

K⁺ channel gating: C-type inactivation is enhanced by calcium or lanthanum outside

Clay M. Armstrong and Toshinori Hoshi

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

Many voltage-gated K⁺ channels exhibit C-type inactivation. This typically slow process has been hypothesized to result from dilation of the outer-most ring of the carbonyls in the selectivity filter, destroying this ring's ability to bind K⁺ with high affinity. We report here strong enhancement of C-type inactivation upon extracellular addition of 10–40 mM Ca²⁺ or 5–50 μM La³⁺. These multivalent cations mildly increase the rate of C-type inactivation during depolarization and markedly promote inactivation and/or suppress recovery when membrane voltage (V_m) is at resting levels (−80 to −100 mV). At −80 mV with 40 mM Ca²⁺ and 0 mM K⁺ externally, ShBΔN channels with the mutation T449A inactivate almost completely within 2 min or less with no pulsing. This behavior is observed only in those mutants that show C-type inactivation on depolarization and is distinct from the effects of Ca²⁺ and La³⁺ on activation (opening and closing of the V_m-controlled gate), i.e., slower activation of K⁺ channels and a positive shift of the mid-voltage of activation. The Ca²⁺/La³⁺ effects on C-type inactivation are antagonized by extracellular K⁺ in the low millimolar range. This, together with the known ability of Ca²⁺ and La³⁺ to block inward current through K⁺ channels at negative voltage, strongly suggests that Ca²⁺/La³⁺ acts at the outer mouth of the selectivity filter. We propose that at −80 mV, Ca²⁺ or La³⁺ ions compete effectively with K⁺ at the channel's outer mouth and prevent K⁺ from stabilizing the filter's outer carbonyl ring.

INTRODUCTION

C-type inactivation is a (usually slow) gating phenomenon originally observed as a conductance decrease that occurs in many voltage-gated K⁺ channels after activating the channels with a depolarizing step (Hoshi et al., 1991). Results of experiments using mutant K⁺ channels show that C-type inactivation is associated with the extracellular mouth of the channel (Hoshi et al., 1991; López-Barneo et al., 1993; Baukowitz and Yellen, 1995; Kurata and Fedida, 2006). Some mutants such as those containing W434F are constitutively almost completely C-type inactivated and generate only gating current under typical recording conditions (Perozo et al., 1993; Yang et al., 1997); the C-type inactivation “gate” is closed, regardless of the voltage and the position of the S4 helices that drive activation/deactivation of the V-gate located at the inner end of the channel (del Camino and Yellen, 2001; Jensen et al., 2012). C-type inactivation is quite distinct from N-type inactivation, which is usually more rapid, and occurs by a ball and chain mechanism that involves the entry of the N terminus of a channel peptide (or a moiety of a β subunit) into the channel's inner mouth from the cytoplasmic side when the V-gate is open (Hoshi et al., 1990; Zagotta et al., 1990; Demo and Yellen, 1991; Zhou et al., 2001a). Functionally, C-type inactivation serves as a form of short-term memory, modifying the availability of the channel based on recent experience.

In the absence of N-type inactivation, ShB, a commonly used splice variant of Shaker K⁺ channels (Timpe et al., 1988), inactivates with a time constant of ~1 s in normal extracellular solution (Hoshi et al., 1991; López-Barneo et al., 1993). Mutation of the threonine residue at position 449 (T449 using ShB numbering), located just external to the channel's outer mouth, has a strong influence on C-type inactivation, which, for example, is virtually eliminated by the mutation of T449 to valine (T449V; López-Barneo et al., 1993). With the mutation W434F, C-type inactivation is essentially permanent. Interestingly, W434F channels (ShBΔN W434F) can be rescued from C-type inactivation by combining the T449V mutation with W434F (Yang, Y., Y. Yan, and F.J. Sigworth. 2002. Biophysical Society Annual Meeting. 1138-Pos Board #B190). Mutating T449 to alanine, glutamate, lysine, or serine shortens the time constant of inactivation to milliseconds (López-Barneo et al., 1993). Except in mutants such as W434F, recovery from C-type inactivation is facilitated by repolarization (Kurata and Fedida, 2006).

C-type inactivation has proven to be a challenge to define mechanistically (Kurata and Fedida, 2006) and has been traditionally explained as a constriction of the channel's outer mouth (Yellen et al., 1994; Cuello et al.,

Correspondence to Clay M. Armstrong: carmstro@mail.med.upenn.edu

2010). More recently, this inactivation has been proposed to result from a dilation of the channel's outer mouth (Hoshi and Armstrong, 2013) that destroys the ability of the selectivity filter's outermost site (Åqvist and Luzhkov, 2000; Morais-Cabral et al., 2001) to complex K^+ . This outermost site is called FS1 (filter site 1) hereafter. The crystal structure of a Shaker-like voltage-gated K^+ channel (Kv1.2-2.1 chimera; Long et al., 2007) has been an enormous aid in understanding permeation and channel gating. However, the requirement for high concentrations of K^+ in the crystallization mix has so far limited its usefulness in understanding this gating phenomenon, at least in voltage-activated channels, because high concentrations of K^+ antagonize C-type inactivation (Choi et al., 1991; López-Barneo et al., 1993; Baukowitz and Yellen, 1995; Kurata and Fedida, 2006). Aside from K^+ , many previous studies show that other ions also affect C-type inactivation (Choi et al., 1991; López-Barneo et al., 1993; Loboda et al., 2001; Kurata and Fedida, 2006). We report here the surprising effects of high concentrations of extracellular Ca^{2+} or low concentrations of extracellular La^{3+} on C-type inactivation; both Ca^{2+} and La^{3+} drastically enhance C-type inactivation when V_m is negative. Here we use these multivalent cation effects as tools for unraveling the mechanism of C-type inactivation.

MATERIALS AND METHODS

Channel expression

All experiments were performed in cells expressing various mutants of the Shaker K^+ channel called ShB Δ 6-46; i.e., a segment of the N terminus of the ShB channel was deleted to remove N-type inactivation (Hoshi et al., 1990). Mutants of this base channel (e.g., T449A, meaning that the threonine residue at position 449 was changed to alanine) were transiently expressed in human embryonic kidney (HEK) or mouse Neuro2a (N2a) cells by transfection with plasmid DNAs using FuGene6 (Roche) as described previously (Hoshi and Armstrong, 2012). GFP was used as the transfection marker.

