

Potassium Blocks Barium Permeation through a Calcium-activated Potassium Channel

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ABSTRACT Single high-conductance Ca^{2+} -activated K^+ channels from rat skeletal muscle were inserted into planar lipid bilayers, and discrete blocking by the Ba^{2+} ion was studied. Specifically, the ability of external K^+ to reduce the Ba^{2+} dissociation rate was investigated. In the presence of 150 mM internal K^+ , 1–5 μM internal Ba^{2+} , and 150 mM external Na^+ , Ba^{2+} dissociation is rapid (5 s^{-1}) in external solutions that are kept rigorously K^+ free. The addition of external K^+ in the low millimolar range reduces the Ba^{2+} off-rate 20-fold. Other permeant ions, such as Tl^+ , Rb^+ , and NH_4^+ show a similar effect. The half-inhibition constants rise in the order: Tl^+ (0.08 mM) < Rb^+ (0.1 mM) < K^+ (0.3 mM) < Cs^+ (0.5 mM) < NH_4^+ (3 mM). When external Na^+ is replaced by 150 mM *N*-methyl glucamine, the Ba^{2+} off-rate is even higher, 20 s^{-1} . External K^+ and other permeant ions reduce this rate by ~ 100 -fold in the micromolar range of concentrations. Na^+ also reduces the Ba^{2+} off-rate, but at much higher concentrations. The half-inhibition concentrations rise in the order: Rb^+ (4 μM) < K^+ (19 μM) \ll Na^+ (27 mM) < Li^+ (>50 mM). The results require that the conduction pore of this channel contains at least three sites that may all be occupied simultaneously by conducting ions.

INTRODUCTION

High-conductance Ca^{2+} -activated K^+ channels (BK channels) are found in a great variety of tissues (Marty, 1981; Barrett et al., 1982; Latorre et al., 1982; Schwartz and Passow, 1983; Trautmann and Marty, 1984; Cecchi et al., 1986). This channel is particularly fascinating because it displays two apparently contradictory characteristics: very high conductance ($\geq 200 \text{ pS}$ in symmetrical 150 mM K^+) and strong selectivity for K^+ over other cations (Blatz and Magleby, 1984; Yellen, 1984a; Eisenman et al., 1986). To account for this unusual combination, it was proposed that BK channels might possess a short narrow conduction pathway that can be occupied by at most one ion at a time (Latorre and Miller, 1983; Blatz and Magleby, 1984). However, recent evidence has ruled out this simple picture. First, the fact that external K^+ has the ability to relieve internal Na^+ block by accelerating the blocker's

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dissociation rate, implies that both of these ions reside in the channel simultaneously (Yellen, 1984*b*). Second, the voltage dependence of Cs⁺ block is too strong to be consistent with a single-ion conduction mechanism (Cecchi et al., 1987). Lastly, measurements of channel conductance in the presence of mixtures of ions showed pronounced "anomalous mole fraction" effects (Eisenman et al., 1986). These three independent results demonstrate that several ions can simultaneously occupy the conduction pathway of BK channels.

In this study, ionic interactions inside the conduction pathway of rat muscle BK channels are probed with Ba²⁺, a high-affinity blocker of numerous K⁺ channels (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Armstrong et al., 1982; Vergara and Latorre, 1983; Benham et al., 1985; Miller et al., 1987). In the case of the BK channel, Ba²⁺ block has been characterized at the single-channel level (Vergara and Latorre, 1983; Miller, 1987; Miller et al., 1987). Ba²⁺ acts as a reversible blocker that resides on its blocking site for about 5 s, on average. The blocker is effective from either side of the membrane, but is much more potent when applied to the internal solution. At positive potentials, the association rate constant for internally applied Ba²⁺ is 10,000 times higher than that for external Ba²⁺, while the Ba²⁺ dissociation rate does not depend on the side of application.

Three lines of evidence support the idea that the Ba²⁺ blocking site is located inside the channel's conduction pathway. First, Ba²⁺ block is relieved by K⁺ (Vergara and Latorre, 1983); increasing the K⁺ concentration decreases the binding rate of internally applied Ba²⁺. Second, the channel must be in its "open" conformation to allow Ba²⁺ binding or dissociation (Miller et al., 1987). Finally, binding rates of Ba²⁺ applied internally as well as externally are voltage dependent in a way that indicates that the blocker, in the process of binding to the channel, traverses part of the applied voltage drop through the channel.

In this and the following paper, we investigate the interactions between Ba²⁺ and permeant cations inside the channel's pore. First, we show that the kinetics of Ba²⁺ block are strongly affected by the presence, at micromolar concentrations, of K⁺ in the external solution. Our results demonstrate that the conduction pathway of a Ba²⁺-blocked channel carries an externally facing binding site of extremely high affinity for K⁺ and other permeant cations. We also show that occupancy of this site by K⁺ prevents Ba²⁺ dissociation to the external solution, as would be expected if K⁺ and Ba²⁺ lie in single file within the conduction pathway. In the following paper, we show that at much higher concentrations, external K⁺ speeds up Ba²⁺ dissociation from the channel, and the internal K⁺ affects Ba²⁺ block kinetics in a way that is analogous to the effects of external K⁺. By examining these K⁺-Ba²⁺ interactions, we argue that the BK channel can be simultaneously occupied by one Ba²⁺ and at least three K⁺ ions.

MATERIALS AND METHODS

Biochemical

Plasma membrane vesicles containing BK channels were prepared from rat skeletal muscle as described (Moczydlowski and Latorre, 1983) and stored in 0.4 M sucrose at -70°C. The lipids used were 1-palmitoyl,2-oleoyl phosphatidylethanolamine (POPE) and the analogous

phosphatidylcholine (POPC), obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Lipids were stored in stock solutions in chloroform/methanol, 2:1, under N₂ at -70°C. Ultrapure NaCl (Alpha Inorganics, Danvers, MA) was used to avoid contamination of NaCl solutions and of agar bridges by K⁺. Ultrapure KCl (Johnson Matthey Chemicals, Ltd., Royston, England) was used to minimize contamination of the internal solutions by undesired Ba²⁺. *N*-methyl-D-glucamine (NMDG) was obtained from Sigma Chemical Co. (St. Louis, MO). All "K⁺-free" solutions contained <5 μM contaminating K⁺, while "Ba²⁺-free" solutions contained ~0.25 μM Ba²⁺.

