Flow and shortcuts along the Shaker Kv channel slow inactivation gating cycle

Valerie Abigail Nirenberg and Ofer Yifrach

Slow inactivation gating in Kv channels natively or artificially lacking the fast inactivation “ball and chain” segment is usually described by a simple four-state gating cycle describing the dependence of one gate opening on the conformational state of the second. In the current issue of the Journal of General Physiology, Szanto et al. address flow along the gating cycle of the Shaker-IR channel during the onset of slow inactivation at negative membrane potentials and the consequences of locking open inactivated channels on recovery from slow inactivation. The authors demonstrated that the onset of slow inactivation in the Shaker-IR Kv channel occurs through the open state at either negative or positive voltages and that O1–CI transition is mandatory for recovery from slow inactivation. The results provide essential missing evidence that contribute to more complete understanding of Shaker channel slow-inactivation gating.

Voltage-dependent potassium ion currents through the membrane play fundamental roles in generating and shaping action potentials, as well as in determining their conductance properties, frequency, and firing modes (Jan and Jan, 2012; Bean, 2007; Zeberg et al., 2010). Such potassium currents are regulated in both spatial and temporal dimensions, as respectively determined by the clustering (Nirenberg and Yifrach, 2020) and gating mechanisms of Kv channel proteins (Yellen, 1998), through which such currents pass. Three primary gates along the ion conduction pore domain of Kv channels control the timing and amplitude of current flow through the channel: the activation gate (A) and the fast (N-type) and slow (C-type) inactivation gates (\( I_{fast} \) and \( I_{slow} \), respectively). These gates undergo structural changes between open (O) and closed (C) states to allow or block K\(^+\) ion flow through the channel. Taking a reductionist view of Kv channel gating, and assuming that the inactivation “ball” can only “sneak” into its receptor site upon activation gate opening, six distinct but connected gating states are possible for a Kv channel, of which only one is conductive (\( A_{0,0} I_{fast} I_{slow} \), Fig. 1 A). This thermodynamic view allows for addressing of the coupling between the activation and slow inactivation gates (left cycle) and between the two, fast and slow, inactivation mechanisms of the channel (right cycle). This Kv channel gating scheme can be simplified by assuming a Kv channel natively or artificially (\( \Delta I \) or IR) lacking the fast inactivation ball and chain N-terminal module (Fig. 1 B; Hoshi et al., 1991). In this issue of the Journal of General Physiology, Szanto et al. (2020) studied slow inactivation gating in the prototypical Shaker-\( \Delta I \) Kv channel using the “duo modal” gating mechanism described in Fig. 1 B (Yellen, 1998; Panyi and Deutsch, 2006; Cuello et al., 2017) to address flow and shortcuts along the cycle during onset and recovery from slow inactivation at negative membrane potentials. Their results, obtained through an elegant experimental design, provide essential missing evidence that contributes to a more complete understanding of Shaker channel slow inactivation gating.

Initially, the authors asked whether the sequence of gating transitions leading to entry into and recovery from slow inactivation (Fig. 1 B) occurs along the same path at both extreme depolarizing and hyperpolarizing potentials. This may seem to be a trivial question at first. However, since voltage affects the stability of the different gating states along the cycle to different extents (and hence, the probability of the different gating transitions), a distinct slow inactivation gating sequence could well occur at very negative voltages, possibly through the closed channel state (i.e., the C–CI transition). The possibility of closed-state inactivation seems reasonable, considering the authors’ observation that steady-state slow inactivation in the Shaker-\( \Delta I \) channel seems to occur even at hyperpolarizing voltages where no detectable activation was noted (see Fig. 2 in Szanto et al., 2020). To discern whether slow inactivation of the Shaker-\( \Delta I \) channel at negative voltages occurs through the closed or open state, given how minute K\(^+\) currents traverse the channel in this voltage range, a sensitive quantitative handle to monitor the open state is needed. For this, the authors used the T449A;V474C double-mutant Shaker-\( \Delta I \) channel that accelerates slow inactivation to a reasonable rate during recordings (T449A) (Hoshi and Armstrong, 2013) and which reacts with Cd\(^{2+}\) ions through cysteine-474 only in the open state (Liu et al., 1997; Panyi and Deutsch, 2006). Cd\(^{2+}\)-binding blocks K\(^+\) currents through the channel in a manner proportional to the probability...
of the channel being open (del Camino and Yellen, 2001). The characteristic steady-state activation and slow inactivation gating parameters of this mutant are comparable to those of the WT channel (see Fig. 2 in Szanto et al., 2020).

The authors devised an elegant three-pulse protocol and used inside-out patch clamp electrophysiological recordings to evaluate the pathway for onset of slow inactivation in the Shaker-ΔI 

The diagram shows four states of the Kv channel: C (closed), O (open), CI (closed-inactivated), and OI (open-inactivated). The authors evaluate the pathway for slow inactivation onset and discuss the role of Cd²⁺ ions in blocking potassium currents.

