its inactivating counterpart, but it was not possible to distinguish the currents on the basis of the measured steady-state activation and inactivation of Na current (○) and window current, an unequivocal answer regarding its origin could not be arrived at by a consideration of macroscopic voltage step responses or current fluctuations alone. How-
To facilitate the analysis of threshold, FitzHugh (1960) utilized the difference in timescales of the gating variables to reduce the Hodgkin–Huxley equations to a two-dimensional system, which allows a clear understanding of the determinants of threshold. The system is referred to as the $V$–$m$ system because only voltage ($V$) and $m$ (Na channel gating) are dependent variables. This set of equations maps nicely onto the system under study here, and the equations can be written as

$$\frac{dV}{dt} = \frac{1}{C} [I - g_{\infty}^m P(V - E_N) - g_L(V - E_L)]$$

$$\frac{dP}{dt} = \frac{P_r(V) - P}{\tau_m}$$

where $P_\infty$ is given by Eq. 3 and $\tau_m$ is the time constant of activation (which is assumed constant, $\tau_m = 0.8$ ms).

The intersections of the two nullclines (i.e., $dV/dt = 0$ and $dm/dt = 0$) demarcates three singular points: the filled circle and the solid circle are stable and correspond to the rest state and excited state, respectively, whereas the filled triangle is a saddle point that essentially determines the threshold properties of the system (Fig. 12A). If the system is perturbed from rest by an infinitesimally short current pulse it will be driven to a point parallel to the $V$ axis (horizontally).

**Effect of $I_{PNa}$ on neuronal threshold**

Neurons do not, as was first demonstrated by Pecher (1939), exhibit a precise threshold. If the probability of firing is estimated as a function of input amplitude, the resulting curves do not show a steplike transition to unity firing but typically show a graded transition. It was perhaps first appreciated by Verveen and Derksen (1966) that the probabilistic nature of firing results from channel noise, providing the first evidence for the discrete stochastic nature of ion channels. Lecar and Nossal (1971a,b) incorporated channel noise into a Hodgkin–Huxley model of reduced dimension and derived a relationship for the relative spread (RS) in terms of channel parameters. It was shown by Sigworth (1980) that their model accounted well for the experimentally observed RS in node of Ranvier.
zontal arrow in Fig. 12A, inset), and then its motion on the phase plane will be determined by the dynamics of the system. If the system is deposited to the right of the separatrix it will be swept to the excited state (open circle). While if it falls short of the separatrix it will return to rest.

The $V$–$m$ system has an infinitesimally sharp threshold, whereas neurons exhibit a smooth transition to unity probability of firing. Lecar and Nossal (1971a,b) extended the $V$–$m$ model by incorporating channel noise and derived a relationship for the RS of the transition to unity probability of firing in terms of the channel parameters. They found that the probability of firing for a short-duration stimulus could be described by the following equation

$$P(\text{fire})|I = 0.5 \left[ 1 + \text{erf} \left( \frac{I - I_0}{\text{RS} - I_0} \right) \right]$$

(11)

where $I$ is the stimulating current and $I_0$ is the threshold current that has the same form as experimental firing probability curves (Verveen 1961) and has derived an analytic expression for RS (Lecar and Nossal 1971b).

The Lecar–Nossal equations were used to compute the RS of the $V$–$m$ system in Purkinje neurons. Average parameters were capacitance = 20 pF, $g_{Na}^{\text{th}} = 8.5$ nS, $g_l = 0.5$ nS, $E_{Na} = 53$ mV, and $E_l = -65$ mV. Parameters for the activation of $I_{Na}^p$ were $V_{10} = -38.0$ mV and $\delta = -5.2$ mV. The conductance of $I_{Na}^p$ at 37°C was estimated with the use of the measured $Q_{10}$ and the average conductance measured at room temperature (1.9 nS). If the persistent Na current were carried by a channel with a maximal probability of activation of unity, the number of channels is estimated to be 162 and the RS would be 0.0097. If as in the cells studied here $\phi = 0.027$, then the number of channels is 6,000 and the RS is 0.0006, 16 times lower than in the unitary probability model. The expected firing probability curves are plotted in Fig. 12B. The utilization of a channel with low probability of activation significantly sharpens the current threshold of the system. This is only an approximation and does not take into account single-channel kinetics. For a more accurate picture the full stochastic differential equation should be studied, as was done by Skauken and Walloe (1979), who showed that if channel numbers are low the stochastic dynamics of single channels can have a significant impact on both the firing dynamics and threshold of neurons.

Functional significance of $I_{Na}^p$

Recently much attention was given to the noninactivating Na currents in central neurons and their possible functional significance (Crill 1996). The precise effect of $I_{Na}^p$ depends on its interaction with the leak conductance, other conductances active in the subthreshold range, and the cells morphology (Cannon et al. 1993; White et al. 1995). At least three distinct properties are conferred by this persistent inward current: nonlinear synaptic gain, plateau potentials, and subthreshold oscillations.

