Initial steps in the opening of a Shaker potassium channel

Toshinori Hoshi and Clay M. Armstrong¹

Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104

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The structural model of a K_V (K^+ -selective, voltage-gated) channel in the open state is known (Protein Data Bank ID code 2R9R). Each subunit of the channel has four negatively charged residues distributed in the transmembrane segments S1, S2, and S3 that bind to and facilitate the movement within the membrane of the positively charged, voltage-sensing residues of S4. When extrapolated to the closed state, the two outermost negatively charged residues are exposed to extracellular fluid and not bound to S4 residues, all of which have theoretically been driven inward by voltage. If this closed state model is correct, these residues are available to bind external cations. We examined the effects of La^{3+} on voltage-gated Shaker K^+ channels. Addition of the trivalent cation La^{3+} (50 μM) extracellularly markedly prolongs the lag that precedes channel opening and slows the subsequent rise of K^+ current (I_K) at all voltages. Decay kinetics of I_K at negative voltages are unaltered. Gating current (I_g) recorded from a nonconducting mutant shows that La^{3+} reduces the initial amplitude of I_g nearly twofold. We postulate that, in the resting state, La^{3+} binds to the unoccupied, outermost negative residues, hindering outward S4 motion, thus increasing the lag on activation and slowing the rise of I_K . In the activated state, La^{3+} is displaced by outward movement of arginine residues in S4; La^{3+} , therefore, is not present to affect channel closing. The results give strong support to the closed state model of the K_V channel and a clear explanation of the effect of multivalent cations on cellular excitability.

calcium | electrophysiology | voltage sensor | surface charge

Voltage-gated Na^+ (Na_V) channels and K^+ (K_V) channels are fundamental components in cellular excitability, responsible for the action potentials in neuronal and other cell types. Regulation of action potential threshold by a wide array of factors, including synaptic inputs and intracellular signaling cascades, underlies the remarkable plasticity of nerve cells, ultimately contributing to higher functions of the brain, including learning and memory as well as the pathogenesis of neurological disorders such as epilepsy (1).

Since the experiments in the work by Hodgkin and Huxley (2), it has been known that several steps are involved in opening what is now called an Na_V or K_V channel. Na_V channels open quickly after a depolarization (positive change of membrane potential), producing an inward current that drives membrane potential (V_m) up to the action potential peak. K_V channels open more slowly to repolarize V_m to the resting potential. The timing of channel opening is, thus, crucial in determining excitability (3). For example, if the sigmoid delay (lag) normally seen in the opening of the K_V channel is decreased substantially (2), development of K^+ current (I_K) will overlap Na^+ current (I_{Na}) and hinder or prevent development of an action potential.

A voltage-gated channel is now known to contain either four subunits (as in K_V channels) or four domains (as in Na_V channels), each of which, in turn, possesses six transmembrane segments, S1–S6 (4). The segments S5 and S6 comprise the ion conduction pore domain and the gate, and segments S1–S4 form the voltage-sensing domain. Of particular importance is the S4 segment, which in the Shaker K_V channel, contains seven positively charged

residues: from outside to inside, four arginines (R1–R4), a lysine (K5), an arginine (R6), and a final lysine (K7) (5).

K_V and Na_V channels are locked closed at the resting potential. Outward motion of the four positively charged S4 segments, one in each subunit/domain of the channel, driven by positive-going membrane (internal) voltage or depolarization, is typically necessary before a channel's gate is unlocked and opens, enabling flow of the selected ion (6). The gate itself is formed by convergence of the inner ends of the S6 segments (6–10).

The atomic crystal structure of a K_V channel (Kv1.2–2.1 chimera; Protein Data Bank ID code 2R9R) is known (11), and the position of the S4 segment in one of the four identical subunits is shown in Fig. 1A. In the open state, the outermost arginines (R1 and R2) are exposed to the external solution, and R3 and R4 are electrostatically bonded, respectively, to E183 in S1 and E226 in S2, whereas K5 (lysine) is bonded to E236 in S2.

The closed state of a K_V channel has not been determined crystallographically but has been speculated on by multiple studies (6, 11–13). Among them, the hypothetical closed state structure in the work by Long et al. (11), showing one voltage-sensing domain in the deactivated resting state, is presented in Fig. 1B. The work by Long et al. (11) postulated that the membrane field is concentrated in a narrow band centered on F233 in S2 (11, 14). In the closed state, R1 is, from the electrical point of view, on the inside. Going from the hypothetical fully deactivated state to the open state would involve movement of R1 through R4 to the (electrical) outside, thus moving 16 elementary charges (e_0) per channel across the electric field. This estimate is relatively close to the biophysical estimates, including those from gating current (I_g) (15–17) and limiting-slope ionic current measurements (15, 16, 18).