Electrophysiology and data analysis

The electrophysiological measurements were performed at room temperature typically 24–48 h after transfection as described

TABLE 1

<i>External solutions used in the whole-cell current measurements</i>				
Solution name	[CaCl ₂]	[KCl]	[NaCl]	[LaCl ₃]
	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
2Ca0K	2	0	157	
2Ca2K	2	2	155	
4Ca2K	4	2	152	
10Ca0K	10	0	145	
10Ca2KLa	10	2	143	0.01
20Ca0K	20	0	130	
28Ca0K	28	0	118	
40Ca0K	40	0	100	
40Ca2K	40	2	98	

Each solution also contained 10 mM Tris and pH 7.0.

previously (Hoshi and Armstrong, 2012). The outside-out and whole-cell configurations of the patch-clamp method were used. Outside-out patch experiments were performed as described previously (Hoshi et al., 2013). Unless otherwise noted, the base external solution for these whole-cell experiments contained (mM) 157 NaCl, 2 CaCl₂, 2 KCl, and 10 Tris, pH 7.0. External solutions used in the whole-cell current measurements are shown in Table 1.

The internal solution for whole-cell experiments contained (mM) 100 K glutamate, 30 KF, 20 KCl, and 10 Tris, pH 7.0. Other solutions used are described in the figure legends. Linear leak and capacitive currents were subtracted using a P/6 protocol in outside-out experiments or, for whole-cell experiments, by scaling membrane current for a step from -100 to -140 mV. Series resistance compensation of $\sim 60\%$ and $\sim 70\text{--}90\%$ was used in outside-out and whole-cell experiments, respectively. Series resistance error was at most 10 mV, which is not of serious concern in our qualitative measurement of outward current. The results presented here are illustrative of numerous experiments: >30 exposures to elevated Ca^{2+} and ~ 10 to La^{3+} . Kinetics of current inactivation was estimated by fitting a single exponential.

Online supplemental material

Fig. S1 calculates the decrease in K^+ -binding affinity caused by moving a coordinating carbonyl group away from the K^+ ion by 1 Å. Fig. S2 calculates the energy change when an empty carbonyl ring (K^+ ion removed) increases or decreases in diameter by 1 Å. Online supplemental material is available at <http://www.jgp.org/cgi/content/full/jgp.201411223/DC1>.

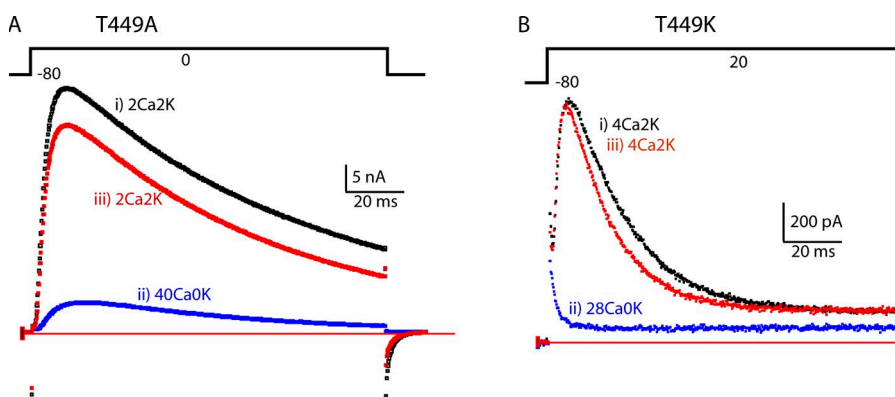


Figure 1. In two mutants that have C-type inactivation, high extracellular Ca^{2+} depresses I_K . (A) The traces show I_K from a cell expressing T449A during a depolarization lasting 110 ms from -80 to 0 mV. The traces were taken in the order i–iii, with a rest of ~ 2 min between them to allow full recovery from C-type inactivation. I_K is strongly depressed by 40Ca0K. Note, however, that the time constant of inactivation of the current remaining in 40Ca0K is similar to that in 2Ca2K. (B) I_K traces from a T449K cell. The extracellular solution was changed from 4Ca2K (i) to 28Ca0K (ii) and then back to 4Ca2K (iii).

RESULTS

C-type inactivation in high extracellular Ca^{2+}

Potassium current (I_K) recorded from a cell expressing the rapidly inactivating mutant T449A (López-Barneo et al., 1993) is shown in Fig. 1 A. In base extracellular medium containing 2 mM Ca^{2+} and 2 mM K^+ (2Ca2K; see Materials and methods and Table 1 for detailed solution composition), I_K during a 110-ms step to 0 mV decayed with a time constant of ~ 90 ms (trace i). 2 min after resting in an extracellular solution containing 40 mM Ca^{2+} and 0 mM K^+ (40Ca0K), the much smaller I_K trace ii was recorded. The reduced I_K was similar in time course to the larger I_K trace, but inactivated with a slightly accelerated time constant of ~ 55 ms. The effect of 40Ca0K was largely reversed on return to 2Ca2K (iii).

I_K from another mutant, T449K (López-Barneo et al., 1993), which inactivates even more rapidly than T449A (inactivation time constant ~ 19 ms), is shown in Fig. 1 B. When the extracellular medium was changed from 4Ca2K to 28Ca0K (28 mM Ca^{2+}), I_K was reduced to a rapidly decaying component that finished within milliseconds of depolarization onset. Similar results were previously observed with the rapidly inactivating mutant E418Q (Ortega-Sáenz et al., 2000).