SYNAPPTIC GAIN MODULATION. Because a graded voltage-gated conductance can effectively interact with ligand-gated potentials, the first possibility to consider is amplification of excitatory synaptic inputs. The clearest result in Purkinje cells is the amplification of the climbing fiber activation. The complex spike burst produced in these cells by the activation of the climbing fiber has been well characterized electrophysiologically (Eccles et al. 1966). As many as 300 separate active zones are simultaneously activated by this unique single fiber input that covers the majority of the smooth dendritic branching system in that cell. Although the dendrites are endowed with voltage-gated calcium conductances (Tank et al. 1988), nevertheless $I_{Na}^p$ and the resurgent current appear to be essential in the generation of the prolonged complex spike waveform at Purkinje cell somata implicating this ionic conductance in spike initiation at the Purkinje cell axon, the ultimate role of the climbing fiber system. Synaptic gain modulation for $I_{Na}^p$ as well as excitability modulation were shown in neocortical neurons (Schwindt and Crill 1995; Stuart and Sakmann 1995).

PLATEAU POTENTIALS. Perhaps equally important is the role of $I_{Na}^p$ in neuronal inhibition. A neuron having a sustained plateau potential would be effectively silenced by the inactivation of $I_{Na}$. Resetting the membrane potential to its resting level by a hyperpolarizing input would lift the inhibition.

OSCILLATORY PROPERTIES OF CENTRAL NEURONS. $I_{Na}^p$ was shown to be essential in subthreshold membrane oscillation and thus on the temporal coherence of neuronal assemblies. A noninactivating membrane oscillation was initially recorded for layer II stellate neurons in the entorhinal cortex (Alonso and Lliná 1989). This TTX-sensitive Na conductance was activated at ~50 mV and was shown to support 10-Hz oscillations, which served as the basis for a 10-Hz, spike-generating attractor. More recently oscillatory events at frequencies ~40 Hz were encountered in cortical interneurons (Gutfreund et al. 1995; Lliná et al. 1991), which are also dependent on $I_{Na}^p$.

A role for persistent Na currents may be anticipated in neuropathies on the basis of clear precedents in muscle. For example, the inherited muscle disorders myotonia and periodic paralysis both result from defects in Na channel inactivation (Cannon 1996). It seems likely that some of the hyperexcitability characteristic of epilepsy and other neuropathies may result from the proteolysis of Na channels. The ease with which inactivating Na conductance can be converted into a persistent conductance (Armstrong et al. 1973) by exogenous proteases suggests that cells might have mechanisms to protect channels from indiscriminate proteolysis by the ubiquitous intracellular proteases.

Origins of $I_{Na}^p$

$I_{Na}^p$ was not an invariant characteristic of all Purkinje cells; 18.9% of 37 neurons exhibited no detectable $I_{Na}^p$. So, if Na channels do exhibit mode switching, there is some degree of variability in the process, perhaps indicating that the probability of mode switching is determined by the state of post-translational modification of the channel. Moreover, if a fixed proportion of Na channels entered the persistent mode, one would expect the steady-state inactivation of $I_{Na}^p$ to parallel that of $I_{Na}$. In practice they differ considerably (Fig. 2B), arguing against a straightforward mode-switching mechanism.

The average maximal conductance of $I_{Na}^p$ in the Purkinje cell somata was 118 nS corresponding to 7,375 channels, with a single-channel conductance of 16 pS (Alzheimer et
al. 1993). With the use of Eq. 4 and the average values of \( \phi \) (0.027), \( g_{Na}^p \) (1.9 nS), and \( \gamma_{Na} \) (11.7 pS), we estimate that there are 6,000 \( I_{Na}^p \) channels. The approximate match in the numbers of channels suggests that, if mode switching is occurring, the channel spends most of the time in the persistent state. It should be noted that Raman and Bean (1997) recorded a single instance of a channel that showed persistent activation during a 40-ms pulse in isolated rat Purkinje cells, but the channel had a conductance of 20 pS.

Although \( I_{Na}^p \) in Purkinje cells exhibits some inactivation, it appears to be less rapid than that observed in other preparations. For example, Fleidervish and Gutnick (1996) found that a prepulse of 10-s duration to 20 mV led to a 70% reduction of the persistent component, whereas in this study during a pulse to –20 mV the current inactivated <10%. Only with prepulses of 50 s did the inactivation of the current approach that observed by Fleidervish and Gutnick.

In considering the origins of \( I_{Na}^p \), it is appropriate to recall that \( I_{Na} \) can be transformed into a persistent current by proteolysis. For example, in both squid axon (Armstrong et al. 1973) and neocortical pyramidal neurons (Brown et al. 1994) treatment with internal protease renders the Na channel nonactivating, and it might be argued that the persistent Na conductance observed in the acutely isolated Purkinje is an artifact of the isolation procedure. However, the presence of \( I_{Na}^p \) in guinea pig cerebellar slices argues against this mechanism.