Our interest here is that, in the hypothetical closed state, E183 and E226 in each of the four subunits are exposed to the external medium and not bound to an S4-arginine (Fig. 1B). These residues, thus, seem potential attractants of multivalent cations, which are known to interfere with opening of both Na_V and K_V channels. We have explored this interference using the trivalent La^{3+} ion, which is similar to Ca^{2+} in its effect on channels and crystal radius (1.02 vs. 0.99 Å) but much more powerful because of its extra charge.

Results

Effects of Extracellular La^{3+} on Gate-Opening Kinetics of Shaker Channels.

In response to step depolarization, I_K through Shaker K^+ channels activates with a noticeable lag; I_K has a prominent sigmoidal time course, which is illustrated for ShBΔ6–46 T449V, a Shaker mutant without N- or C-type inactivation (Fig. 2A) (19). In structural terms, the lag in I_K reflects the fact that a channel has four S4 segments, each of which, in turn, undergoes multiple conformational changes before the ion conduction gate opens. The flow of I_K in a single

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¹To whom correspondence should be addressed. E-mail: carmstro@mail.med.upenn.edu.

Fig. 1. The resting and activated conformations of the Kv1.2–2.1 voltage-sensing domain. (A) Experimentally determined activated conformation (Protein Data Bank ID code 2R9R). Note that R1 in Kv1.2–2.1 is glutamine, whereas R1 in Shaker is arginine. (B) Hypothetical resting/closed conformation. The yellow sphere represents the Ca^{2+} in the hypothetical binding area. The images were created using Mac PyMol v0.9. The residues are labeled using the numbering in Kv1.2–2.1. Modified from Long et al. (11).

channel, thus, usually begins (all or none) after completion of the final motion of the channel's last S4 segment.

Application of La^{3+} (50 μM) to the extracellular side markedly alters the time course of I_K development on depolarization (Fig. 2A, blue). La^{3+} slows down the overall activation kinetics by prolonging the initial lag phase after the onset of depolarization. This effect is particularly noticeable at less depolarized voltages (for example, at -20 mV). The marked prolongation of the initial lag by La^{3+} suggests that either outward motion of S4 is slowed or the final gate-opening step is slowed (20, 21). The prolongation of the initial lag phase by La^{3+} observed here is qualitatively similar to the prolongation seen with Zn^{2+} and a gastropod marine toxin (22).

In addition to slowing of the activation kinetics, La^{3+} inhibits the peak current size at each voltage (Fig. 2A), causing the peak current–voltage (I–V) curve to shift in the positive direction by

Fig. 2. Slowing of I_K activation by extracellular La^{3+} . (A) Representative I_K traces without (black) and with (blue) 50 μM La^{3+} . I_K was recorded from ShBΔ6–46 T449V, and La^{3+} was applied to the extracellular side. Currents were elicited by 6.5-ms depolarization pulses to the voltages indicated from the holding potential of -80 mV. The traces without La^{3+} are the average of a set taken before application of La^{3+} and another closely similar set after washing it away. (B) Peak outward I–V curves without (black) and with (blue) 50 μM La^{3+} . The data points are from the experiment in A and are connected by straight lines for clarity.

~ 15 mV (Fig. 2B). Despite the shift, the maximum slope of the I–V curve remains similar, implying that the maximum macroscopic K^+ conductance is unaltered by La^{3+} .

La^{3+} Slows S4 Motion During Activation. The experimentally determined and inferred structures of the Kv1.2–2.1 channel (6, 11–13) and the idea that the membrane electric field is narrowly focused around one residue (11, 14) together suggest that each S4 segment, with its multiple positively charged residues, moves outward through the membrane electrical field in several steps (14). Which of the S4 steps is slowed by La^{3+} , or is it the final, gate-opening step? Although I_K is a good measure of the last step in activation—opening of the gate—earlier steps representing S4 movements before the gate opens are better inferred from measurements of I_g , which is a direct reflection of composite S4 motion. The effect of La^{3+} on I_g from ShBΔ6–46 W434F, a nonconducting stably C-type inactivated mutant (23, 24), is shown in Fig. 3A. At all four voltages illustrated, the peak outward I_g amplitude, after a transient of ~ 100 μs , is approximately cut to one-half by La^{3+} in a reversible manner. Given a fixed quantity of charges traversing the membrane electric field, the amplitude of I_g is proportional to the rate of charge transfer. Therefore, the reduction of I_g amplitude shows that the earliest resolvable step of S4 motion is slowed by a factor of approximately two by La^{3+} . In addition to the decreased size, the I_g in the presence of La^{3+} has a markedly slower time course (Fig. 3A, blue). At 40 mV, for example, the I_g trace in La^{3+} has a half-decay time of 2.0 ms compared with 0.94 ms for the control trace.

