Brief Communications

Inwardly Permeating Na Ions Generate the Voltage Dependence of Resurgent Na Current in Cerebellar Purkinje Neurons

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Voltage-gated Na channels of cerebellar Purkinje neurons express an endogenous open-channel blocking protein. This blocker binds channels at positive potentials and unbinds at negative potentials, generating a resurgent Na current and permitting rapid firing. The macroscopic voltage dependence of resurgent current raises the question of whether the blocker directly senses membrane potential or whether voltage dependence is conferred indirectly. Because we previously found that inwardly permeating Na ions facilitate dissociation of the blocker, we measured voltage-clamped currents in different Na gradients to test the role of permeating ions in generating the voltage dependence of unblock. In reverse gradients, outward resurgent currents were tiny or absent, suggesting that unblock normally requires "knockoff" by Na. Inward resurgent currents at strongly negative potentials, however, were larger in reverse than in control gradients. Moreover, occupancy of the blocked state was prolonged both in reverse gradients and in control gradients with reduced Na concentrations, indicating that block is more stable when inward currents are small. Accordingly, reverse gradients shifted the voltage dependence of block, such that resurgent currents were evoked even after conditioning at negative potentials. Additionally, in control gradients, peak resurgent currents decreased linearly with driving force during the conditioning step, suggesting that the stability of block varies directly with inward Na current amplitude. Thus, the voltage dependence of blocker unbinding results almost entirely from repulsion by Na ions occupying the external pore. The lack of voltage sensitivity of the blocking protein suggests that the blocker's binding site lies outside the membrane field, in the permeation pathway.

Introduction

Resurgent Na current results from a rapid open-channel block and voltage-dependent unblock of voltage-gated Na channels by an endogenous protein (Raman and Bean, 1997; Grieco et al., 2002). Upon depolarization, Na channels open and bind the blocking protein more rapidly than the fast inactivation gate. Upon repolarization to negative potentials, the blocker unbinds, evoking a resurgent current that flows until channels either close (at strongly negative potentials) or inactivate (at moderately negative potentials) (Raman and Bean, 2001). Resurgent current is maximal near −30 mV, and its rise time decreases with hyperpolarization, indicating that the blocker dissociates from the channel more rapidly at more negative potentials. In cerebellar Purkinje cells, this cycle of opening and blocking with depolarization and rapid unblocking upon repolarization reduces the accumulation of Na channels in inactivated states, thereby facilitating high-frequency firing (Raman and Bean, 2001; Khaliq et al., 2003).

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The molecular mechanism of block remains an open question. The voltage dependence and kinetics of the endogenous open-channel blocker are mimicked by a free peptide replicating part of the intracellular tail of the Na channel β subunit $Na_V\beta4$, giving rise to the hypothesis that $Na_V\beta4$ is the endogenous blocking protein (Grieco et al., 2005). When heterologously expressed with Na channel α subunits, however, the $Na_V\beta4$ subunit cannot reconstitute block (Chen et al., 2008; Aman et al., 2009) suggesting that either $Na_V\beta4$ is not the blocker, or other factors are necessary for the intact subunit to function as a blocking protein. Supporting the latter interpretation, siRNA-mediated knockdown of $Na_V\beta4$ correlates with reduced resurgent currents in cultured cerebellar granule cells (Bant and Raman, 2008).

Because the putative blocking sequence of $Na_V\beta4$ immediately follows the transmembrane segment, however, a major question is how this region might interact with the channel in a voltage-dependent manner. To sense voltage directly, this segment must penetrate the channel sufficiently to detect the membrane field, which seems unlikely given its location. An alternative hypothesis is that the voltage dependence arises indirectly, as it does for other open-channel blocking compounds of both voltage-gated Na and K channels. Raising the extracellular concentration of permeant ions facilitates the expulsion of blockers from these channels via electrostatic repulsion, or "knockoff" (Armstrong, 1971; Shapiro, 1977; Demo and Yellen, 1991; Tang et al., 1996; Afshari et al., 2004). It is therefore possible that the voltage dependence of unbinding may be conferred by knockoff preceding voltage-dependent Na flux.

