

Discrete Ba^{2+} Block as a Probe of Ion Occupancy and Pore Structure in the High-Conductance Ca^{2+} -activated K^+ Channel

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ABSTRACT In this study, high-conductance Ca^{2+} -activated K^+ channels from rat skeletal muscle were incorporated into planar phospholipid bilayers, and discrete blockade of single channels by Ba^{2+} was studied. With 150 mM K^+ held constant in the internal solution, increasing external K^+ over the range 100–1,000 mM raises the rate of Ba^{2+} dissociation. This “enhancement effect,” which operates at K^+ concentrations 3–4 orders of magnitude higher than those required for the “lock-in” effect described previously, depends on applied voltage, saturates with K^+ concentration, and is not observed with Na^+ . The voltage dependence of the Ba^{2+} off-rate varies with external K^+ in a way suggesting that K^+ , entering the channel from the external side, forces Ba^{2+} dissociation to the internal solution. With K^+ held fixed in the external solution, the Ba^{2+} off-rate decreases as internal K^+ is raised over the range 0–50 mM. This “lock-in” effect is similar to that seen on the external side (Neyton and Miller, 1988), except that the internal lock-in site is of lower affinity and shows only a fivefold preference for K^+ over Na^+ . All the results taken together argue strongly that this channel’s conduction pathway contains four sites of very high affinity for K^+ , all of which may be simultaneously occupied under normal conducting conditions. According to this view, the mutual destabilization resulting from this high ionic occupancy leads to the unusually high conductance of this K^+ -specific channel.

INTRODUCTION

Like many K^+ -specific ion channels, the high-conductance Ca^{2+} -activated K^+ (BK) channel operates by a multi-ion conduction mechanism, in which several K^+ ions simultaneously occupy the permeation pathway in their journey across the membrane. In the companion study (Neyton and Miller, 1988), we showed that K^+ and Ba^{2+} strongly interact within this channel’s conduction pore. Specifically, a Ba^{2+} ion entering the channel from the internal solution dissociates to the external side, as long as external K^+ is absent; the addition of K^+ to the external solution prevents

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Ba²⁺ dissociation to that side, and effectively “locks” the Ba²⁺ ion inside the channel. The surprising aspect of this result is the very high affinity with which K⁺ acts as this external “lock-in” site; the concentration for half-inhibition is as low as 20 μM.

We argued that the existence of a single K⁺-binding site of such high affinity within the permeation pathway is inconsistent with the high conductance of this channel, that at least two additional K⁺ sites must be occupied during channel conduction. In this study, we further exploit the phenomenon of discrete block by the Ba²⁺ ion to demonstrate the existence of two additional K⁺-binding sites within this channel. One of these sites, when occupied by K⁺, accelerates the dissociation of Ba²⁺ from the channel, and is located between the Ba²⁺ blocking site and the external lock-in site. The second site is located towards the internal face of the channel, and produces a Ba²⁺ lock-in effect from this side. Taken together, these experi-

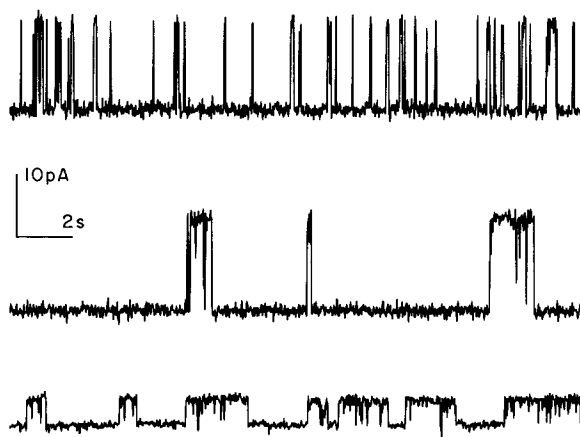


FIGURE 1. Effects of external K⁺ on Ba²⁺ block kinetics. Single BK channels were observed in planar bilayers in the presence of 150 mM internal KCl and 150 mM external NaCl media, with additional KCl added to the external medium. Blocks were induced with internal Ba²⁺, and the holding voltage was 40 mV. Top trace: no added K⁺ in external solution; mean block time, 0.5 s. Middle trace: 10 mM K⁺ added; mean block time, 4 s. Bottom trace: 500 mM K⁺ added; mean block time, 1.7 s. Ba²⁺ concentration was 1 μM in top trace, and 50 μM in two other traces.