High Ca^{2+} acting on a mutant that lacks C-type inactivation
In a cell expressing T449V, which does not C-type inactivate (López-Barneo et al., 1993), changing the external

solution from 4Ca2K to 40Ca0K had very little effect on I_K amplitude (Fig. 2 A), in striking contrast with the current-depressing effect seen with the fast-inactivating mutants T449A and T449K (Fig. 1). Close inspection of the activation kinetics at expanded time scale (Fig. 2, B and C) shows that elevated Ca^{2+} slows activation of I_K and that the initial lag before I_K rises is longer. Fig. 2 D plots the maximum value of I_K as a function of the voltage during the depolarizing step. The curve in 40Ca0K is shifted to the right by slightly less than 20 mV. These familiar effects of increased Ca^{2+} , slower activation of I_K after a longer lag and a right shift of the I-V curve, are not related to C-type inactivation, which does not occur in T449V channels (López-Barneo et al., 1993). The increased lag and slower rise of I_K , sometimes called surface charge or surface potential effects (Frankenhaeuser and Hodgkin, 1957; Campbell and Hille, 1976), have more recently been attributed to di- or trivalent cation binding to negative charges on the migration path of the S4 through the membrane as the channels activate (Hoshi and Armstrong, 2012).

C-type inactivation examined with short pulses

The current-depressing effect of Ca^{2+} on T449A seen in Fig. 1 A was also assessed by repetitively applying 25-ms depolarizing pulses to 20 mV at 0.1 Hz (Fig. 3 A). The experiment began with a large current recorded in 2Ca2K (trace i, black). After changing to 2Ca0K, I_K of the 11th pulse at 0.1 Hz (trace ii, orange) was essentially

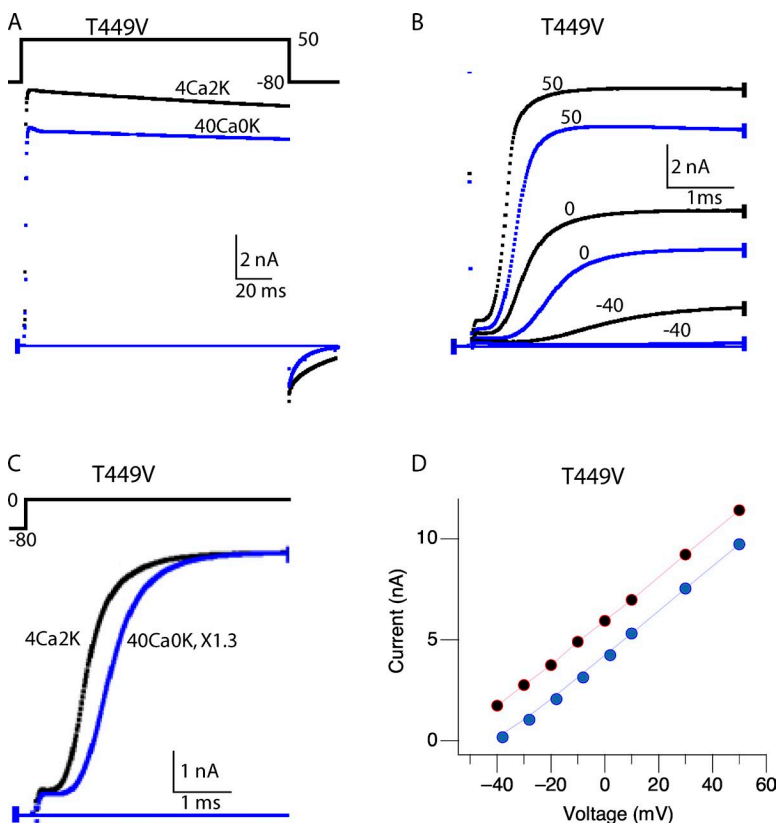


Figure 2. Ca^{2+} does not appreciably inhibit currents through T449V channels. (A) Whole-cell currents elicited by pulses from -80 to 50 mV in 4Ca2K and 40Ca0K. (B) Slower activation time course of T449V in 40Ca0K (blue) compared with those in 4Ca2K (black). (C) The currents for pulses from -80 to 0 mV are scaled by 1.3 times to account for the familiar right shift of the I-V curve caused by high Ca^{2+} , which is shown in D. (Gilly and Armstrong, 1982; Hille, 2001). All results shown are from the same cell.

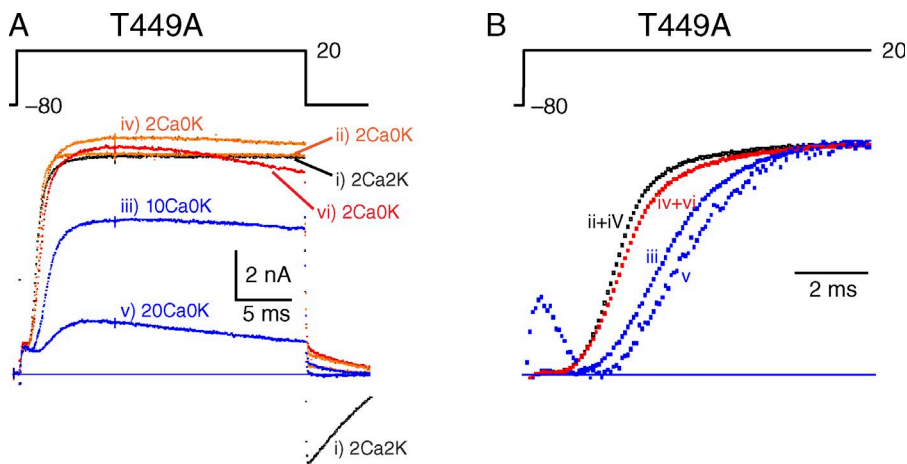


Figure 3. Ca²⁺ action on C-type inactivation examined with short pulses. (A) A cell expressing T449A was stimulated at 0.1 Hz in several solutions, beginning with 2Ca2K. In each solution, maximum I_K was in a steady-state after ~10 pulses or less, and a current record for the 11th pulse in each solution is displayed. The currents were elicited by 25-ms pulses from -80 to 20 mV and then back to -80 mV. (B) Slowing of activation kinetics by 10 and 20 Ca²⁺. The currents from A are scaled vertically for comparison and displayed on a fast time scale. ii+iv, iv+vi: mean of ii and iv or iv and vi, respectively. A gating current transient is visible in trace v, the most amplified.

unchanged except for elimination of the inward tail of current at pulse end seen in 2Ca2K. (Note: Perfusion time was not measured in this experiment.) Changing to 10Ca0K reduced I_K (11th pulse at 0.1 Hz) by about a third (trace iii, blue), with complete recovery on return to 2Ca0K (11th pulse, trace iv, orange). Changing to 20Ca0K reduced I_K to about a third (11th pulse, trace v, blue) and caused a slow decline of I_K during the pulse, probably as the result of somewhat accelerated C-type inactivation. Again, recovery was complete on return to 2Ca0K (11th pulse, trace vi, red), but a hint of C-type inactivation remained.