To investigate the relationship between unblock and permeation, we modified Na gradients to regulate the direction and amplitude of Na current. The results indicate that little resurgent current flows in the outward direction. Moreover, at potentials that favor unblock in normal Na gradients, block persists when the gradient is reversed. These data reveal that dissociation of the blocking protein has no absolute voltage sensitivity and instead depends almost entirely on inward permeation of Na ions.

Materials and Methods

Purkinje neurons were acutely dissociated (Raman and Bean, 2001) from C57BL/6 mice (P14–P21). Mice were anesthetized with halothane and decapitated according to guidelines approved by the Northwestern University Institutional Animal Care and Use Committee. The cerebellar cortex was minced in oxygenated ice-cold dissociation solution (in mM: 82 Na_2SO_4, 30 K_2SO_4, 5 $MgCl_2$, 10 HEPES, 10 glucose, 0.001% phenol red, pH 7.4 with NaOH), incubated with 3 mg/ml protease XXIII (31°C; 7 min), and microdissected in dissociation solution with 1 mg/ml bovine serum albumin and trypsin inhibitor. Neurons were isolated by trituration in Tyrode's solution (in mM: 150 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4 with NaOH).

Parafilm-wrapped borosilicate pipettes (1.5–3 MΩ; A-M Systems) were filled with intracellular solution (in mM: 108 HCH_3SO_3, 9 NaCl, 1.8 $MgCl_2$, 9 HEPES, 1.8 EGTA, 48 sucrose, 4.5 tetraethylammonium (TEA)-Cl, 14 Tris-creatine PO_4, 4 MgATP, and 0.3 TrisGTP) titrated to pH 7.4 with CsOH (control gradients) or NaOH (reverse gradients, total Na = 116 mM). Whole-cell voltage-clamped currents (series resistance compensated >85%) were recorded with an Axopatch 200B amplifier and pClamp 9.0 (Molecular Devices). Control extracellular solution contained the following (in mM): 150 NaCl, 10 TEA-Cl, 2 $BaCl_2$, 0.3 $CdCl_2$, 10 HEPES, 10 glucose, pH 7.4 with NaOH (total Na = 154 mM) ± 900 nM TTX. For reverse gradients, Cs replaced all but 15 mM extracellular Na (pH 7.4 with CsOH). "Near-symmetric" gradients consisted of reverse intracellular and control extracellular solutions. For "reduced control" gradients, Cs replaced all but 10% of the intracellular and extracellular Na. To improve voltage-clamp in control gradients, activation curves were recorded in 30–60 nM TTX. The K_d of TTX was estimated at 38 nM by measuring transient currents (0 mV) in 0–60 nM TTX, and conductance in 0 TTX was calculated. TTX-sensitive Na current was isolated by subtraction of recordings in 900 nM TTX from those in 0–60 nM TTX. Control and reduced control gradients gave predicted and measured reversal potentials of +73 mV. Reverse and near-symmetric gradients gave predicted reversal potentials of −53 and +7 mV, respectively, but measured reversals of −45 and +20 mV, consistent with 1% Cs permeation (Hille, 2001) and possibly some action of pumps and exchangers. Drugs were from Sigma-Aldrich, except TTX (Alomone).

Data were analyzed with IGOR-Pro (Wavemetrics) and reported as mean ± SEM. Resurgent current amplitudes were measured as the maximal current evoked upon repolarization, less the steady-state current after complete current decay. Conductance-voltage plots (from measured reversal potentials) were fit with Boltzmann functions, $G/G_{max} = 1/(1 + \exp(-(V - V_{1/2})/k))$, with conductance G, maximal conductance G_{max}, half-maximal activation voltage $V_{1/2}$, and slope factor k. Current decays were fit with single exponential functions, $I = A^*\exp(-t/\tau) + y_0$, with current amplitude A, time constant τ, and steady-state current y_0. Statistical significance was assessed with Student's two-tailed t tests. Asterisks and p values <0.05 indicate statistical differences from control. Capacitative artifacts were digitally reduced.