Bilayer Formation and Channel Incorporation

BK channels were inserted into planar lipid bilayers as described previously (Latorre et al., 1982; Miller et al., 1987). Bilayers were formed by applying a drop of lipid solution (14 mM POPE + 6 mM POPC in *n*-decane) to a 250-μm-diam hole in the plastic septum separating two chambers filled with an aqueous solution. Before the formation of the bilayer, both chambers contained the same solution (10 mM HEPES, 5 mM NMDG; pH 7.4). After the formation of the bilayer, 150 mM KCl and 200 μM CaCl₂ (final concentrations) were usually added to one of the chambers defined as "the internal chamber." This procedure, and the systematic use of NaCl agar bridges, was used to avoid contamination of the "external" chamber by K⁺, and to keep the Na⁺ concentration lower than 1 mM in both chambers. In the presence of the transbilayer salt gradient, channel insertion occurred spontaneously after the addition of 1–5 μg/ml of the plasma membrane vesicles to the internal solution, which was continuously stirred. As soon as a single BK channel appeared (recognizable by its characteristic rapid fluctuations of 15 pA at zero voltage), the stirring of the internal solution was stopped and, depending on the experiment, either 150–170 mM NaCl, or 150 mM NMDG-Cl (final concentrations) was added to the external solution. The internal chamber was then perfused with a solution containing 150 mM KCl, 10 mM HEPES, 5 mM KOH; pH 7.4. CaCl₂ was added to the internal solution to achieve the desired level of channel activation. With this preparation of plasma membrane vesicles from rat skeletal muscle, BK channels almost always inserted with the cytoplasmic face of the channel facing the internal solution, as was confirmed by the polarity of voltage-dependent gating, and by the sidedness of activation by Ca²⁺ (Latorre et al., 1982) and of blocking by Ba²⁺ (Vergara and Latorre, 1983). All experiments were carried out at room temperature (20–25°C).

The electronics have already been described (Hanke and Miller, 1983). A desired voltage was applied across the bilayer, and the transmembrane current was measured with a low-noise current-to-voltage converter circuit. The current signal was filtered at 1 kHz using an eight-pole Bessel filter and stored on FM tape. The voltage command was applied by a laboratory computer (Indec, Sunnyvale, CA), which was also used for data analysis. The electrophysiological voltage convention is always used here, with the external side of the channel defined as zero voltage. Voltages have not been corrected for liquid junction potentials, since these were always <5 mV.

Analysis of Ba²⁺ Block Kinetics

This study analyzes the effects of externally applied permeant cations on Ba²⁺ block kinetics, which were observed as discrete blocking events at the single-channel level. Under the conditions used in previous work (100–150 mM internal K⁺, 5–300 mM external K⁺; Vergara and Latorre, 1983; Miller, 1987; Miller et al., 1987), Ba²⁺ block events could be easily distinguished from closures because of the channel gating process; the mean block time was ~5 s, while closed times were in the millisecond range. In such conditions the rates of Ba²⁺ interaction can be measured by analyzing the slow transitions between long-lasting nonconducting

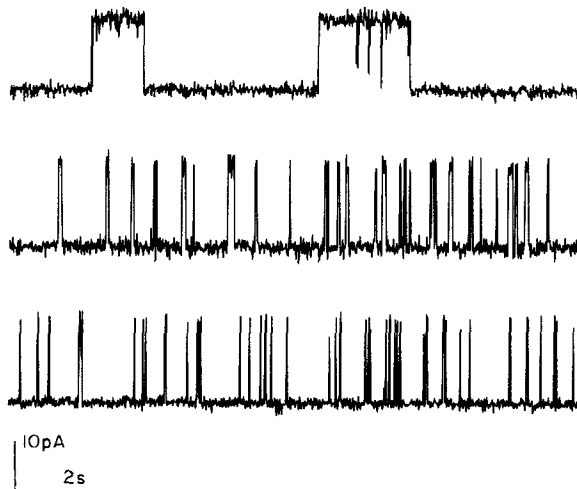
periods (blocks) and periods of rapid fluctuations between open and closed states (bursts). This is done by setting a "cutoff time" (100–300 ms) above which any nonconducting event is defined as a block.

The present experiments show that when external K^+ falls below 1 mM, the Ba^{2+} blocks become much more short-lived and closer to the time domain of channel gating. Under these conditions, therefore, it is invalid to define block events with a cutoff time. However, closed time histograms show two clearly separated components, a fast component that arises from gating, in the 1–20-ms range, and a slower component dependent on Ba^{2+} , that has a 50–500 ms time constant. Our analysis is focused on the slow component, which we will show is due to Ba^{2+} blocks. The time constant of the Ba^{2+} -dependent slower component, τ_b , is related to the dissociation rate constant for Ba^{2+} , k_{off} , by:

$$k_{off} = \tau_b^{-1} \quad (1)$$

Determination of the pseudo-first-order association rate constant, k_{on} , raises the problem

FIGURE 1. Effect of removing external K^+ on Ba^{2+} block kinetics. Single BK channels were recorded in planar bilayers with K^+ either present or absent in the external solution, at 50 mV applied voltage. In all cases, the internal solution contained 150 mM KCl, 100–200 μM Ca^{2+} , and 0.5–2 μM Ba^{2+} . Top trace: external solution contained 150 mM NaCl + 15 mM KCl and internal solution contained 1 μM Ba^{2+} . Mean block time was 4.8 s. Middle and lower traces: external solution contained 150 mM NaCl. Ba^{2+} concentrations were 0.5 μM (upper trace) and 2 μM (lower trace). Mean block time for recordings in K^+ -free external solutions was 400 ms. In this and all succeeding channel records, opening is upward.



of defining a burst, given the fact that blocking events are partly contaminated with channel closures. Our procedure is to use the double-exponential nonconducting time cumulative histogram to choose a cutoff time, t_c , that is long enough so that >98% of the events longer than this are blocks. *Apparent bursts* are defined as periods of gating separated by nonconducting intervals longer than t_c , and we always find the time distribution of bursts thus defined to be single exponential. The time constant of this distribution is spuriously high, because of missed short blocks. However, since the apparent burst distribution is single exponential, it is easy to show that the observed mean burst time, $\tau_u(\text{obs})$, can be used to calculate the true association rate constant (Blatz and Magleby, 1986):

$$(k_{on})^{-1} = \tau_u(\text{obs}) \exp(-t_c/\tau_b) - \tau_b[1 - (1 + t_c/\tau_b) \exp(-t_c/\tau_b)] \quad (2)$$

Since Ba^{2+} binding and dissociation rates both vary with the unblocked channel's open

probability (Miller et al., 1987), it was necessary to insure that this open probability, p_o , remained constant during an experiment. Therefore, we always worked at high enough Ca^{2+} so that p_o was always >0.9 . In most experiments, 100–200 μM Ca^{2+} was used, but in cases using highly negative voltages, Ca^{2+} concentrations as high as 10 mM were required. By working with a high p_o , we never observed spontaneous long-lasting “shifts” of p_o .