ments strongly suggest that the BK channel contains at least four K⁺-binding sites within the conduction pathway, at least three of which are nearly always occupied under normal conducting conditions. The results reconcile this channel's puzzling combination of high ionic selectivity and high conductance.

MATERIALS AND METHODS

Details of the experimental measurements were identical to those described in the companion paper (Neyton and Miller, 1988) except for the following point. In this study, because many experiments were carried out at negative potentials, we kept the channel highly activated by a combination of high Ca²⁺ and Mg²⁺ in the internal solution. Mg²⁺ is known to act allosterically to raise the affinity of this channel for activator Ca²⁺ (Golowasch et al., 1986; Oberhauser et al., 1988). Therefore, many experiments reported here were performed with 10 mM Mg²⁺ along with 1–10 mM Ca²⁺ in the internal solution.

RESULTS

External K^+ at High Concentration Speeds Dissociation of Ba^{2+}

This study was initiated by the observation illustrated in Fig. 1. In this experiment, the channel was held at +40 mV and Ba^{2+} block kinetics were analyzed as the external K^+ concentration was varied. An increase in external K^+ from 0 to 10 mM produced the "lock-in" effect documented in detail in the preceding paper (Neyton and Miller, 1988): a profound *decrease* in the rate of Ba^{2+} dissociation. As external K^+ was raised above 100 mM, however, the opposite effect was observed: an *increase* in Ba^{2+} off-rate. An acceleration of a blocker's dissociation rate by raising the concentration of permeant ions is not in the least surprising. This sort of effect has been observed in the case of Na^+ block of the delayed rectifier K^+ channel of squid axon (Bezanilla and Armstrong, 1972) and of the BK channel in bovine chromaffin cells (Yellen, 1984). Indeed, this is an expected property of multi-ion channel. The

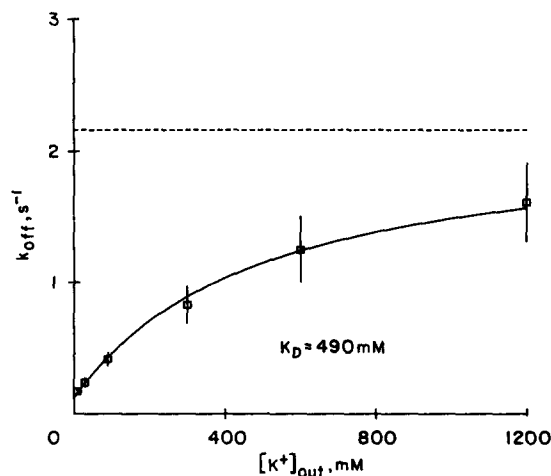


FIGURE 2. K^+ enhancement of Ba^{2+} dissociation. The Ba^{2+} dissociation rate (k_{off}) was measured at different concentrations of external K^+ . Conditions were as described in Fig. 1, except that the holding voltage was -20 mV, and internal Ba^{2+} was varied from 0.2 to 10 mM to obtain a high frequency of blocking events. Experimental points were fitted with Eq. 1, where $K_D = 490$ mM.

binding in a blocked channel of an additional ion destabilizes the blocker on its binding site due to electrostatic repulsion between the two ions (Hille and Schwarz, 1978). Notice that for the BK channel, an increase in Ba^{2+} off-rate is observed above 100 mM external K^+ , i.e., under conditions of almost complete saturation of the external lock-in site ($K_D = 0.3$ mM). This strongly suggests that it is the entry of a second K^+ ion into the external part of the conduction pathway that is responsible for this "enhancement effect," which is the enhanced rate of Ba^{2+} dissociation induced by external K^+ .