Results

To investigate the basis of the voltage dependence of resurgent current, we measured Na currents in reverse Na gradients. We reasoned that if the endogenous blocking protein itself senses the membrane field, the resurgent conductance at any potential should be independent of the direction of current flow. If, however, unblock instead depends on Na ions occupying the extracellular permeation pathway and expelling the blocker, then outward resurgent current might be minimal. In control gradients, after 10 ms conditioning steps to +30 mV, robust inward resurgent currents were evoked by repolarization to a range of voltages (Fig. 1*A*, top). In reverse gradients, however, outward resurgent currents were tiny and often undetectable (Fig. 1*A*, middle), although inward resurgent currents were consistently evoked at potentials negative to the reversal of −45 mV. To account for differences in driving force, conductance was computed, illustrating that reversing the gradient greatly reduced outward resurgent conductance (Fig. 1*B*). To test the possibility that outward resurgent current might be absent because the blocking protein does not bind the channel effectively when current is outward, we recorded currents in a "near-symmetric" gradient with a reversal of +20 mV, in which transient currents at +30 mV were outward but currents at negative potentials were inward. Under these conditions, robust resurgent currents were elicited, often exceeding those in control (Fig. 1*A*, bottom), indicating that outward current does not prevent association of the blocker with the channel (Fig. 1*B*, gray symbols).

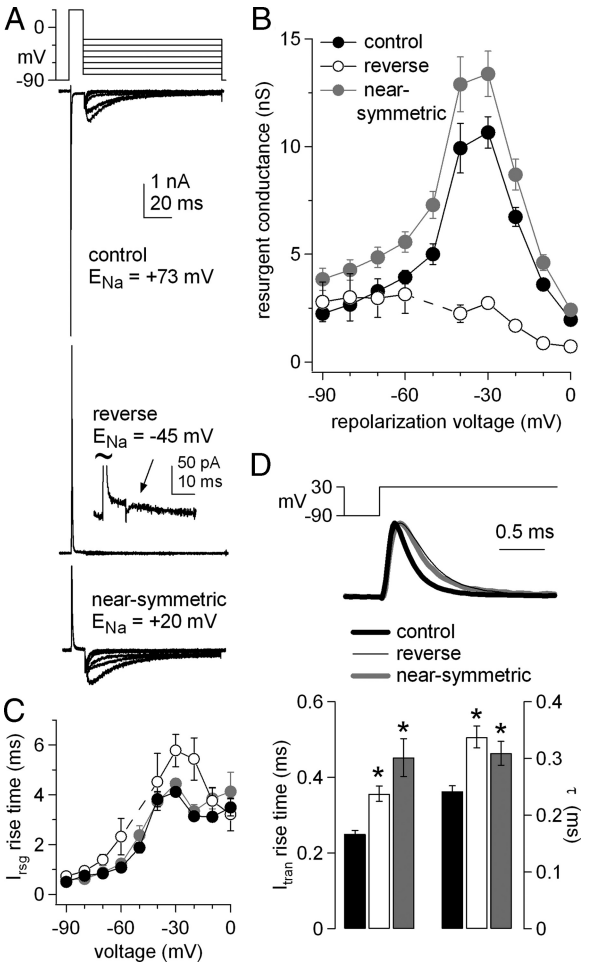

Figure 1. Reverse Na gradients reduce outward resurgent currents. ***A***, Voltage protocol and representative traces of resurgent currents. Inset, Outward resurgent current at −30 mV in reverse gradient at high gain. ***B***, Mean resurgent conductance versus voltage for control (n = 17), near-symmetric (n = 10), and reverse (n = 8) gradients. ***C***, Resurgent current rise time versus voltage for control (n = 16), reverse (n = 8), and near-symmetric (n = 10) gradients. Symbols as in ***B***. ***D***, Voltage protocol and normalized representative traces (top). All traces are plotted upward for comparison. Bottom, mean rise time (left) and decay τ (right) for control (n = 17), reverse (n = 8), and near-symmetric (n = 7) gradients.