The high internal Ca^{2+} concentrations used in our experiments raised another potential

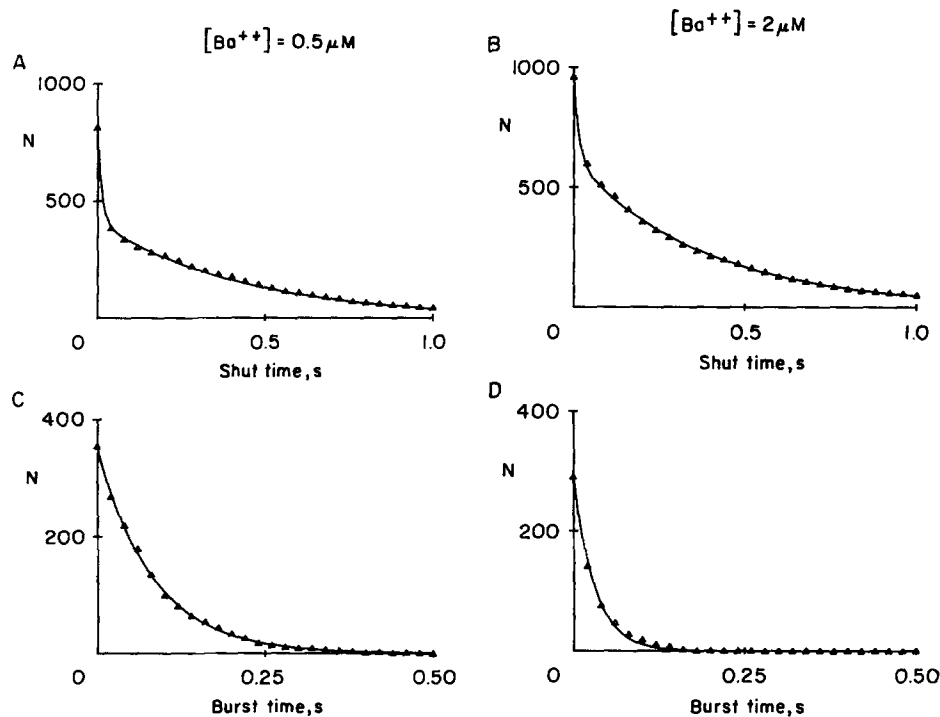


FIGURE 2. Kinetic analysis of Ba^{2+} blocks in absence of external K^+ . The cumulative distributions of nonconducting times or burst times are shown for single channels in K^+ -free external solutions, at two different Ba^{2+} concentrations, 0.5 μM (A, C) and 2 μM (B, D). Data are taken from the same experiment as that displayed in Fig. 1. Nonconducting (or “shut”) time distributions (A, B) were fitted by two exponentials with time constants of 12 ms (51%) and 420 ms (49%) in A, and of 16 ms (36%) and 390 ms (64%) in B. The fast exponential time constants were first determined by fitting expanded time scale histograms (not shown). Burst-time histograms (C, D), calculated as described in Methods, using a closed-time cutoff of 50 ms, were fit with single exponentials of time constants 83 ms in C and 31 ms in D. Mean burst times for these records, corrected for missed events, were 70 and 24 ms, respectively, and they differed from the spuriously high time constants of the distributions for reasons described in Methods.

problem. Vergara and Latorre (1983) showed that internal Ca^{2+} , at high concentrations (>50 μM) and depolarized potentials (>30 mV), induces blocking events very similar to the Ba^{2+} blocks. Indeed, we also observed occasional blocks in nominally Ba^{2+} -free solutions. While a portion of these may represent block by Ca^{2+} , the majority are due to low levels of Ba^{2+} contaminating our solutions (see below). For this reason, we always added Ba^{2+} to a concen-

tration high enough so that at least 80% of the observed blocks were due to the Ba^{2+} actually added in the experiment, with most of the remaining 20% still being true Ba^{2+} blocks.

RESULTS

Ba²⁺ Block Kinetics in the Absence of External K⁺

This study was provoked by the observation that the removal of the last traces of K^+ from the external solution dramatically alters the kinetics of Ba^{2+} block. Fig. 1 illustrates this effect; Ba^{2+} was present in the internal solution to induce block. In the top trace, we see the pattern of Ba^{2+} block that was originally described by Vergara and Latorre (1983), which was observed with at least 2 mM K^+ present in the external solution. The channel record is interrupted by long-lived (5 s) Ba^{2+} blocking events that separated "bursting" intervals; since high concentrations of Ca^{2+} were

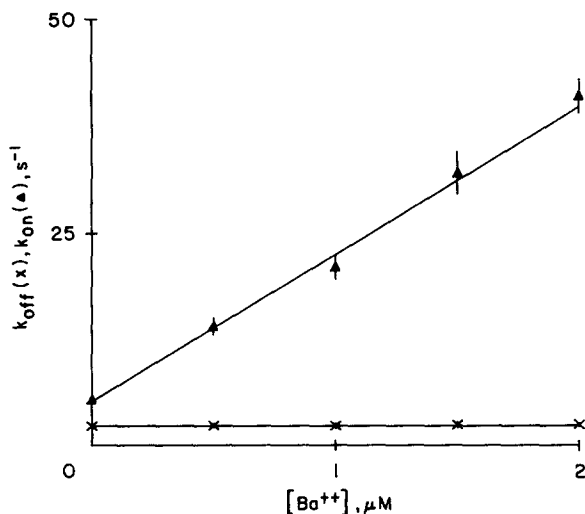


FIGURE 3. Bimolecular kinetics of Ba^{2+} block at zero external K^+ . Association (\blacktriangle) and dissociation (\times) rates (k_{on} and k_{off}) for Ba^{2+} were measured according to the procedure described in Methods for single channels in zero external K^+ solutions, under the conditions described in Fig. 2. Internal Ba^{2+} concentration was varied as indicated.

always used here, the channel was nearly always open during a burst, and closures due to channel gating were in the millisecond range. When K^+ is removed from the external solution (Fig. 1, lower traces), Ba^{2+} -induced blocks and bursts are still apparent, but now they operate on a much faster time scale, 50–500 ms. The two lower traces of Fig. 1 show qualitatively that increasing internal Ba^{2+} concentration shortens the burst times, but leaves the block times unaffected, as is expected if the blocks are caused by individual Ba^{2+} ions binding to the channel.

Examination of the nonconducting dwell-time distributions under zero external K^+ conditions confirms our interpretation of the blocking behavior. Fig. 2, A and B illustrate this distribution at two Ba^{2+} concentrations. At both concentrations of Ba^{2+} , the distributions are fit by two well-separated exponentials of time constants of ~ 15 and 400 ms. When the Ba^{2+} concentration is raised, the fraction of long-lived nonconducting events increases, but the time constant of this fraction remains the same. This result argues that the slow fraction of the nonconducting time distri-

bution represents discrete Ba²⁺ blocking events. Although this picture of Ba²⁺ block has been confirmed repeatedly in the past (Vergara and Latorre, 1983; Miller et al., 1987), it is necessary to go through the argument again now, since the rate of Ba²⁺ dissociation is so much faster in the absence of external K⁺. This picture is further confirmed qualitatively by the burst-time distributions, seen in Fig. 2, C and D. These burst times are single exponential, and the time constant decreases as the Ba²⁺ concentration is raised. As described in Methods, the Ba²⁺ association rate constant is not measured directly from this distribution, but rather from the mean burst time corrected for missed events.