The slowing of K⁺ channel activation seen in T449V (Fig. 2 B) is also found in the T449A mutant, as illustrated in Fig. 3 B in which the traces from Fig. 3 A have been scaled to the same amplitude to facilitate temporal comparison and shown at an expanded time scale. At elevated Ca²⁺, I_K develops more slowly and the initial lag in its development is longer.

Another experiment using short repetitive pulses with several solution changes is summarized in Fig. 4 A. A cell expressing T449A was held at -80 mV and pulsed

to 20 mV at 0.1 Hz. The extracellular Ca²⁺ and K⁺ concentrations are shown above, and peak I_K amplitude recorded for each pulse is plotted below. The experiment begins in 2Ca2K, with a change to 40Ca2K at (1). This increase in [Ca²⁺] inhibited peak I_K from ~20 nA to ~13 nA. Pulsing was suspended for 120 s (2), and on resumption of pulsing I_K remained partially inhibited (~13 nA); there was no recovery of I_K amplitude during the suspension of pulsing. The solution was then changed back to 2Ca2K (3), and I_K fully recovered to ~20 nA (4). A suspension of pulsing caused no change in I_K (4 to 5). At 440 s (5), the solution was changed to 40Ca0K, increasing [Ca²⁺] concurrent with removal of K⁺. I_K progressively decreased to ~4 nA, and suspension of pulsing did not alter I_K. Decreasing extracellular [Ca²⁺] to 2Ca0K (6) restored I_K to ~15 nA. Full restoration to the original current size of ~20 nA was seen when K⁺ was added back to the extracellular medium, making the final solution of 2Ca2K (7).

Is the depression of I_K by high Ca²⁺ in fact the result of enhanced C-type inactivation, as strongly suggested by its occurrence only in mutants that C-type inactivate?

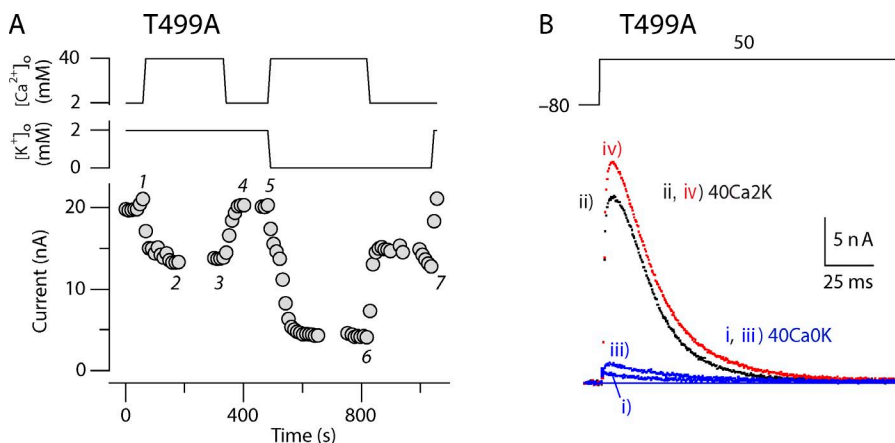


Figure 4. Enhancement of C-type inactivation by high extracellular Ca²⁺ is antagonized by extracellular K⁺. (A) Whole-cell currents from a T449A cell were elicited by 25-ms voltage pulses from -80 to 20 mV applied at 0.1 Hz. Peak outward current in nanoamperes is plotted as a function of time as Ca²⁺ and K⁺ concentrations in the extracellular solutions were changed as indicated at the top. Solution changes were completed over the course of several pulses. The numbers (1-7) are explained in the section C-type inactivation examined with short pulses. (B) Whole-cell currents from a cell expressing T449A channels during exposure to 40Ca0K or 40Ca2K. The currents were elicited by pulses from -80 to 50 mV after a rest of 2 min.

To further support this idea, we compared (a) the recovery time of I_K after exposure to elevated Ca^{2+} ($n = 4$) with (b) the recovery time of I_K after depolarization-induced C-type inactivation ($n = 4$). Recovery was monitored in 2 mM Ca^{2+} by applying short pulses at 0.1 Hz. The recovery time constant was ~ 20 s for both experiments, supporting the idea that high Ca^{2+} depresses I_K by enhancing C-type inactivation.

The results in Fig. 4 A clearly show that extracellular Ca^{2+} causes substantial inactivation in 40Ca2K, but much more inactivation when extracellular K^+ is zero (40Ca0K; compare 3 with 6 in Fig. 4 A). Thus, a small extracellular K^+ concentration largely reverses the effects of 40 mM Ca^{2+} on C-type inactivation. The antagonism between extracellular Ca^{2+} and K^+ is also seen in Fig. 4 B, using currents elicited by longer depolarizing pulses to 50 mV. Each trace (in the order i–iv) was recorded after a 2-min rest in the designated solution to allow full equilibration. In traces i and iii, recorded in 40Ca0K, C-type inactivation was nearly complete, resulting in a small I_K . Note that C-type inactivation had occurred at -80 mV, during a 2-min period with no pulsing. After a return to 40Ca2K with a 2-min rest, I_K was much larger and C-type inactivated in the usual way (traces ii and iv). The addition of only 2 mM K^+ thus was sufficient to substantially antagonize the Ca^{2+} effect on C-type inactivation. Judging from the results shown in Fig. 4 A, I_K in Fig. 4 B would have been still larger had extracellular Ca^{2+} been reduced from 40 mM as K^+ was added. The results thus reiterate the strong and reversible effect of elevated extracellular Ca^{2+} on C-type inactivation and show that even a low concentration of extracellular K^+ antagonizes the current-depressing effect of Ca^{2+} . In the Discussion, we argue that Ca^{2+} and K^+

complete for occupancy of a site at the outer mouth of the K^+ channel. Additionally, the results show that with high concentrations of Ca^{2+} , C-type inactivation develops at the resting potential even without pulsing.