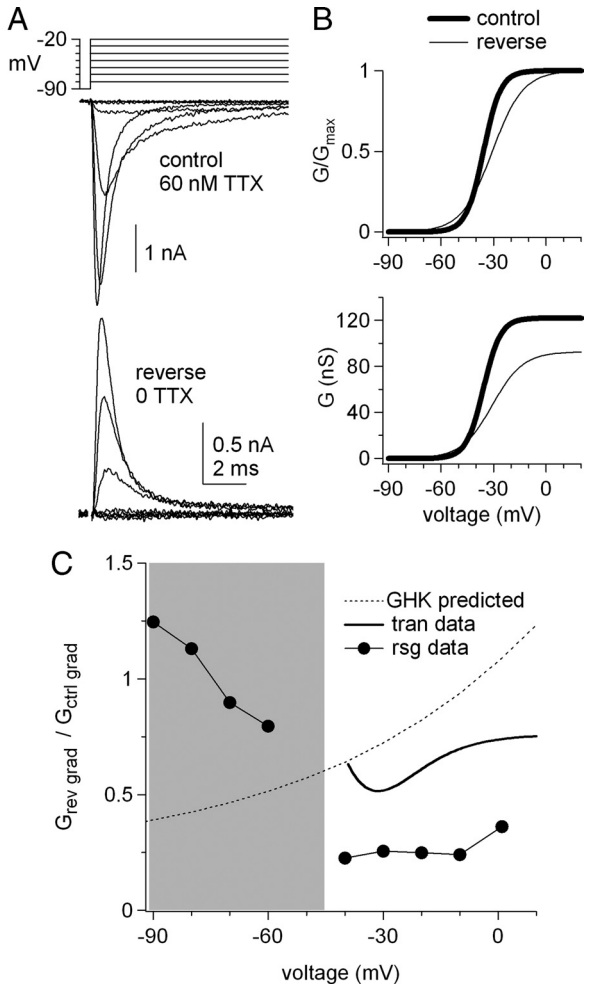

Figure 2. Reverse Na gradients differentially affect transient and resurgent Na currents. ***A***, Voltage protocol and representative traces for Na current activation. ***B***, Activation curves with mean fit parameters for control and reverse: $V_{1/2} = -34.3$, -30.7 mV; $k = 5.1$, 8.8 mV; $G_{max} = 122, 92$ nS; $n = 16, 8$. Conductance is normalized (top) for comparison of $V_{1/2}$ and k, and raw (bottom) for comparison of G_{max}. ***C***, Conductance ratio of reverse to control gradients for predictions by the GHK current equation, and measured transient and resurgent conductances. Shaded area indicates voltages below reversal potential in reverse gradients.