The voltage dependence of the charge movement (Q_g) estimated from the integral of I_g is displaced in the positive direction by ~ 15 mV by La^{3+} (Fig. 3B). However, the total Q_g at very positive voltages (≥ 20 mV) remains essentially unaltered by La^{3+} . Thus, the same number of charges moves on depolarization, but the kinetics are slowed by La^{3+} .

La^{3+} Interacts with E183 in S1 and E226 in S2? In terms of the model in the work by Tao et al. (14), multiple sequential steps generate I_g . The first event is the transfer of R1 across the membrane field narrowly centered on F233; R1 moves from E236, past F233, and to E226 (Fig. 1). La^{3+} slows this first step of S4 motion and thus, reduces initial I_g by competing with R1 for occupancy of the site at E226 on the extracellular side. In the second step, R1 moves from E226 to E183, and R2 crosses the field to E226. The negative residues E226 and most probably, E183 can interact with S4 or La^{3+} but not both contemporaneously.

La^{3+} Does Not Alter Gate Closing on Repolarization. After R1 and R2 have successfully moved outward beyond F233 and occupied E226 and E183, La^{3+} must have been displaced: its binding site near these negative charges has been preempted by S4 residues. A consequence would be that La^{3+} should have no effect on the kinetics of gate closing. Fig. 4 shows this prediction to be the case. On repolarization to -80 mV after opening the channel gates with a 12-ms step to 20 mV, the tail currents with and without La^{3+} have a very similar time course. In La^{3+} , the tail is smaller because fewer channels open during the activating pulse (Fig. 2B, I–V curves). When the current recorded with La^{3+} is scaled up, the tails almost superimpose (Fig. 4). The effect of La^{3+} on tail I_K is clearly much less than its effect on opening (Fig. 2A).

La^{3+} and S4 Motion During Deactivation. Unlocking the gate of a channel typically follows activation of its S4s; K5 must be bound to E236 in all subunits to unlock the gate, after which it opens as depicted in the crystal structure of Kv1.2–2.1. After the gate has opened, the inward motion of S4 on repolarization is slow for two reasons. (i) Gating motion is completely paralyzed as long as the gate is open, and closing of the gate is relatively

After a depolarization to -30 mV, there is considerable S4 motion, but few of the S4s activate completely. As a result very few of the gates open, and most S4 motion remains free rather than paralyzed. The partially activated S4s begin to rapidly move back to the resting position, generating a large inward peak of I_g followed by a quasiexponential tail as the S4s deactivate (Fig. 5). In the presence of La^{3+} , the I_g tail is somewhat smaller, because

In summary, the inward S4 motion reflected in I_g at -80 mV after 14-ms activating pulses of various sizes has two kinetic components. (i) Partially activated channels generate a fast exponential tail of I_g , seen in almost pure form after the -30 -mV activating pulse. (ii) Fully activated channels with open gates generate a slow wave of I_g with zero initial amplitude: inward S4 motion in a channel with open gate is paralyzed. I_g then grows as channels close; however, because gate closing and therefore, S4 motion are slow, the amplitude is never very large. As closing continues, the amplitude of I_g slowly decays. This component is seen in pure form after the 40-mV activating pulse in the absence of La^{3+} . The tails following steps to -10 and 10 mV are composed of the same two components: the fast component that decreases as more gates open and the slow component, with zero initial amplitude, that increases. In all cases, total charge movement during deactivation equals total charge movement during activation within experimental accuracy.

Our results are consistent with the open state structure determined in the work by Long et al. (11) and their hypothetical closed state structure. In terms of the closed state model in their work (11), we postulate that La^{3+} slows activation by competing

Fig. 5. I_g kinetics on repolarization. Representative I_g values from ShBΔ6–46 W434F at -80 mV after 14-ms activating pulses to the voltages indicated are shown. The black and blue traces show the results obtained without and with La^{3+} ($50 \mu\text{M}$), respectively. In addition, the results obtained after a pulse to -30 mV were scaled and are shown in *inset*.

with R1 for E226 and E183, two negative residues in the channel facing the extracellular medium (Fig. 1). In the initial step of S4 motion, R1 is driven by depolarization outward from its resting position at E236 through the membrane electric field, which is centered at F233. When R1 reaches E226, the energy of La^{3+} binding and the probability of La^{3+} occupancy of the E226–E183 neighborhood decrease sharply because of the competing charge, R1, which occupies E226. Thus, after the initial step, (i) subsequent activation steps are, in theory, much less affected by La^{3+} , and (ii) La^{3+} has no effect on inward S4 motion and gate closing on repolarization. The first postulate remains to be rigorously tested, but the second postulate is clearly established by the experiments presented here. Our model of regulation of S4 dynamics by extracellular Ca^{2+} is reminiscent of the scheme proposed in the work by Gilly and Armstrong (26) in 1982. To explain the activation-slowing effect of Zn^{2+} on squid voltage-gated K^+ currents, these authors, in pre-cloning days, hypothesized that negative charges on one helix are paired to positive charges on another helix and that activation involves relative outward motion of the positive helix. In the resting state, the outermost negative charge in their scheme is vacant and available for Zn^{2+} binding, which hinders activation.