La^{3+} also enhances C-type inactivation

La^{3+} has almost the same ionic radius (1.17 Å) as Ca^{2+} (1.14 Å; Shannon, 1976) and has a Ca^{2+} -like effect on C-type inactivation of T449A channels but at much lower concentrations. On depolarization to 0 mV (Fig. 5 A), I_K activated quickly in 4Ca2K and then inactivated with a time constant of ~ 106 ms (mean before and after). Addition of 50 μM La^{3+} to the external solution markedly depressed I_K . After washing away the La^{3+} with 4Ca2K, I_K recovered completely. A lower concentration of La^{3+} (5 μM) was also effective in progressively depressing currents through T449A K^+ channels (Fig. 5 B). In our outside-out patch experiments, solution change typically required 2–5 s, which is much faster than the time course of the fall in I_K in this experiment. It can be seen in Fig. 5 B that the time course of inactivation was not markedly changed by La^{3+} , as is also true for elevated Ca^{2+} (Fig. 1 A).

In Fig. 5 C, La^{3+} was applied to wild-type ShB ΔN channels with a threonine at position 449. These channels normally inactivate with a time constant of ~ 1 s (Hoshi et al., 1991). At “a” the patch was depolarized to 0 mV for 3 ms, too short a time to cause appreciable C-type inactivation. That little inactivation occurred is shown by the tail of outward I_K seen on repolarization to -60 mV. Immediately after the pulse, 5 μM La^{3+} was added externally. In the next pulse, I_K was completely suppressed. We interpret this to mean that La^{3+} within seconds caused complete C-type inactivation at the holding potential of

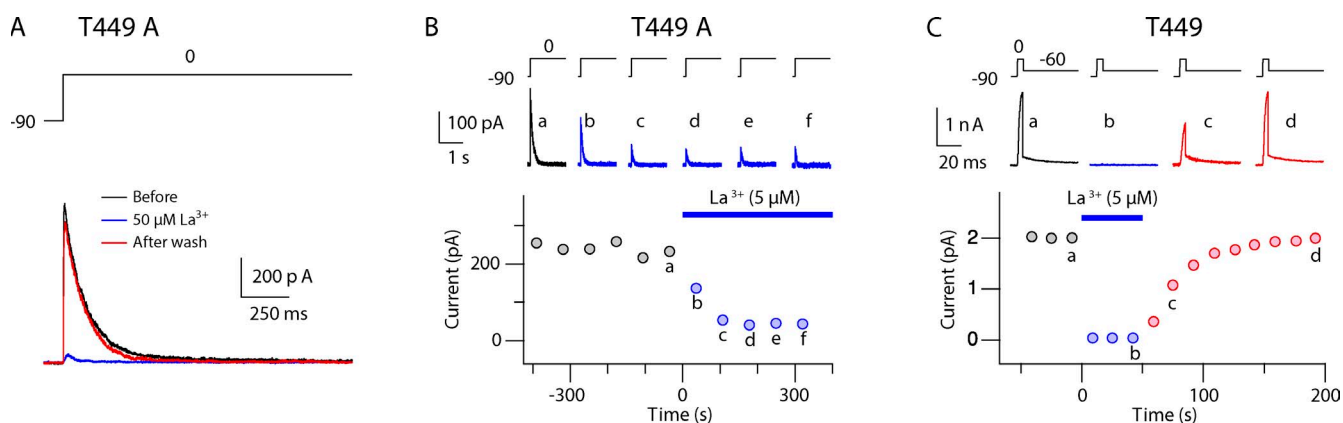


Figure 5. Extracellular La^{3+} enhances C-type inactivation in T449A and T449T (i.e., wild type) channels. (A) The black and the red trace are I_K from an outside-out patch before and after exposure to 50 μM La^{3+} , which virtually eliminated I_K (blue trace). I_K was elicited by 1.5-s pulses from -90 to 0 mV every 62 s. (B) Peak I_K through T449A channels before and during exposure to 5 μM La^{3+} in the extracellular solution. Similar results were obtained in four patches. (C) Peak I_K through T449T (wild type) channels before, during, and after washout of 5 μM La^{3+} in the extracellular solution. The patch was held at -90 mV except to measure the currents as illustrated in the insets. Similar results were obtained in four patches. The extracellular solution contained (mM) 130 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.4, plus 5 μM La^{3+} in blue traces. The internal solution contained (mM) 140 KCl, 2 $MgCl_2$, and 10 HEPES, pH 7.2.

−90 mV, with no pulsing. I_K recovered completely when La^{3+} was removed.

La^{3+} action on T449V channels that do not C-type inactivate
 In contrast to its strong effect on T449T and rapidly inactivating T449A (Fig. 5), La^{3+} , like Ca^{2+} , has only mild effects on I_K of T449V, as illustrated in Fig. 6. These effects, slow activation and a positive shift of the I-V curve, are similar to the effects of much higher concentrations of Ca^{2+} (Fig. 2) and are not related to C-type inactivation. Note that the inward tail of I_K seen in 10Ca2K is suppressed by La^{3+} , showing that at negative voltages K^+ channels are blocked by this trivalent ion. A similar block at negative voltage by high external Ca^{2+} has been previously reported (Tytgat and Daenens, 1997; Gomez-Lagunas et al., 2003).

DISCUSSION

This paper presents what we think are novel aspects of the interaction of two extracellularly applied multivalent cations, Ca^{2+} and La^{3+} , with voltage-gated K^+ channels. These effects are used here as tools to refine ideas regarding the mechanism of C-type inactivation. Ca^{2+}/La^{3+} effects on this inactivation (Hoshi et al., 1991; Kurata and Fedida, 2006) are quite distinct from the effects of multivalent cations on the voltage-controlled activation gate (Figs. 2 and 6). We think that the changes of channel properties caused by these ions are related to C-type inactivation. The main reason is that the deep depression of conductance caused by Ca^{2+}/La^{3+} is seen only in