The Na gradient, however, affected multiple aspects of channel gating, including the kinetics of resurgent as well as transient currents: In the reverse gradient, the rise times of resurgent currents were prolonged relative to control and near-symmetric gradients (Fig. 1*C*). Likewise, the rise time of transient currents at +30 mV was increased when currents were outward (reverse, near-symmetric) and the decay time constant was concomitantly prolonged (Fig. 1*D*). The parallel sensitivity of resurgent and transient kinetics to the gradient raised the possibility that the changes in resurgent conductance simply reflected changes in the transient conductance resulting from shifts in voltage dependence, single-channel conductance, or the properties of inactivation and block. To explore this possibility, we examined the voltage dependence of activation of transient currents in control and reverse gradients (Fig. 2*A*). Activation curves were calculated for individual cells and normalized curves with average parameters were compared (Fig. 2*B*, top). In reverse gradients, the slope factor, k, but not the $V_{1/2}$, was increased (control, $k = 5.1 \pm 0.2$, reverse, 8.8 ± 0.8 mV, $p < 0.05$; control, $V_{1/2} = -34.3 \pm 1.1$, reverse, -30.7 ± 1.4 mV, $p > 0.05$), and G_{max} was reduced by approximately 25% (Fig. 2*B*, bottom, calculated control, $G_{max} = 122 \pm 5.3$, reverse, 92.7 ± 10.7 nS). To assess whether the reduction of outward currents resulted from the changes in ionic concentrations, we compared the relative conductance in reverse and control gradients predicted by the Goldman-Hodgkin-Katz current equation with the experimental data. Plotting predicted and measured conductance ratios illustrated that both the transient and resurgent outward conductances were reduced more than predicted by ionic conditions alone (Fig. 2*C*). The extra decrease in conductance can be partly attributed to a decreased single-channel conductance, γ, as nonstationary variance analysis, validated by cell-attached recordings, estimated that γ at 0 mV was ~15 pS in control and ~5 pS in reverse gradients (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Nevertheless, at voltages above the reversal potential for reverse gradients, the resurgent conductance ratio was reduced more than the transient conductance ratio. In contrast, at voltages below the reversal potential for reverse gradients, where transient currents did not activate, the resurgent conductance ratio far exceeded the GHK prediction. These data demonstrate that the channels generally function less efficiently in reverse gradients above the reversal potential, as manifested by the slow rise times and small conductance. Although multiple mechanisms may underlie this behavior, the reduction of transient currents is consistent with the idea that blocked states are favored when currents are outward. An increased occupancy of blocked states can also account for the larger resurgent currents elicited upon repolarization below the reversal potential.

To examine more directly whether the stability of block is affected by knockoff by inwardly permeating Na, we made use of previous evidence that the blocking protein binds rapidly upon depolarization to any potential, but unbinds over tens of milliseconds, and then is replaced by the fast inactivation gate (Raman and Bean, 2001; Aman and Raman, 2007). Consequently, resurgent current amplitudes in control gradients become smaller as conditioning step depolarizations are progressively prolonged. We therefore tested whether blocked states indeed last longer in reverse gradients by applying 5–50 ms conditioning steps to +30 mV and then repolarizing cells to −90 mV (Fig. 3*A*). Because channels that inactivate during the step remain unavailable upon repolarization, whereas those that remain blocked can reopen, the amplitude of resurgent current evoked at −90 mV gives a measure of the proportion of channels in blocked states at the end of the conditioning step. Consistent with previous results (Aman and Raman, 2007), resurgent current in control gradients decreased to ~50% over the first 50 ms of depolarization. In reverse gradients, however, resurgent currents did not decrease and even enlarged slightly, suggesting that binding of the blocker persisted throughout the 50 ms. To test whether exit from blocked states in control solutions depended directly on knockoff, we decreased the concentration of permeant ions while keeping reversal potential constant, by substituting 90% of the intracellular and extracellular Na with Cs. Indeed, this manipulation made resurgent current amplitudes remain high, supporting the idea that the transition to inactivated states depends on displacement of the blocker by external Na entering the channel (Fig. 3*A*).

Because reducing extracellular permeant ions promotes slow inactivation (Baukrowitz and Yellen, 1995; Townsend and Horn, 1997), we tested whether slow inactivation influenced these measurements by applying an additional test depolarization to 0 mV after the 20 ms repolarization to −90 mV (Fig. 3*B*). This interval allows recovery from blocked and fast inactivated states entered during the conditioning step to +30 mV, but not slow inactivated states (Aman and Raman, 2007). Transient currents evoked by