The difference in temperature dependence of the amplitudes of the persistent and inactivating currents does not unequivocally point to different channels because the current amplitudes are complex functions of the gating rates, and these may show differential sensitivities to temperature. The marked difference in temperature dependence of the conductances may have important consequences for the physiology because the ratio of the two currents can have a powerful effect on the neuronal dynamics.

We did not consider in any detail whether the two components of the inactivating Na conductance correspond to two distinct channels. Similar kinetics were observed in heterologously expressed Na channels and were attributed to mode switching (Moorman et al. 1990), and a similar mechanism may account for the components observed in this study. It does not appear to be the case for the Na channels in Purkinje cell somata that the kinetic components interconvert. So, if the slow component declines, there is no proportionate increase in the fast component, as was seen to be the case for rat skeletal muscle Na channels expressed in oocytes (Ji et al. 1994).

Anomalous tails, similar to the ones seen in this study, were observed by Raman and Bean (1996) in acutely isolated rat Purkinje cells. They found that hippocampal CA3 neurons did not display the resurgent tails, suggesting the presence of a distinctive Na conductance in cerebellar Purkinje cells. Evidence for passage through the open state during recovery from inactivation was also found in calcium (Slesinger and Lansman 1991) and potassium channels (Demo and Yellen 1991). Resurgent currents may be anticipated to endow cells with unique firing properties. During the repolarization phase of the action potential, resurgent currents should be activated, leading to an afterdepolarization that should predispose the cell to fire a burst of action potentials.

**Conclusions**

In the absence of specific channel blockers, it is seldom possible from macroscopic currents or noise alone to arrive at a clear attribution of the molecular origins of the various kinetic components. We find nothing in our data inconsistent with a mode-switching mechanism; however, if this mechanism does prevail, our results suggest that it does so in a fashion that is more complicated than a fixed failure rate of the inactivation gate. Our results could be explained equally well by a channel distinct from the inactivating Na channel.

The tendency of a Na channel to exhibit persistent activation may be associated with certain channel variants or with some of the ancillary proteins (e.g., \( \beta \) subunits) (Isom et al. 1994) associated with the \( \alpha \) subunit, which forms the core of the Na channel. The molecular biology of Na channels has a number of gaps. Five neuronal rat \( \alpha \) subunits were cloned (types I, II, IIA, and III and rat NaCh6), but there are indications that there are \( \approx 10 \) Na channel genes in the rat (Schaller et al. 1995). The recent finding by Raman et al. (1997) that both the persistent and resurgent current are greatly diminished in \( Scn8a \) (orthologous to rat NaCh6) null mice might be taken to indicate that both currents can be attributed to a single channel; however, a number of findings stand in the way of this interpretation. Raman and Bean’s (1997) single-channel recordings provided evidence that the same channel that supports a transient component is also responsible for the resurgent component but not for the persistent component. At the molecular level evidence was found for at least two transcripts of the \( Scn8a \) gene (Plummer et al. 1997). Although in hippocampal CA3 pyramidal neurons \( Scn8a \) is transcribed (Schaller et al. 1995), no resurgent current is observed (Raman and Bean 1997). Single-cell polymerase chain reaction studies in adult guinea pig Purkinje cells identified orthologs of type I and NaCh6 (De Miera et al. 1997). Recently, Smith and Goldin (1998) successfully expressed the rat I Na channel, one that resisted expression for over 10 years, and their results support the notion that rat I is the rapidly inactivating component of the Na current in Purkinje cells. All these observations suggest that \( I_{Na}^p \) and the resurgent current may indeed arise from a

**Origins of the anomalous noise characteristics of \( I_{Na}^p \)**

From published records it appears that switching from the inactivating to noninactivating state seldom occurs during the voltage-clamp pulses (Alzheimer et al. 1993). Therefore, in a mode-switching mechanism, the variance would be expected to be dominated by channels resident in the slowly or noninactivating state that are switching between closed and open states.

The precise origins of the excess noise remain unresolved in light of the available data. The model whereby the channels have a maximal probability of activation less than unity accounts for the form of the variance-voltage relationship; however, the fit to this model is not precise. Although we were not able to account fully for the form of the variance, it seems relevant that the noise close to threshold is far less than that predicted for conventional gating schemes. Teleologically speaking the channel seems to be tailored to minimize the noise at threshold and hence reduce the RS.

Downloaded from http://jn.physiology.org/ by 10.220.33.1 on May 6, 2017
single gene (Scn3a) but may ultimately be distinct channels that result from the alternative splicing, posttranslational modification or perhaps association with ancillary subunits that modify the channel gating. Resolution of these questions must await expression of Scn3a in a heterologous system.

This work was supported by grants from the National Institutes of Health and the Office of Naval Research to A. R. Kay, M. Sugimori, and R. Llinás. Address for reprint requests: A. R. Kay, Biological Sciences, 138 BB, University of Iowa, Iowa City, IA 52242, email: alan-kay@uiowa.edu

Received 17 October 1997; accepted in final form 11 May 1998.

REFERENCES


RAMAN, I. M., SPRUNGER, L. K., MEISLER, M. H., AND BEAN, B. P. Alternate...