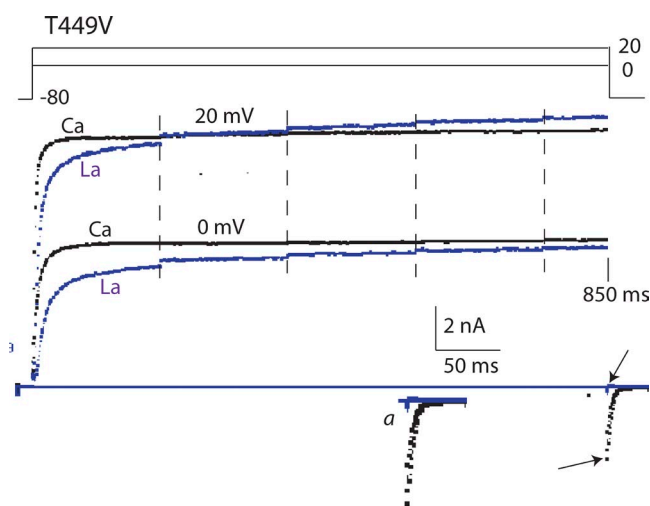


Figure 6. Extracellular La^{3+} (10 μ M) slows activation of T449V channels but causes no inactivation. I_K is compared in 10Ca2K (black traces) and in 10Ca2K + La^{3+} (10 μ M La^{3+} ; blue traces). Sampling was interrupted at each of the vertical dashed lines for 100 ms and then resumed. Total sweep duration was 850 ms. On return to −80 mV, there was a clear inward I_K tail without La^{3+} (bottom arrow), but none in the presence of La^{3+} , which blocks K^+ channels at negative voltage (top arrow). The tail is shown at slightly higher gain in inset a.

channels that have what is classically defined as C-type inactivation, a relatively slow current decay that follows activation of a K^+ channel's V-gate (Hoshi et al., 1991; López-Barneo et al., 1993). Additionally, the recovery from classical, depolarization-induced C-type inactivation is the same within the accuracy of the measurement as recovery for Ca^{2+}/La^{3+} -induced depression of I_K . The C-type inactivated state can also be accessed without experimental depolarization by the mutation W434F (Perozo et al., 1993; Yang et al., 1997) and rescued by the mutation T449V (Yang, Y., Y. Yan, and F.J. Sigworth, 2002. Biophysical Society Annual Meeting. 1138-Pos Board #B190). We think Ca^{2+}/La^{3+} , similar to W434F, facilitates entry of the channels into the C-type inactivated state, without depolarization.

The exact nature of C-type inactivation continues to be debated and has been attributed to either pore constriction (Liu et al., 1996; Cuello et al., 2010) or dilation of the outer pore (Hoshi and Armstrong, 2013). Fig. 7 (abstracted from the chimera analyzed by Long et al. [2007]) shows a slice through the selectivity filter and some surrounding residues known to be important in C-type inactivation. For convenience, the residue numbering of the chimera has been converted to that appropriate for ShB simply by adding 72 (see the alignment of chimera and Shaker in Long et al. [2007]); in ShB, residues 451 and 452 are valine and glycine. All others are the same as in the chimera.

Within the selectivity filter, there are four ion-binding sites (FS1 through FS4), of which only two are occupied by dehydrated K^+ ions at any instant. When conducting, occupancy oscillates between FS1 and FS3 (Fig. 7 A) and FS2 and FS4 (Fig. 7 B). External to FS1 is a hydration/dehydration site (FS0); a fully hydrated K^+ ion is shown just above this site in Fig. 7 A and partially hydrated in Fig. 7 B. (A partially dehydrated K^+ ion in FS0 can be seen in Zhou et al. [2001b].) Precise positioning of the carbonyls is required for effective permeation: a rough electrostatic calculation shows that moving a single carbonyl 1 Å away from the K^+ ion, in, e.g., FS1, decreases the binding affinity by a factor of roughly 50,000 according to Hille [2001] (Figs. S1 and S2), greatly raising the energy barrier to ion permeation.

The stability of the filter depends on the K^+ ions bound within it. Each ion is bound with an energy approximating the hydration energy of a K^+ ion in water (~ 77 kcal/mol or 323 kJ/mol [Burgess, 1978]), making the energy barrier for entering the filter from aqueous solution low. Removing a K^+ ion from a site would lead to a large mutual repulsive force among the carbonyls, tending to dilate the site. In fact, prolonged removal of all K^+ ions from both sides of the membrane is known to completely destroy conduction through K^+ channels (Gomez-Lagunas, 1997). In Fig. 7 A, all of the carbonyl rings except the innermost are bound electrostatically to K^+ . When the internal voltage is negative,

the ions in the filter are drawn by the electric field to positions FS2 and FS4 (Fig. 7 B). In this case, every carbonyl is bound to an ion in the filter, except for the outermost ring of FS1, which is contributed by Y445 residues. When voltage is negative, this outer ring depends for its stability on electrostatic binding to a partially dehydrated ion in FS0 (Fig. 7 B).

The selectivity filter is surrounded by residues that support the precise positioning required for selective K^+ permeation. To the left of the filter is W434, which has a close contact (Fig. 7 A, black dot) with the ring of Y445. The mutation W434F causes permanent C-type inactivation of the channel: only gating currents can be recorded (Perozo et al., 1993; Yang et al., 1997). Undoubtedly important in the positioning of W434 is a hydrogen bond from the carboxyl of D447 to the ring nitrogen of W434. The mutation D447E alters this bonding and speeds C-type inactivation (Molina et al., 1997).

To the right of the filter in the figure is V449 (T449 in wild-type ShB), which has close contact with Y445, on the side of the ring opposite to the W434 contact. Residue 449 is perhaps the most studied of the residues that affect C-type inactivation (López-Barneo et al., 1993). A449, K449, and E449 make C-type inactivation much faster, whereas V449 prevents it (summarized in Kurata and Fedida [2006]). To the right of residue 449 is a chain of contacting residues that stretches all the way to R365, which is in the voltage-sensing S4 helix (Tao et al., 2010). In the diagram, residue 449 connects to residues 450–452, which are in the loop joining the filter to S6. Both threonines in the crystal structure (ShB positions 451 and 452) form H-bonds to E418 at the top of S5 (one H-bond is shown with a yellow bar in Fig. 7 A). E418 connects to A417 and F416; the latter is in close contact (Fig. 7 A, black dot) with R365 in S4. This chain provides a likely basis for the observed coupling of C-type inactivation to S4 motion (Hoshi and Armstrong, 2013). In the resting state, the contact between F416 and R365 would not exist: R365 would be displaced inward by ~ 14 Å (Jensen et al., 2012).