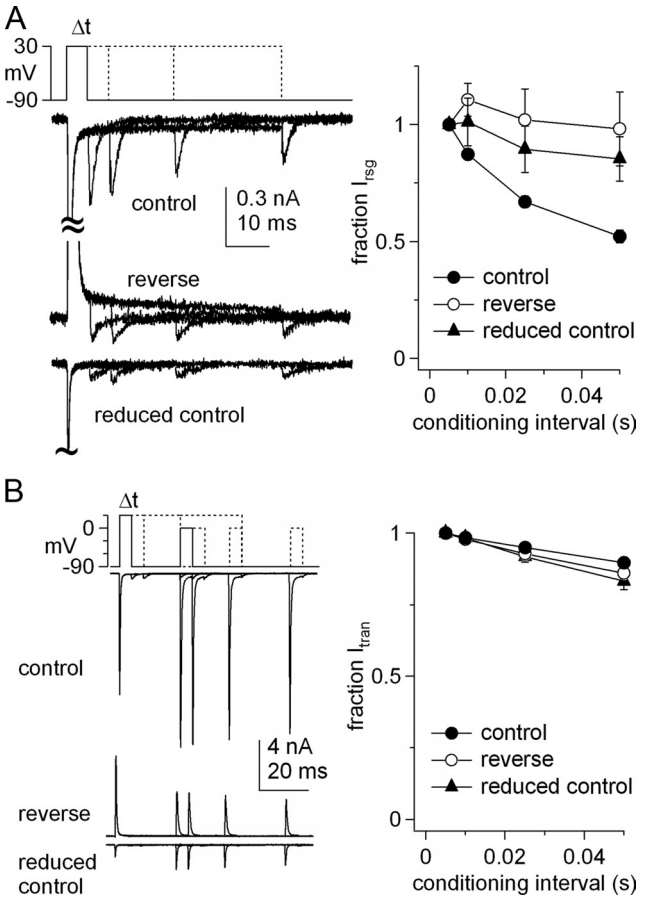

Figure 3. The blocker binds channels stably in reverse Na gradients. ***A***, Voltage protocol and representative resurgent currents after 5–50 ms conditioning steps (left). Normalized resurgent currents versus conditioning duration for control ($n = 6$), reverse ($n = 9$), and reduced control ($n = 9$, right). ***B***, Voltage protocol and representative transient currents at 0 mV to assess slow inactivation after 5–50 ms conditioning steps (left). The protocol is an extension of that in ***A***. Normalized transient currents versus conditioning duration for control ($n = 6$), reverse ($n = 7$), and reduced control ($n = 7$).

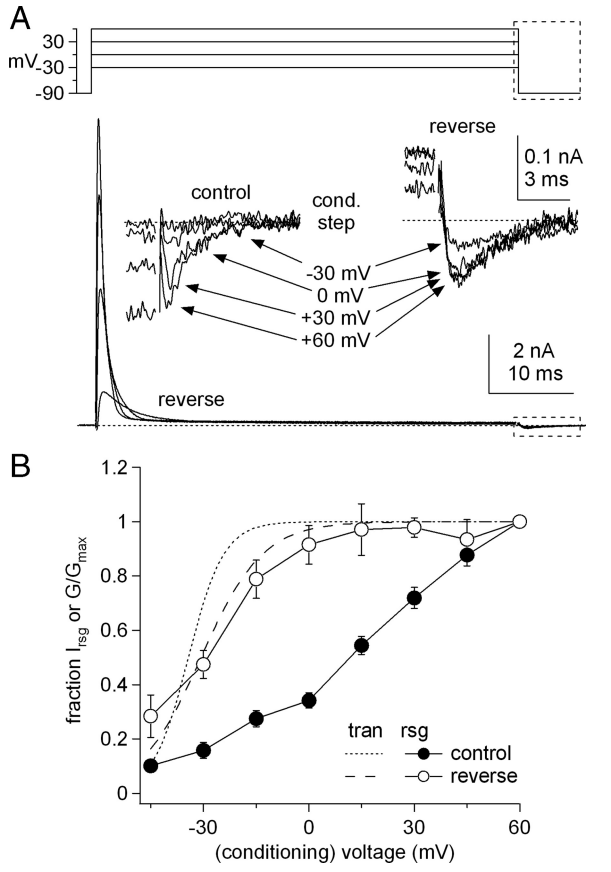

Figure 4. The blocker requires knockoff by inwardly permeating Na to unbind. ***A***, Voltage protocol (top) and representative traces (bottom and middle at high gain). ***B***, Mean fractional resurgent currents versus voltage for control ($n = 9$) and reverse ($n = 6$) gradients. Activation curves of transient current (Fig. 2) are replotted for comparison.

the test step decreased by ~10% in control gradients and ~15% in the other conditions (Fig. 3*B*), indicating that the larger reduction of resurgent current in the control condition likely results from the dissociation of the blocker and subsequent fast inactivation rather than from slow inactivation. Conversely, the small decrease of resurgent current after the longest steps in reverse and reduced control gradients likely reflects slow inactivation rather than loss of block. The results provide evidence that the blocking protein binds stably to the channel, preventing fast inactivation, when the probability of inward flux is decreased.