The results presented here provide a markedly different picture of what have been called surface charge effects. An increase in extracellular Ca^{2+} has been long known to stabilize or inhibit membrane excitability by shifting the voltage dependence of a variety of voltage-gated channels, including Na_V and K_V channels, in the positive direction (4, 27, 28). The survey of the literature by Hille (4) led to the conclusion that all voltage-gated channels on the surface membrane, but not inward rectifiers, are affected by

Ca²⁺. This excitability stabilizing effect of extracellular Ca²⁺ has usually been explained as neutralization by Ca²⁺ of diffusely distributed negative charges on the membrane surface, resulting in a less intense membrane voltage field which equally affects activation and deactivation kinetics (27). The results described here necessitate a reinterpretation of this long-held view. The charges involved are not distributed uniformly over the membrane surface but instead, have an intimate connection with the gating apparatus. An earlier theoretical study hinted that negatively charged residues in the pore domain of K_V channels may play a role (29), but the involvement of the negative charges in the voltage-sensing domain was not suspected. Our results provide an explanation for the familiar stabilizing effects of Ca²⁺ on the closed state of K_V channels (4, 26, 27) and by extension, Na_V channels that have a basic similarity. Ca²⁺ ion affects Na_V channels essentially in the same way (27, 28), and low extracellular Ca²⁺ concentration leads to the hyperexcitability of nerve fibers seen in hypocalcemic tetany (30): Na_V channels open more easily when lowered Ca²⁺ provides less hindrance to S4 motion.

In conclusion, La^{3+} ion slows the activation of K_V channels by occupying negatively charged glutamate residues that form an essential part of the S4 migration pathway, thus inhibiting the first step in the outward motion of the positively charged S4 segments. Our results provide a simple explanation for the suppressive effects of divalent cations on K_V channel opening. By extension to the similar S4 segments of Na_V channels, the results also provide an explanation for the hyperexcitability of nerve fibers seen in hypocalcemic tetany. Finally, our findings lend strong support for aspects of the model in the work by Long et al. (11) and open the path for additional analysis of S4 motion by performing gating current experiments on channels with strategically placed mutations.

Materials and Methods

Channel Expression. Shaker channels were heterologously expressed in human embryonic kidney (HEK) or mouse neuroblastoma Neuro2A cells obtained from American Tissue Type Collection by transient transfection of cytomegalovirus promoter-containing plasmid DNAs with FuGene 6 (Roche) as described (31). In most of the experiments, enhanced GFP was used as a transfection marker. Two Shaker channel mutants were primarily used: ShBΔ6–46 T449V, lacking both N- and C-type inactivation (19), and ShBΔ6–46 W434F, a stably C-type inactivated mutant (23, 24). Transfected cells were plated at a low density on poly-L-lysine coated coverslips and used for electrophysiological measurements 24–48 h later without any additional proteolytic digestion.

Electrophysiological Data Acquisition and Analysis. Data were acquired in the whole-cell configuration using pipettes of $\sim 1\text{ M}\Omega$ initial resistance when filled with the solution below. The homemade voltage-clamp amplifier had three main characteristics: (i) a $10\text{-M}\Omega$ feedback resistor on the head stage for fast frequency response, (ii) supercharging (32) for quick voltage steps, and (iii) series resistance compensation, with a tuning circuit to allow near complete compensation. Linear leakage and capacitance were subtracted by scaling current recorded during a negative-going control step; from -80 to -140 mV for I_K experiments and from -130 to -160 mV for I_g experiments. The external recording solution for I_K experiments contained 146 mM NaCl , 10 mM CaCl_2 , 2 mM KCl , 5 mM Tris , $\text{pH } 7.0$, $\pm 50\text{ }\mu\text{M LaCl}_3$; for I_g experiments, the solution contained 146 mM NMGC (*N*-methyl-*D*-glucamine *Cl*), 10 mM CaCl_2 , 2 mM KCl , 5 mM Tris , $\text{pH } 7.0$, $\pm 50\text{ }\mu\text{M LaCl}_3$. The internal solution contained $112\text{ mM K gluconate}$, 30 mM KF , 20 mM KCl , 5 mM Tris , $\text{pH } 7.0$. Representative results of several independent measurements are shown.

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