Further validation of this view is shown in Fig. 3. Here, we measure the blocking and dissociation rates as a function of Ba²⁺, using the slow fraction of the nonconducting time distribution to determine off-rate, and the corrected mean burst time to calculate on-rate. As demanded by a bimolecular blocking process, the off-rate is independent of Ba²⁺ concentration, while the on-rate increases linearly with blocker concentration. Close inspection of Fig. 3 reveals that the on-rate intercept at zero-

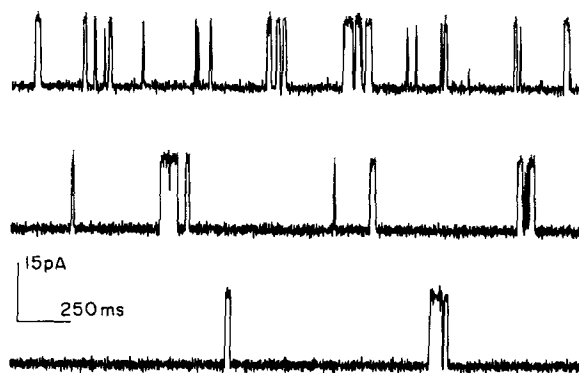


FIGURE 4. Ba²⁺ block kinetics: variation with external K⁺. A single channel was studied under the conditions described in Fig. 1, with the external solution initially K⁺-free (150 mM NaCl medium). The internal solution contained 2 μM Ba²⁺. After records were taken in K⁺-free external solution (top trace), KCl was added externally to final concentrations of 0.3 mM (middle trace) and 2 mM (lower trace).

added Ba²⁺ is not zero. Partly, this is because of slow blocking by Ca²⁺, but most of this blocking is probably due to Ba²⁺ contaminating our solutions; when 75 mM K₂SO₄ is used in the internal solution, so as to maintain Ba²⁺ concentration below 1 nM, the blocking rate is 5–10-fold lower than that seen in KCl medium with no added Ba²⁺. In all experiments, we used Ba²⁺ at concentrations high enough so that over 80% of the blocking events observed were caused by the Ba²⁺ added to the medium.

External K⁺ Locks Ba²⁺ in the Channel

We have established that when Na⁺ is the only cation present in the external solution, internal Ba²⁺ induces relatively short blocks. Previous work performed in the presence of 5–300 mM K⁺ in the external solution (Vergara and Latorre, 1983; Miller et al., 1987) has described Ba²⁺ blocks of 10–20-fold longer duration. Over what concentration range does external K⁺ exert its effect on the block times? Fig. 4 shows recordings of Ba²⁺ blocking events at varied external K⁺ concentrations, in

the presence of 150 mM external Na^+ . A clear lengthening of the block time can be seen at 0.3 mM K^+ , and nearly a full effect of K^+ in slowing Ba^{2+} dissociation is expressed by 2 mM. Block-time distributions (Fig. 5), are single-exponential over this range of external K^+ concentration.

The effect of external K^+ on the Ba^{2+} dissociation rate is reported in Fig. 6 A. The off-rate falls from a value of $\sim 5 \text{ s}^{-1}$ at zero external K^+ , to about 5% of that value by 10 mM K^+ . The inhibition of Ba^{2+} off-rate follows a simple rectangular hyperbolic function of the external concentration of K^+ , the concentration of half-inhibition, K_i , being 185 μM . The Ba^{2+} association rate also decreases dramatically as external K^+ is raised (Fig. 6 B), although the K_i for the on-rate, 450 μM , is 2–3-fold higher than the K_i for the off-rate. This is a particularly notable effect of external K^+ at submillimolar levels, since Ba^{2+} acts from the internal solution, which always contains 150 mM K^+ . The remainder of this study addresses only the off-rate; effects of K^+ on the association rate will be discussed in the following report (Neyton and Miller, 1988).

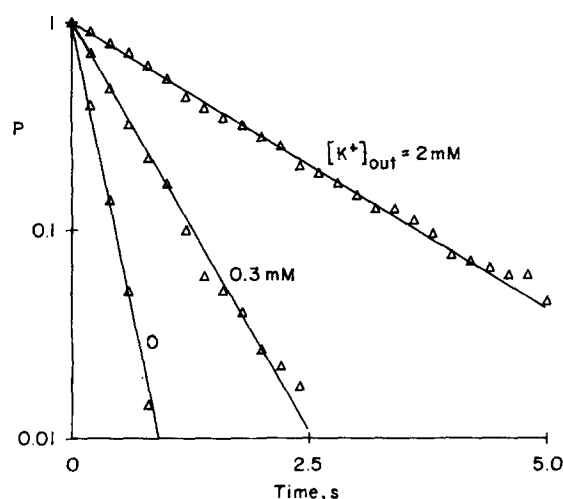


FIGURE 5. Ba^{2+} block-time distributions at varying external K^+ . Block-time distributions were measured from single-channel records as in Figure 4, at the external K^+ concentrations indicated. A cutoff time for blocks of 50 ms was chosen to eliminate the short-lived closures, and the distributions were accordingly single exponential. Time constants were 200 ms at zero K^+ , 530 ms at 0.3 mM K^+ , and 1.6 s at 2 mM K^+ .

A Possible Explanation

The inhibition of Ba^{2+} dissociation by external K^+ is an unexpected and startling result. Two lines of evidence had suggested that Ba^{2+} leaves the channel to the internal solution when it dissociates from its blocking site (Vergara and Latorre, 1983; Miller et al., 1987). First, although Ba^{2+} is able to block the channel from the external solution, the apparent affinity of external Ba^{2+} is much lower than that of internal Ba^{2+} at depolarized potentials. This suggested that the channel's outer mouth presents a high energy barrier that limits the entry of Ba^{2+} from the external solution. Such a barrier did not seem to exist in the inner mouth of the channel. The second point is that the same blocking site was attained, regardless of the side of Ba^{2+} application. Thus Ba^{2+} was postulated to exit primarily to the internal side, avoiding the externally facing energy barrier. In these previous experiments, exter-

nal K⁺ was always >5 mM, a concentration which we now know gives nearly a maximal inhibition of the off-rate. The ability of external K⁺ to slow down Ba²⁺ dissociation cannot be reconciled in a simple way with the idea that Ba²⁺ always dissociates to the internal solution. This idea, along with the simple picture of ion-ion repulsion within a single-file pore, predicts that external K⁺ would accelerate the dissociation of the blocker, as has been seen in several other cases (Armstrong et al., 1982; Yellen, 1984b; Cecchi et al., 1987; MacKinnon and Miller, 1988). Our observations run directly counter to this prediction.