If unblock depends solely on knockoff, then changing the reversal potential should change the voltage dependence of resurgent current. Specifically, block should persist at negative potentials, as long as net currents (through available channels) are outward. To test this idea, we applied 50 ms depolarizations to a range of potentials and assayed resurgent currents in both control and reverse gradients (Fig. 4*A*). Resurgent currents evoked at −90 mV were normalized to those elicited after the largest conditioning step (+60 mV; Fig. 4*B*). Reverse gradients shifted the plot of resurgent current versus conditioning voltage in the hyperpolarizing direction. For instance, after conditioning at 0 mV, only 35% of maximal resurgent current was evident under control conditions, whereas it remained nearly 100% in reverse gradients, consistent with dissociation of the blocker resulting almost exclusively from inward permeation. At more negative voltages, however, normalized resurgent currents decreased even in reverse gradients, indicative of less open-channel block at the end of those steps. The maximal occupancy of blocked states that can develop during depolarization, however, is limited by the channel activation during each step. We therefore superimposed the mean activation curves of Figure 2 onto the plot of resurgent current versus conditioning step. This comparison revealed a match between the extent of activation and the amplitude of resurgent current for the reverse gradient where currents were outward, indicating that nearly all channels that opened remained blocked throughout the step. For example, at −30 mV, where Na channels were half-maximally activated, the resurgent current was also half-maximal, and when activation was maximal (>0 mV), so was resurgent current. In contrast, in control gradients, after complete activation (>0 mV), the amount of resurgent current increased linearly as the reversal potential was approached. In other words, the amount of unblock varied directly with the driving force during the conditioning step, suggesting that the voltage dependence of resurgent current is determined almost exclusively by the amplitude of inward Na flux predicted by driving force and concentration.

Discussion

These data provide evidence that the endogenous blocking protein responsible for resurgent current requires knockoff by permeating Na ions to unbind normally. First, in reverse gradients, the amplitude of the outward resurgent conductance is much

smaller than in control gradients. Second, after channels become blocked upon depolarization, the blocker remains bound in reverse gradients when currents are outward, whereas it gradually dissociates in control gradients when currents are inward. Likewise, when Na concentrations are reduced without changing the gradient, the displacement of the blocker during depolarization is diminished. Third, because blocked states persist as long as transient current is outward, repolarization elicits resurgent current in reverse gradients even after conditioning at negative potentials. Together, these results suggest that the blocking region itself does not sense the membrane field. Instead, the voltage dependence of blocker unbinding, and consequently of macroscopic resurgent current, is conferred indirectly by the voltage dependence and magnitude of current through the channel.

The open-channel blocking protein thus resembles the fast inactivation gate, whose voltage-dependent interactions with the channel arise largely from channel gating, rather than from voltage-sensing by the fast inactivation sequence itself (Armstrong and Bezanilla, 1977; Aldrich et al., 1983; Sheets and Hanck, 1995). Unlike recovery from fast inactivation, however (Kuo and Bean, 1994; Tang et al., 1996), recovery from blocked states is greatly facilitated by inward permeation, making resurgent conductances far greater for inward than for outward currents. Occasionally, however, small outward resurgent currents are detectable. Possibly, as the membrane is hyperpolarized, a movement of voltage-sensing domains toward a resting position modifies the exposure of the binding site, favoring dissociation of the blocker, as proposed for Shaker K channels (Gómez-Lagunas and Armstrong, 1994). Additionally, although net Na current is outward just positive to the reversal potential, the occasional occupancy of the external pore by Na may sometimes be sufficient to displace the blocker.