We postulate that S4 voltage-sensor activation perturbs the amino acid residues surrounding the ion conduction pore allosterically, destabilizing its outer mouth and allowing it to dilate, thus facilitating C-type inactivation. In normal function, we postulate, outward movement of S4 brings R365 in contact with F416 (Fig. 7 A, black dot), perturbing it and starting a wave of conformational changes. The conformational wave spreads to E418, which is hydrogen-bonded to V451 ShB (Fig. 7 A, yellow bar at right). The mutations E418C and E418Q remove this hydrogen bond and greatly accelerate inactivation (Larsson and Elinder, 2000). The wave continues from V451 to D447. All of the residues in the 450–447 segment change conformation upon C-type inactivation (Liu et al., 1996; Molina et al., 1998). Based on study of the crystal structure, homology modeling, and

mutation experiments, we propose that the propagated changes cause the side chain of D447 to move outward, breaking a hydrogen bond (Fig. 7 A, yellow bar at left) to the ring nitrogen of W434. Deprived of this stabilizing H-bond, W434 rotates and its ring pushes on the ring of Y445 (Fig. 7 A, black dot), tending to cause rotation of Y445 and movement of its carbonyl away from the pore axis (all seen in homology modeling [Hoshi and Armstrong, 2013]). If the residue at position 449 is a valine, close contact (Fig. 7 A, black dot) between one of the two γ -carbons of the valine and the Y445 tyrosine ring prevents rotation of the ring. This firmly stabilizes the outer carbonyl ring, explaining why T449V does not undergo C-type inactivation and why Ca^{2+}/La^{3+} affects only S4 motion in this mutant. If the residue at position 449 is alanine, lysine, or one of numerous other residues, rotation is possible. If Y445 rotates, its carbonyl recedes ~ 1 Å from the pore axis (seen in homology modeling [Hoshi and Armstrong, 2013]), producing a

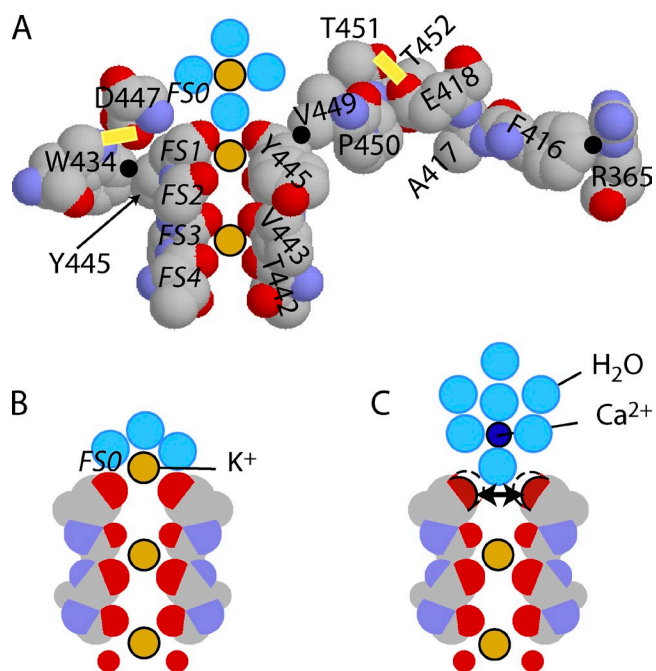


Figure 7. K^+ occupancy of FS1 is affected by voltage and extracellular Ca^{2+}/La^{3+} . (A) A slice through the selectivity filter and some surrounding residues. Points of significant close contact between residues are shown by black dots. Two significant H-bonds are indicated by the yellow bars. The spheres on the pore axis are dehydrated K^+ ions in filter sites FS1 and FS3 and a hydrated K^+ in FS0, just outside the filter (water molecules are light blue). (B) The filter only, in occupancy state FS2–FS4. When conducting, occupancy alternates between FS1–FS3 and FS2–FS4. At rest, when the channel is closed and V_m is negative, FS2–FS4 is the preferred state. In this state, the outermost carbonyls are stabilized by attraction to a partially dehydrated K^+ ion in FS0. (C) FS0 is occupied by a Ca^{2+} ion. Its hydration shell is too firmly attached to allow close approach to the outer carbonyls of FS1. As a result, they repel, as indicated by the two-headed arrow, leading to dilation and C-type inactivation. Residue numbering is appropriate to ShB Δ N.

high-energy barrier for K^+ entry into FS1. This greatly decreases the probability of FS1 occupancy, resulting in increased repulsion between the three other carbonyls of the outer ring, further dilating FS1 and completing C-type inactivation.

Although C-type inactivation is normally started by R365–F416 contact (we postulate), appropriate mutation of almost any of the residues in the chain from R365 to D447 can increase its likelihood (Hoshi and Armstrong, 2013). The residues in this postulated chain seem closely linked in a favored conformation, activated or inactivated: modification of one residue can affect the entire chain and, indirectly, the conformation of FS1. Furthermore, the stability of the outer carbonyl ring depends, in some cases crucially, on the presence of a K^+ ion in FS0 or FS1; removing external K^+ may affect the entire chain, all the way to F416 in the voltage-sensor domain.

The slowing of C-type inactivation by extracellular K^+ or TEA^+ is an almost defining characteristic (Choi et al., 1991; López-Barneo et al., 1993). The importance of internal K^+ in slowing C-type inactivation is shown by the finding that C-type inactivation is much faster in channels that are N-type inactivated, i.e., when the N terminus of any one of the four peptides forming a Shaker K^+ channel enters the inner end of an open channel and blocks I_K (Hoshi et al., 1990, 1991). The importance of intracellular K^+ was discovered and neatly explained by Baukrowitz and Yellen (1995): “N-type inactivation...inhibits outward K^+ flux, which [i.e. the flux] normally fills an external ion site and thus prevents C-type inactivation.” The “external ion site,” in our opinion, is FS1 in the selectivity filter.