We therefore propose the preliminary model illustrated in Fig. 7. The channel is viewed as having two sites: a Ba²⁺-blocking site (which might bind K⁺ when Ba²⁺ is not in the channel), and a K⁺-binding site located externally to the blocking site. In the absence of external K⁺, as soon as a Ba²⁺ ion reaches the blocking site, the

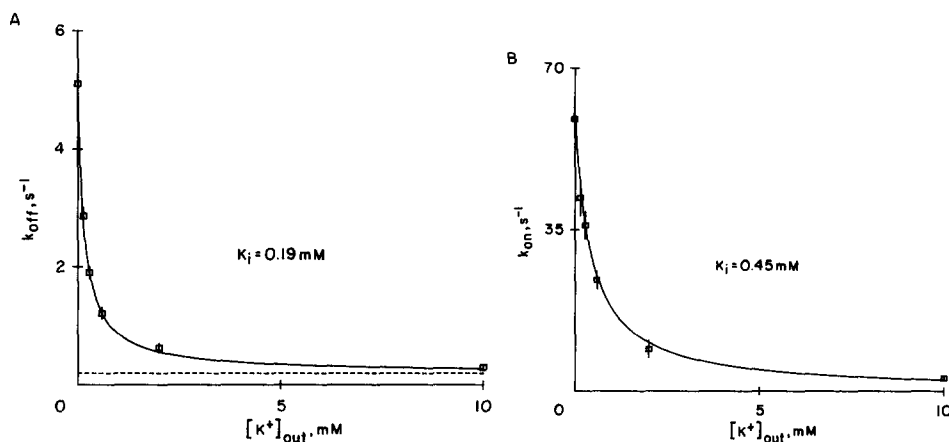
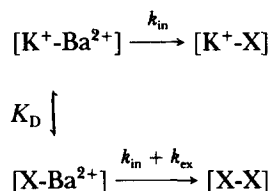


FIGURE 6. Slowing of Ba²⁺ block kinetics by external K⁺. Ba²⁺ dissociation and association rates were measured, in external 150 mM NaCl medium, as a function of external K⁺ concentration, under conditions described in Figs. 4 and 5. (A) Dissociation rate. Solid curve is plotted according to Eq. 3, with $K_i = 185 \mu\text{M}$. (B) Association rate. Solid curve is a rectangular hyperbola with $K_i = 450 \mu\text{M}$.

external K⁺ site becomes empty, since now the internal K⁺-containing solution is cut off from communication with the external K⁺ site. In other words, blocking the channel puts this site into a true equilibrium with the external solution. We propose that with an empty external K⁺-binding site, Ba²⁺ now has the opportunity to dissociate to the external solution. Moreover, we assert that this externally directed dissociation must occur at a much higher rate than inward dissociation. With K⁺ in the external solution, the external K⁺-binding site will tend to be occupied by K⁺ during a Ba²⁺ block, depending on the K⁺ concentration. The blocking Ba²⁺ ion then must either dissociate to the internal solution, or wait for the external K⁺ site to become empty; once K⁺ dissociates from this external site, Ba²⁺ may then occupy it, and thus be in a position to dissociate to the external solution. This model merely points out the primary characteristic of single-filing behavior, that Ba²⁺ can exit only after

a vacancy becomes available. Thus, external K^+ ions "lock" Ba^{2+} inside the channel.

This model can be formalized by the following scheme:



Here, the brackets indicate the occupancies of the two sites within the channel (X indicating no occupancy), and K_D represents the dissociation constant for K^+ at the external binding site. This scheme explicitly assumes that the externally directed dissociation rate, k_{ex} , is zero when K^+ occupies this site, and that the internally directed

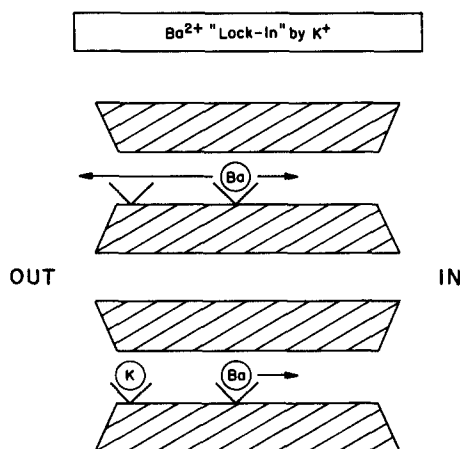


FIGURE 7. A model for K^+ influence on Ba^{2+} dissociation. Cartoon of the BK channel occupied by a Ba^{2+} ion, drawn to illustrate the effect of external K^+ in reducing the rate of Ba^{2+} dissociation. When the external "lock-in" site is unoccupied by a K^+ ion, Ba^{2+} can dissociate either to the inside or the outside solution. Occupancy of the lock-in site by K^+ prevents externally directed dissociation, and thus reduces the net dissociation rate.

dissociation rate, k_{in} , is the same regardless of whether the external K^+ -binding site is occupied or not. Though this latter assumption may not be strictly true, as long as outward dissociation is much faster than inward, this assumption will not compromise the interpretation. It then follows that the observed dissociation rate varies with external K^+ concentration, according to a rectangular hyperbola:

$$k_{off}([K^+]_{out}) = k_{in} + k_{ex}/(1 + [K^+]_{out}/K_D) \quad (3)$$

We have seen (Fig. 6) that this expectation is verified. In terms of this model, the measured K_i is identical to the dissociation constant for K^+ on the lock-in site.

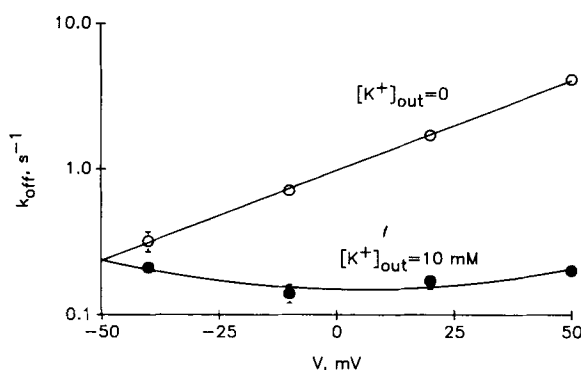
External K^+ Ions Affect the Voltage Dependence of k_{off}

Since $k_{in} \ll k_{ex}$, the "lock-in" model predicts that when no K^+ is present in the external solution, Ba^{2+} dissociates mainly to this side. In contrast, when the external concentration of K^+ is in the range 5–50 mM, and as k_{off} approaches its minimum value,

a significant proportion of the Ba²⁺ dissociation events proceed to the internal solution. As Ba²⁺ bears two positive charges, transbilayer voltage is expected to affect the rates of Ba²⁺ dissociation to the internal and external solutions in opposite ways. We therefore expected to find that k_{off} would display a different voltage dependence in the presence of external K⁺ than in its absence.

Fig. 8 shows that the expected effects were actually observed. In this experiment, with a K⁺-free external solution, the Ba²⁺ dissociation rate increased when the internal chamber was made more positive (e-fold increase in k_{off} for ~30 mV depolarization). A similar voltage dependence, of the same polarity, was seen for the Ba²⁺ association rate (e-fold increase in k_{on} for 23 mV depolarization). After the addition of 10 mM external K⁺, k_{off} became virtually independent of voltage, while the voltage dependence of k_{on} remained the same (data not shown). In a series of nine experiments in zero external concentration of K⁺, k_{off} was found to increase e-fold for 27 ± 1 mV depolarization, while k_{on} increased e-fold for 21 ± 1 mV depolarization.