Changes in Na gradients also influence transient conductances. At any potential, the transient conductance depends on the single channel conductance, extent of activation, latency to opening, open probability, and onset of open-channel block; reversing the gradient affected multiple parameters. The prolonged rise and decay times and the apparently weaker voltage-sensitivity of gating reflected by larger k-values, suggest direct effect of gradients or flux on activation properties. The reduced outward transient conductance may result partly from these changes in gating, as well as from the diminished single-channel conductance of outward currents. Because the concentrations of permeant ions predict a conductance at 0 mV that is nearly identical to that in control gradients, the decreased γ suggests either an intrinsic rectification or filtering by rapid channel blockade by one or more factors. In other preparations, macroscopic transient Na currents in low external Na inwardly rectify because of pore blockade by intracellular polyamines (Huang and Moczydlowski, 2001), although it is unknown whether this block is sufficiently rapid to affect the single-channel conductance.

Resurgent conductances depend on the same parameters as transient conductances, but the extent of activation, latency to opening, and open probability are directly affected by the unbinding properties of the blocker. The greater reduction of resurgent than transient outward conductance in reversed gradients supports the idea that the stability of blocked states is increased in low external Na. Moreover, resurgent conductance increased with all manipulations predicted to raise inward current at negative but not positive potentials, suggesting that unbinding of the blocking protein is promoted by inwardly permeating Na ions repelling and displacing the blocker. Such knockoff mechanisms have been described for the expulsion of exogenous and endogenous factors, including quaternary ammonium ions from K channels (Armstrong, 1971), peptide blockers from Na channels (Tang et al., 1996), the Shaker inactivation gate (Demo and Yellen, 1991), and the blocker responsible for resurgent Na current (Afshari et al., 2004; the present data). Given the opposite directions of Na and K gradients, however, it is resurgent Na current that is most likely to be affected by inwardly permeating ions *in vivo*. In fact, even above 0 mV, the proportion of blocked channels is probably limited by inwardly flowing Na ions repelling the blocking protein. Consistent with this idea, when control concentrations were reduced tenfold, displacement of the blocker at positive voltages was minimal. Thus, during the upstroke of the action potential, Na influx likely antagonizes blocker binding, even as protein-protein interactions stabilize it (Grieco et al., 2005). The net block is substantial, however, as indicated by the large resurgent currents elicited after spike waveforms or brief steps to 0 mV (Raman and Bean, 1997; Aman and Raman, 2007). After prolonged hyperpolarizations, however, increased spike amplitudes may permit even more channel blockade during depolarization, followed by more reopening of channels upon repolarization, possibly facilitating burst firing.

These results have implications for the hypothesis that the $Na_V\beta4$ subunit is the natural open-channel blocker in Purkinje neurons (Grieco et al., 2005). The overlap of the resurgent current amplitude and transient conductance curves in Figure 4 indicates that the amount of block is directly proportional to the fraction of opened channels in reverse gradients, consistent with the idea that channel opening is a prerequisite for block. Moreover, the driving force on inward Na current during depolarization is directly proportional to resurgent current amplitude, indicating that the dissociation of the blocker is a direct consequence of knockoff by inwardly flowing Na. This idea is further supported by the rise times of resurgent currents: low external Na prolongs the rise time, both in reverse (Fig. 1) and reduced control gradients (rise time 0.9 ± 0.01 ms at -90 mV, $n = 9$). Given the sensitivity of the onset of block to channel activation, and unbinding to the concentration of external Na, the simplest interpretation is that the blocker associates with a site in the permeation pathway that is exposed upon channel opening. Because the putative blocking sequence of $Na_V\beta4$, KKLITFILKKTREK, directly follows the transmembrane segment, the present data suggest that it may obstruct the permeation pathway by simply bending and binding near the inner pore of the open channel, e.g., near the S6 cytoplasmic ends. Thus, despite the relatively restricted movement expected for the sequence, $Na_V\beta4$ remains a reasonable candidate for the endogenous blocking protein.

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