The authors elegantly demonstrate how closed- or open-state inactivation at negative potentials occurs solely through the closed state, then I₁ should be equal to I₃, as all channels are in the closed state before the third 50 mV voltage shift (blue box under H3). Whether or not Cd²⁺ is present in the bath solution during H2 does not matter, as Cd²⁺ can block potassium currents only when the channel is open (Liu et al., 1997). If, however, at this voltage, slow inactivation occurs through the open channel state and all available open channels are modified by Cd²⁺ ions, then I₁ should be smaller than I₁ yet equal to I₂ (I₁ > I₂). I₅ would be lower than I₁ because, given the long (20 s) -90-mV holding interval (segment H2), Cd²⁺ would bind to all available open channels and block their ion conduction pathways, making this channel fraction transparent in electrophysiological recordings, irrespective of whether the bound channel is open, closed, or in the inactivated state. At the ~120-mV holding interval before the third pulse (segment H3), this fraction of modified channels would assume the closed state (red box under H3) and not contribute to observed peak current during the onset of the third pulse, thus rendering I₅ = I₃.

These considerations are summarized by the quantity of recovered current fraction (RCF) defined by the authors as RCF = (I₁ - I₃) / (I₁ - I₂) (see Fig. 3 in Szanto et al., 2020). This quantity elegantly demonstrates how closed- or open-state inactivation at negative potentials can be discriminated using the three-pulse protocol. RCF equals 1 when slow inactivation at negative potentials occurs solely through the closed state, in which case I₁ = I₃. At the other extreme, an RCF of 0 reflects the case where slow inactivation occurs predominantly through the open state and peak current amplitude (I₂) at a depolarizing voltage of +50 mV (P2) following a long ~90-mV holding interval (H2) and in the presence of Cd²⁺, relative to two identical peak current amplitudes (I₁ and I₃) obtained upon a ~120 to +50 mV pulse, with one preceding the second pulse (P1) and the other (P3) following P2.
assuming all channels open at −90 mV are Cd²⁺-bound ($I_3 = I_2$). RCF values close to 0 (0 < RCF << 1) reflect slow inactivation through the open or closed states. The voltage and duration of each holding (H) or pulse (P) step is indicated, as is when Cd²⁺ ions (black circle) were applied (solid line above segment H2). Below each H or P segment, the probable channel states are indicated, as principally and qualitatively determined using the law of mass action along the slow inactivation gating cycle of the Shaker-$\Delta I$T449A channel (Fig. 1 B). The red or blue gating sequences along the cycle shown below the −90-mV holding segment (H2) correspond to the onset of slow inactivation through the open or closed state, respectively. Cd²⁺-binding steps or gating transitions among Cd²⁺-bound states are indicated by black arrows. Channel gating states in which Cd²⁺ is bound are indicated by an asterisk. The magnitudes of forward and backward rate constants, determining the prevalence of each state, are qualitatively indicated by the length of each arrow. Note that the timescales of the different holding intervals are not scaled. To monitor whether Cd²⁺-modification depends on the probability of the channel being open, the protocol was repeated, setting the H2 holding voltage to either −80 or −70 mV. (B) The protocol used by Szanto et al. (2020) to investigate whether the OI–CI gating transition of the Shaker-$\Delta I$T449A channel is mandatory for recovery from slow inactivation. Locking the channel in the OI state is achieved by applying Cd²⁺ toward the end of the pulse protocol (solid line). The creation of a Cd²⁺-mediated inter-subunit metal ion cross-bridge locks the channel open (OI), thus preventing the OI–CI transition and providing a shortcut for flow along the slow inactivation gating cycle (see text for further details). $n$ represents the multiple times the protocol was repeated (represented by the letter x).

Following steady-state characterization of the activation and slow inactivation gating properties of the Shaker-$\Delta I$T449A; V474C channel (see Fig. 2 in Szanto et al., 2020) and proper controls (see Fig. 4 in Szanto et al., 2020), the authors showed that at a negative holding potential (−70, −80, or −90 mV) applied in H2 before the middle P2 test pulse (in the presence of 20 µM Cd²⁺ in the bath solution), RCF values between 0.1 and 0.4
were obtained for the double-mutant channel (see Fig. 5 in Szanto et al., 2020). Furthermore, the RCF is both voltage- and [Cd²⁺]-dependent with increased depolarization or increased Cd²⁺ concentrations, resulting in lower, close to 0 values (see Fig. 5 C in Szanto et al., 2020). Lastly, whether Cd²⁺ modification of the double mutant at a given negative voltage (e.g., −90 mV) achieved through one long exposure or multiple cycles of short exposures yields identical results with respect to entry into slow inactivation (see Fig. 6 in Szanto et al., 2020). Given the close to 0 RCF values obtained, the results revealed that the onset of slow inactivation in the Shaker-ΔI T449A;V474C channel at negative membrane potentials occurs through the open channel state. Thus, despite the lack of observable activation currents at −90 mV (see Fig. 2 in Szanto et al., 2020), the pathway for onset of slow inactivation at negative voltage passes through the C→O→OI gating sequence, as observed at depolarizing voltages (Panyi and Deutsch, 2006; Kurata and Fedida, 2006). Here, however, the sequence of steps continues onto the CI state, since at −90 mV the lower activation gate is predominantly stabilized in its closed state. Thus, unlike other Shaker Kv channel mutants that exhibit dramatically altered gating properties, no evidence for direct C→CI closed-state slow inactivation gating transition was found for the WT-like double-mutant channel studied by Szanto et al. (2020).