FIGURE 8. Effect of K⁺ on voltage dependence of Ba²⁺ dissociation. Voltage dependence of Ba²⁺ dissociation was measured in either zero external K⁺ (O) or 10 mM external K⁺ (●), with 150 mM NaCl external medium. To keep the channel open probability high across the entire voltage range, the internal solution contained 10 mM Ca²⁺ and 10 mM Mg²⁺. Blocks were induced by internal Ba²⁺ in the range 10–100 μM. In the absence of external K⁺, k_{off} increased e-fold per 30 mV depolarization; this voltage dependence was lost in 10 mM external K⁺.



The fact that k_{off} increases with depolarization is a strong argument for the idea that Ba²⁺ dissociates to the external solution when this solution is K⁺-free. The loss of voltage dependence of k_{off} in the presence of 10 mM K⁺ also agrees with our model, which asserts that when the external K⁺-binding site is occupied, dissociation will tend to occur more often to the internal solution. Indeed, we thought that with external K⁺ present, the voltage dependency of k_{off} might actually reverse polarity. Such an effect is observed, but it develops only at very high external K⁺ concentrations (>100 mM), and will be discussed in the following paper (Neyton and Miller, 1988).

Selectivity of the External "Lock-In" Site among Permeant Cations

The reduction in Ba²⁺ dissociation rate by external K⁺ is also seen with other permeant cations. Fig. 9 shows that substantial selectivity among cations is seen in the apparent affinity for the "lock-in" site. Table I summarizes apparent inhibition constants (K_i) which rise in the order Tl⁺ (0.08 mM) ~ Rb⁺ (0.10 mM) < K⁺

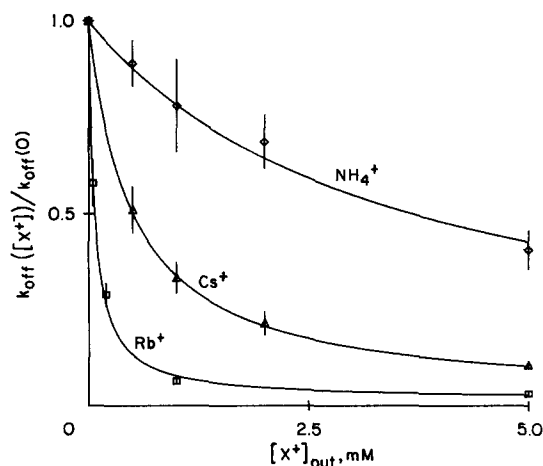


FIGURE 9. Inhibition of Ba^{2+} dissociation by permeant cations. Ba^{2+} dissociation rate was measured in the external NaCl medium as a function of added external permeant ions (X^+), under those conditions found in Fig. 6. Inhibition curves are fit to Eq. 3, with K_i values given in Table I.

(0.3 mM) < Cs^+ (0.5 mM) < NH_4^+ (3 mM). All of these cations are known to permeate this channel (Blatz and Magleby, 1984; Yellen, 1984a; Eisenman et al., 1986), even Cs^+ , which appears to require very high voltages to do so (Cecchi et al., 1987).

The lock-in effect was observed with permeant cations in the submillimolar range, in the presence of a massive excess of the impermeant ion Na^+ , 150–170 mM. Moreover, the addition of 50 mM Li^+ , another impermeant cation, failed to reduce the Ba^{2+} off-rate discernably (data not shown). These results demonstrate that the lock-in site is strongly selective for permeants over nonpermeants, but the protocol used so far (high external NaCl present) prevents a quantitative analysis of this question.

External Na^+ Also Locks Ba^{2+} in the Channel

To determine the affinity of the external lock-in site for Na^+ , experiments had to be performed in the presence of a low initial Na^+ concentration in the external solution. But, to avoid possible changes in local surface potentials near the channel mouth, it was necessary to maintain a high ionic strength throughout the experiment. Therefore, we sought another cation having a lower affinity than Na^+ for the

TABLE I
Ion Selectivity of External Lock-In Effect

Ionic composition of external solution	K_i (μM)					K_i (mM)	
	Tl^+	Rb^+	K^+	Cs^+	NH_4^+	Na^+	Li^+
150 mM Na^+	80 ± 10	100 ± 20	270 ± 40	520 ± 10	$2,900 \pm 300$	—	—
150 mM NMDG		4 ± 1	19 ± 2			27 ± 3	>50

The abilities of various cations to lower the dissociation rate for Ba^{2+} were compared. Values of inhibition constants, K_i , were measured for the indicated cations that were added to the external solution, as in Figs. 9 and 12. Cations were added as the Cl^- salts, except for Tl^+ , for which the acetate salt was used. Each value represents the mean \pm SE of three to five separate determinations in different bilayers.

FIGURE 10. Ba²⁺ block kinetics in the absence of external group IA cations. Records of a single channel were collected in an external medium free of all small cations. The external solution was 150 mM NMDG-Cl, 10 mM HEPES-NMDG, pH 7.4. The standard 150 mM KCl internal medium was used with 100 μM Ca²⁺ present.



Holding voltage was 50 mV. (A) No added Ba²⁺. Blocks and bursts are due to contaminating levels of Ba²⁺ (~0.2 μM), with a mean block time of 68 ms, and a mean burst time of 55 ms. (B) 1 μM Ba²⁺ added. The mean block time was 56 ms and the mean burst time was 11 ms.

external lock-in site. Assuming that the external lock-in site is located inside the narrow conduction pathway of the channel, we guessed that large organic cations, like NMDG⁺ or arginine, might be unable to reach this site. Fig. 10 shows recordings obtained in an experiment where the external solution contained 150 mM NMDG⁺, and no other added cations. Again, in low Ba²⁺ (upper trace), the record consists of bursts and blocks, but now the blocked times are even shorter than with only Na⁺ present, as in Fig. 1. The addition of 1 μM Ba²⁺ to the internal solution (lower trace) induced a pronounced decrease in the burst duration, whereas the mean block times were not significantly affected. Fig. 11 shows that a “slow” component could be easily isolated in the nonconducting dwell-time histogram under such conditions. This slow component can be fitted by a single-exponential of 60–70 ms time constant. An analysis of the variation of blocking and unblocking rates as a function of internal Ba²⁺ concentration, as in Fig. 3, demonstrates that most of the long closures observed in Fig. 10 are actually Ba²⁺ blocks (data not shown).

Comparing experiments performed in, respectively, 150 mM external Na⁺ and 150 mM external NMDG⁺ shows that Ba²⁺ dissociation is about five times faster in

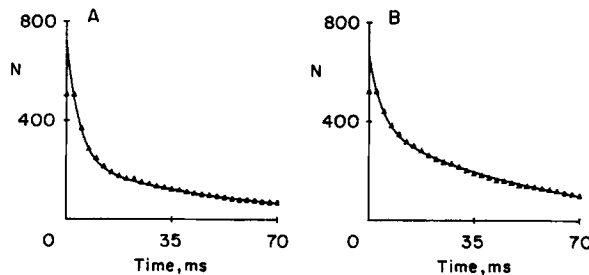


FIGURE 11. Ba²⁺ block-time distribution in zero external K⁺ and Na⁺. Cumulative nonconducting dwell-time distributions were measured using the same experiment as that shown in Fig. 10, at two different Ba²⁺ concentrations. Distributions were double exponential, with a fast time constant of 4 ms in both cases. (A) No added Ba²⁺, the time constant of the slow fraction was 70 ms. (B) 1 μM Ba²⁺ was added and the slow fraction time constant was 59 ms.