As noted above, negative internal voltage draws the two ions in the filter into FS2 and FS4, leaving the outer carbonyl ring, contributed by Y445, dependent for stabilization on a partially hydrated K^+ ion in FS0 (Fig. 7 B). At negative voltage, K^+ efflux is vanishingly small. With no K^+ ions in the external solution, K^+ occupancy of FS0 is thus unlikely at negative voltage, and the carbonyl groups of FS1 thus repel each other, making dilation of the outer carbonyl ring and C-type inactivation more probable as observed experimentally (Choi et al., 1991; López-Barneo et al., 1993; Kurata and Fedida, 2006).

When both K^+ and Ca^{2+}/La^{3+} are present externally, we propose that they compete with K^+ for occupancy of FS0 (Fig. 7 C), particularly at negative voltage. Evidence for this competition is provided by the block of inward I_K observed in high Ca^{2+} for voltage negative to approximately -60 mV (Gomez-Lagunas et al., 2003) and by the block of inward I_K by La^{3+} seen in Fig. 6 (also see Tytgat and Daenens [1997]). In K^+/Ca^{2+} competition, K^+ is strongly preferred over Ca^{2+} : the enhancement of C-type inactivation caused by 40 mM Ca^{2+} is largely overcome by only 2 mM K^+ (Fig. 4). The relatively low affinity of Ca^{2+} for FS0 likely arises from its strong hydration (hydration enthalpy for Ca^{2+} is approximately

-380 kcal/mol vs. approximately -77 kcal/mol for K^+ [Burgess, 1978]). A firmly fixed hydration shell, we postulate, prevents close approach of Ca^{2+} to the outer carbonyls of FS1 (Fig. 7 C). Failure of Ca^{2+} to closely approach the carbonyls makes it ineffective in overcoming their mutual repulsion (Fig. 7 C, two-headed arrow): the force vector of carbonyl- Ca^{2+} attraction, rather than effectively pulling the carbonyls together, is predominantly outward in direction.

Once the outer carbonyls are diluted, it may be that FS1 is occupied by a partially hydrated Na^+ or Ca^{2+} ion. It is important in this argument that FS2 and FS4 remain K^+ selective and are occupied by K^+ even though the outermost carbonyl ring is diluted. Thus, for example, an Na^+ ion occupying the distorted FS1 cannot move inward to replace the securely complexed K^+ ion in FS2. Similarly, a K^+ ion in FS2 cannot move outward because of the energy barrier constituted by the dilated outer carbonyls.

Despite its higher hydration enthalpy, La^{3+} has higher apparent affinity for FS0 than does Ca^{2+} because of its triple charge. The probable mean hydration number is not very different for Ca^{2+} , ~ 4 – 12 , and La^{3+} , ~ 6 – 8 (Burgess, 1978); so each is separated from the outer carbonyls of the channel by, we postulate, a single water molecule in the firmly attached inner shell surrounding the ion (Fig. 7 C for Ca^{2+}). If both Ca^{2+} and La^{3+} have only one firmly attached hydration shell and are thus nearly equidistant from the outer carbonyls, the important factor determining relative Ca^{2+}/La^{3+} affinity for FS0 will be the higher charge on La^{3+} . Based on these ideas, we predict that any trivalent cation with a crystal ionic radius near 1.1 Å (e.g., Gd^{3+} and Tl^{3+}) will enhance C-type inactivation.

The effects of Ca^{2+} on the rate of C-type inactivation, large at negative voltage, are small at positive voltages where there is a strong efflux of K^+ through FS1 (Figs. 1 A and 3 B). It is known that C-type inactivation can occur at a near normal rate when the extracellular solution contains no external divalent cations (López-Barneo et al., 1993), consistent with the finding that the effects of Ca^{2+} are mainly at negative voltage. Some indications exist in the literature that strong K^+ efflux at very positive voltage decreases C-type inactivation. This may be related to a phenomenon observed in some K^+ channels called U-type inactivation (Klemic et al., 2001; Jamieson and Jones, 2014). The rather modest effect of TEA^+ in slowing C-type inactivation during a depolarizing pulse remains to be explained (Choi et al., 1991). We postulate that TEA^+ binds near T449 in ShB, trapping K^+ in FS0 or FS1 (Fig. 7 B) where it prevents dilation of the outermost carbonyls, a modified “foot-in-the-door” hypothesis (Armstrong, 1971; López-Barneo et al., 1993).

In summary, we believe that the requirements and conditions for a cation promoter of C-type inactivation are (a) an extensively hydrated cation (therefore di- or

trivalent) of the appropriate size ($\sim 1.1\text{\AA}$ ionic radius), too big to permeate the K^+ channel when hydrated, but (b) a good fit for FS0, where (c) it binds electrostatically (especially if trivalent) to the outer carbonyl ring of FS1, if (d) there is no K^+ efflux through the channel (closed V-gate or N-type inactivated) and (e) if FS1 is not K^+ occupied, as would be the case at sufficiently negative voltage.

Finally, are the observations in this article more compatible with pore constriction (Yellen et al., 1994; Cuello et al., 2010) or pore dilation (Hoshi and Armstrong, 2013) as the mechanism of C-type inactivation? Before the crystal structure was available, Yellen et al. (1994) originally proposed a constriction at the level of residue 449, which is outside the filter. In the crystal structure (2R9R; Long et al., 2007), the structural components postulated to be involved in the constriction are too widely separated for the required constriction to be a reasonable possibility (Hoshi and Armstrong, 2013). A subsequent proposal (for the bacterial KcsA channel [Cuello et al., 2010]) moved the constriction into the filter itself, at the position equivalent to G444 in ShB. We think the idea of constriction at G444 is unreasonable. Clearly constriction cannot occur if either FS1 or FS2 is K^+ occupied: G444 contributes a carbonyl to K^+ binding in both sites, and there is simply no room for constriction when K^+ (or a water molecule or a hydronium ion) is present. Removing a K^+ from FS1 or FS2 would require ~ 77 kcal/mol. The carbonyls of a K^+ -occupied site are stabilized by this binding energy, which draws them close to the K^+ . In the absence of a K^+ , the electronegative carbonyls would repel, as shown in Fig. S2: decreasing the diameter of the carbonyl ring by 1 \AA is less probable than increasing it by 1 \AA , by 49 kJ/mol, a factor of $\sim 3 \times 10^8$. In short, our evidence is compatible with dilation for the reasons cited above, and the idea of constriction is physically implausible.

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