To investigate whether OI→CI transition is mandatory for Shaker-ΔI recovery from slow inactivation (Panyi and Deutsch, 2006; Kurata and Fedida, 2006), the authors used the T449A; V476C double mutant. Previous results indicated that the V476C mutant enables locking the activation gate of the channel in the open state, even at negative membrane potentials, by forming an inter-subunit Cd²⁺-mediated metal cross-bridge with H486 at neutral pH (~7.4; Holmgren et al., 1998; Webster et al., 2004). This Shaker-ΔI T449A;V476C double mutant is thus suitable for realizing the pathway for channel recovery from slow inactivation, and in particular, the role of the OI→CI transition in this process. Following biophysical characterization of this mutant channel expressed in HEK cells using an inside-out patch-clamp configuration (see Fig. S3 in Szanto et al., 2020), the authors evaluated the consequences of locking the activation gate of inactivated channel (OI) open on recovery from inactivation (Fig. 2 B). Onset and recovery from slow inactivation in the double mutant were induced by a 2-s-long square pulse from −120 to +50 mV and back. Control experiments performed in the absence of Cd²⁺ ions revealed a similar value for the peak current when the protocol was repeated every 60 s several times while holding the potential at −120 mV (see Fig. 7 A in Szanto et al., 2020). Thus, under these conditions, the Shaker-ΔI T449A; V476C channel fully recovers from slow inactivation. When, however, Cd²⁺ was applied for a short time toward the end of the depolarizing pulse (i.e., when the channel is deep in the OI state), and the pulse was repeated every 60 s, as before, an exponential decrease in the peak current was observed as function of cumulative Cd²⁺ exposure time (see Fig. 8 in Szanto et al., 2020). This reduction in peak current amplitude was completely abolished when the experiment was repeated in acidic bath solution (pH 5.3, but with Cd²⁺ ions; see Fig. 9 in Szanto et al., 2020), a condition which facilitates H486 protonation and prevents metal cross-bridge formation with C476 (Webster et al., 2004). Thus, metal bridge-mediated locking down of the Shaker channel in the OI state (O→I) prevents OI→CI transition and completely abolishes channel recovery from slow inactivation. This transition occurs very fast in the WT channel upon membrane repolarization and is mandatory for recovery to the closed state, where the slow inactivation gate is open (CI→C transition, induced by sufficiently negative and prolonged repolarization; Panyi and Deutsch, 2006). This locked-open inactivated channel further prevents O→I transition, a manifestation of the strong negative coupling between the lower activation and upper slow inactivation gates, as manifested by channel-gating states with opposite gate configurations (e.g., the A_{C}C_{O} [C] or A_{O}C_{L} [OI] states; Panyi and Deutsch, 2006; Ben-Abu et al., 2009). These results thus provide clearcut evidence that recovery in the Shaker-ΔI channel occurs through the OI→CI→C transition sequence, thus completing flow along the Shaker-ΔI slow inactivation gating cycle.

To summarize, the study by Szanto et al. (2020) teaches that directionality and flow along the slow inactivation gating cycle of the Shaker-ΔI Kv channel (Fig. 1 B) is preserved during onset and recovery from slow inactivation at both positive and negative potentials. Onset of slow inactivation is achieved only following activation gate opening. No detectable closed-state inactivation occurs for the Shaker channel, even at negative membrane potentials. Furthermore, shortcircuiting the gating cycle of the Shaker channel by locking the channel in the inactivated open state (OI) prevents forward flow along the cycle, resulting in an inability of the channel to recover from slow inactivation and returning to the closed starting state. These results add to previous seminal reports on slow inactivation in this prototypical channel (Panyi and Deutsch, 2006; Kurata and Fedida, 2006) and, together with the wealth of structural, functional, and dynamics data recently obtained on the intensively studied KcsA model channel (Cuello et al., 2010a, 2010b; Cuello et al., 2017; Labro et al., 2018), provide coherent atomic and mechanistic-level understanding of slow inactivation gating in potassium channels.

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