NMDG⁺ than in Na⁺. This observation intimates that Na⁺ is also able to lock Ba²⁺ inside the channel to some extent. We carried out a series of experiments to determine the apparent dissociation constant of the external lock-in site for Na⁺. These experiments were performed as reported above for permeant cations, except that now the external solution contained 150 mM NMDG⁺ rather than Na⁺ at the beginning of the experiment. As Fig. 12 A demonstrates, the Ba²⁺ off-rate is reduced by Na⁺ in a way qualitatively similar to the actions of the permeant cations. From a series of five experiments performed in the same conditions, the average value of the apparent dissociation constant of the external lock-in site for Na⁺ was found to be 27 ± 4 mM. Similar experiments with Li⁺ show that this ion has an even lower affinity, with $K_i > 50$ mM.

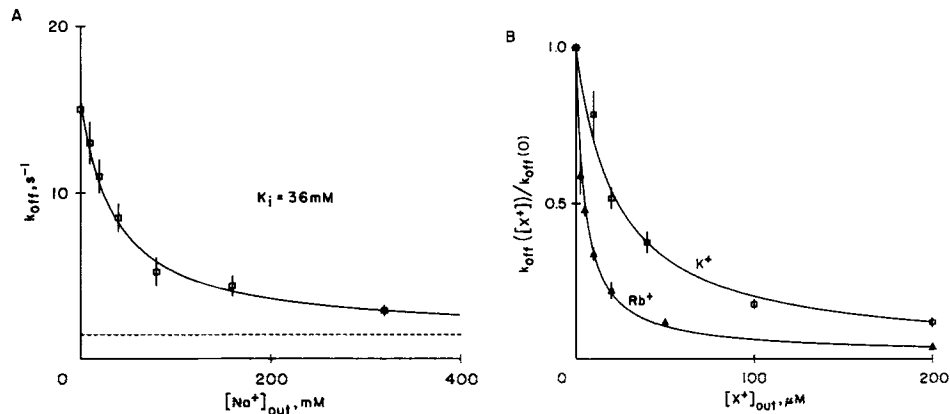


FIGURE 12. Lock-in of Ba²⁺ by external cations. Inhibition of Ba²⁺ dissociation by external cations was studied in external NMDG medium, under conditions similar to those described in Fig. 10. (A) Lock-in by Na⁺. (B) Lock-in by K⁺ and Rb⁺. Variation of k_{off} with external cations was fit with a solid curve according to Eq. 3, with K_i equaling 36 mM for Na⁺, 20 μM for K⁺, and 5 μM for Rb⁺.

A More Realistic Measure of the Affinities for Permeant Cations

The constants of the external lock-in site for permeant cations reported above (Table I) were measured in the presence of high external Na⁺. We have just seen that Na⁺ itself binds to a lock-in site when K_i is ~ 30 mM. If Na⁺ and the permeant cations compete for the lock-in site, then the permeant cation affinities in Table I are serious underestimates. We therefore measured the K_i of the external lock-in site for K⁺ and Rb⁺ in a series of experiments performed in Na⁺-free, NMDG⁺-containing external solutions. The inhibition of k_{off} by these two cations is shown in Fig. 12 B. Under these Na⁺-free conditions, the values of K_i were 15–20-fold lower than those seen in Na⁺ medium (19 ± 2 μM for K⁺, five experiments; and 4 ± 1 μM for Rb⁺, four experiments). It is noteworthy that these represent affinities that are 3–4 orders of magnitude higher than that measured above for Na⁺ under identical conditions.

DISCUSSION

This paper presents two main results concerning ionic interactions inside the conduction pore of the high-conductance BK channel. We have first shown that the binding of a blocker Ba²⁺ ion can be stabilized by the additional binding of a K⁺ or similar cation. These experiments examined the effects of cations added to the external side of the membrane, and so we term the cations' site of action the "external lock-in site." Second, in a blocked channel, this site displays a surprisingly high affinity and selectivity for permeant cations, with apparent dissociation constants in the micromolar range for Rb⁺ and K⁺, and ~30 mM for Na⁺. These results allow us to refine our picture of the mechanism by which Ba²⁺ blocks the BK channel. Moreover, they lead to a more precise view of K⁺ permeation mechanisms in this channel.

The Mechanism of Ba²⁺ Lock-In by External Cations

Ba²⁺ is known to block BK channels via a bimolecular reaction scheme: a single Ba²⁺ ion enters inside the pore where it tightly binds and therefore blocks the K⁺ flow (Vergara and Latorre, 1983). As reviewed in the Introduction, this channel permits several ions to occupy its conduction pathway simultaneously. The ability of external permeant cations to lock Ba²⁺ into the channel adds a further strong indication of the multi-ion nature of the permeation mechanism. The central point here is that occupancy of the lock-in site by K⁺ stabilizes the binding of Ba²⁺ by retarding its dissociation from the channel. We interpret this result to mean that Ba²⁺ in the internal solution actually permeates the channel in the absence of external K⁺, and that K⁺ "blocks" this permeation at an externally facing site. To be sure, Ba²⁺ currents (20 Ba²⁺/s, 10⁻⁶ pA) are small in comparison to K⁺ currents (10⁸ K⁺/s, 10 pA), and indeed can be observed only as the "shot noise" of individual Ba²⁺ ions moving through the single channel. But the clear effect of K⁺ in reducing the flow of Ba²⁺ through the channel is strictly analogous to classical rapid blocking reactions, such as tetraethylammonium block of K⁺ currents (Vergara et al., 1984).

According to the model proposed here, Ba²⁺ can leave the channel either to the internal or external solution. In the absence of external K⁺ ions, Ba²⁺ dissociation proceeds mainly to the external solution; in the presence of external K⁺, occupancy of the lock-in site prevents Ba²⁺ exit to the external solution. The effect of externally added K⁺ on the voltage dependence of k_{off} strongly supports this interpretation. First, the increase of k_{off} induced by depolarization when no external permeant cation is present is a good indication of a preference for Ba²⁺ dissociation to the outside. Second, the disappearance of voltage dependence of k_{off} in the presence of external K⁺ is simply explained by the proposed blocking mechanism, since now Ba²⁺ dissociation is more evenly split between both sides. Even at external K⁺ concentrations as high as 10 mM, with the external lock-in site highly occupied, Ba²⁺ dissociation is not exclusively directed towards the inside, as the voltage independence of the off-rate indicates. The reason for this, as well as for the fact that inward dissociation is slow under the conditions used here, is that the internal side of the channel also carries a lock-in site, as will be described in the companion paper (Neyton and Miller, 1988).

A fundamentally different mechanism was proposed by Armstrong and colleagues (1982) to account for similar "lock-in" effects of external Rb^+ , Cs^+ , or NH_4^+ on Ba^{2+} block of squid axon delayed rectifier K^+ channels. They proposed that these cations stabilize the closed conformation of the Ba^{2+} -blocked channel. This mechanism does not operate in the BK channel, however, since it is now known that the Ba^{2+} -blocked channel's open conformation is stabilized when external K^+ is present (Miller et al., 1987).

The question immediately arises: where is the external lock-in site located? We cannot answer this question with certainty, but the evidence here points strongly towards the idea that the lock-in site lies within the K^+ conduction pathway. We already know that this is a multi-ion channel that must have enough room to accommodate at least two cations within the conduction pore (Yellen, 1984*b*; Eisenman et al., 1986; Cecchi et al., 1987). It is, of course, possible that the lock-in site is located not in the pore at all, but on the external face of the channel, and that binding of an ion here leads to a conformational change preventing Ba^{2+} exit. It is the ionic selectivity of the lock-in phenomenon that makes this "conformation change" hypothesis unpalatable. The impressively high selectivity of the lock-in site, which is of the same order of magnitude as the selectivity of permeation itself, argues strongly that the lock-in site is intimately involved in the permeation process, and is therefore physically located inside the conduction pathway.

Given this picture, the block of Ba^{2+} permeation by K^+ argues that these two ions lie in single file within the channel, with K^+ occupying a position external to the Ba^{2+} site. This conclusion provides further evidence that the lock-in site is located in the narrow part of the conduction pathway, and not, for example, in the externally-facing "mouth." The lock-in site cannot be located very far into the channel, however, since the K_i is only very weakly voltage dependent, with an e-fold increase per 140 mV depolarization (Neyton and Miller, 1988), which suggests that this site feels <20% of the applied voltage drop through the channel.

Selectivity of the External Lock-In Site

The fact that we were able to observe a "lock-in" effect of K^+ in the submillimolar range in the presence of 150 mM external Na^+ was already indicative of a strong selectivity of this site for permeant cations. By studying the effects of Na^+ and Li^+ in the presence of the organic cation NMDG^+ , we found that these impermeant cations bind to the lock-in site, but with affinities 1,400- and 10,000-fold lower, respectively, than that for K^+ .

This very high selectivity of the external lock-in site strongly indicates that at least part of the selectivity in the conduction mechanism of the channel may be associated with structures located near its external mouth. A similar conclusion was reached by Yellen (1984*b*) in his study of the relief by external cations of the block induced by internal Na^+ . However, as was also pointed out by Yellen (1984*b*), the selectivity found in the external part of the channel cannot account for the whole selectivity mechanism of the channel; Cs^+ , which has very low permeability in this channel (Blatz and Magleby, 1984; Yellen, 1984*a*; Cecchi et al., 1987), has an affinity for the external lock-in site similar to K^+ 's. Thus the selectivity against Cs^+ must be exerted somewhere more deeply in the pore.

The Ba²⁺ lock-in effect also reveals a selectivity among K⁺ analogues, with inhibitory potency following the sequence: Tl⁺ > Rb⁺ > K⁺ > Cs⁺ > NH₄⁺. This does not follow the sequence of zero-voltage conductance in symmetrical solutions (Eisenman et al., 1986), nor that of biionic reversal potential (Blatz and Magleby, 1984; Yellen, 1984a; Eisenman et al., 1986). It is, however, similar to the sequence of ion binding to the channel, as measured by the ability of low-conductance permeants to "block" K⁺ currents (Eisenman et al., 1986).

The External Lock-In Site Has a High Affinity for Permeant Cations

All ions able to permeate the BK channels show an astonishingly high affinity for the external lock-in site. This is the case for K⁺, Rb⁺, Tl⁺, and NH₄⁺, which show measurable currents through the channel (Blatz and Magleby, 1984; Yellen, 1984a; Eisenman et al., 1986), and for Cs⁺, which permeates at too low a rate to reveal an observable current (Cecchi et al., 1987). Even the impermeant ions Na⁺ and Li⁺ produce the lock-in effect, but with far lower affinities than the permeant ions. In fact, we consider that the "true" affinity of the lock-in site is even higher than that measured here by the K_i . We must remember that the inhibition constant of this site is measured with a Ba²⁺ ion inside the channel. This divalent cation will exert electrostatic repulsion on a permeant cation willing to bind to the external lock-in site, and thus the dissociation constant for a permeant cation in an unoccupied channel would be well below the 4–20 μM values obtained for K⁺ and Rb⁺. There are many examples, particularly in the literature of membrane transporters, of proteins that bind K⁺ selectively over Na⁺, but none to our knowledge with such a high absolute affinity for K⁺ and its close analogues.

The very high affinity of a site within the K⁺ conduction pore directly contradicts conventional wisdom about ion conduction mechanisms, which argues that ion channels are able to exhibit such high unitary transport rates simply because they bind the permeating ions weakly (Armstrong, 1975; Hille, 1975; Miller, 1986). How can we reconcile our measurement of an apparent dissociation constant for K⁺ in the micromolar range with a turnover rate of 10⁸ K⁺ ions/s? The situation is reminiscent of Ca²⁺ conduction in Ca²⁺ channels (Almers and McCleskey, 1984; Hess and Tsien, 1984), where two equivalent high-affinity Ca²⁺-binding sites are postulated inside the pore. The first of these binds Ca²⁺ with high affinity, and thus cannot let the ion dissociate rapidly enough to carry significant current; a second Ca²⁺ ion can then enter the pore and bind to the second site, with an affinity greatly lowered by the presence of the first ion. Mutual repulsion of Ca²⁺ ions in the doubly occupied channel thus speeds the exit of the ions, and allows high transport rates.

However, the BK channel must be more complex than the Ca²⁺ channel. Indeed, the affinity of the external lock-in site for K⁺, disconcertingly high when a Ba²⁺ ion is in the channel, can hardly be expected to decrease if the divalent Ba²⁺ ion is replaced with a monovalent K⁺ ion. Instead, we suggest that the conduction pathway of the BK channel possesses a central "binding region" that can accommodate more than two ions. When Ba²⁺ is bound, a K⁺ ion may bind with micromolar affinity to produce the lock-in effect. But this would not represent the situation during K⁺ conduction, in which we would envision the simultaneous binding of at least three K⁺ ions. Under such conditions, the binding of all three ions would be

mutually destabilized by electrostatic repulsion, and high conduction rates could be achieved.

Although this picture is speculative, the high affinity of "K⁺ block" of Ba²⁺ permeation, combined with the high transport rate of K⁺ conduction, are unequivocally inconsistent with the idea that at most two ions can simultaneously occupy the channel. In the following study (Neyton and Miller, 1988), we confirm our prediction of an additional K⁺-binding site, and provide direct evidence for occupancy of this channel by a Ba²⁺ and at least three additional K⁺ ions.

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