If this picture is correct, then the rate constant of Ba^{2+} dissociation should depend on the occupancy of the enhancement site, and hence should saturate with external K^+ concentration. We used external K^+ concentrations above 10 mM, so that the lock-in site could be considered saturated, with Ba^{2+} dissociating to the internal solution exclusively (Neyton and Miller, 1988). We assume that Ba^{2+} dissociates at a lower rate when the enhancement site is empty, k_1 , than it does when it is

occupied, k_2 . Therefore, the observed dissociation rate, k_{off} is the occupancy-weighted average of these two rates:

$$k_{\text{off}} = k_1 + (k_2 - k_1)([K^+]/K_D + [K^+]), \quad (1)$$

where $[K^+]$ represents the external K^+ concentration, and K_D is the dissociation constant for K^+ . Fig. 2 confirms that the enhancement effect does follow this expected saturating behavior, and that it requires high K^+ , with K_D in the range of 300–600 mM. The maximum effect at very high K^+ is a 3–10-fold increase in Ba^{2+} dissociation rate.

Voltage Dependence of Ba^{2+} Dissociation

We showed in the preceding paper that Ba^{2+} can dissociate from the channel to either the internal or the external solution. With high internal K^+ and very low

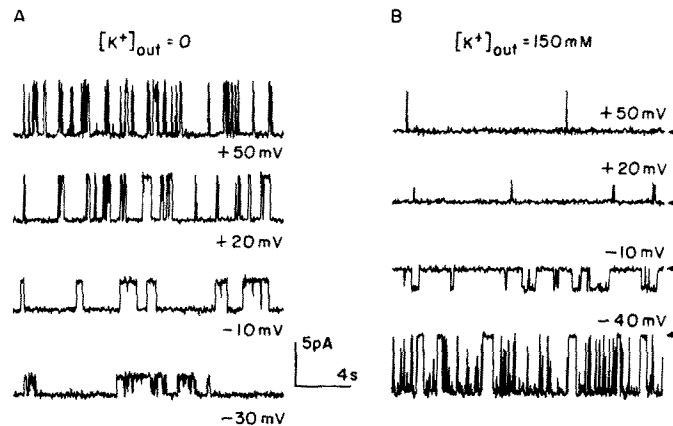


FIGURE 3. Voltage dependence of Ba^{2+} dissociation. Ba^{2+} -blocking records were collected in two separate single-channel experiments, using different external K^+ concentrations, and at the indicated holding voltages. Ionic conditions were as described in Fig. 1. (A) No K^+ added to external solution, 1 μM Ba^{2+} . (B) 150 mM K^+ added to external medium, 3 mM Ba^{2+} . Arrows mark the zero-current levels.

external K^+ , Ba^{2+} nearly always dissociates to the external solution, and as external K^+ is raised, dissociation to the internal solution is progressively favored. We therefore expected that most of the Ba^{2+} dissociation events observed at high external K^+ would proceed to the internal solution. This expectation was confirmed by analysis of the voltage dependence of the Ba^{2+} off-rate, shown in Fig. 3. In K^+ -free external solutions Ba^{2+} block times become shorter with depolarization (Fig. 3 A); in contrast, with 150 mM external K^+ present, the Ba^{2+} blocks are lengthened by depolarization (Fig. 3 B).

Fig. 4 A shows these effects of voltage in more detail. The Ba^{2+} dissociation rate varies exponentially with applied voltage at both high and low external K^+ , as expected for a reaction in which an effective charge, z , moves through a fraction of

the membrane field, δ (Woodhull, 1973):

$$k_{\text{off}}(V) = k_{\text{off}}(0) \exp(-z\delta FV/RT). \quad (2)$$

The figure shows that as external K⁺ is raised, the effective valence of the dissociation reaction, $z\delta$, changes sign. In other words, high external K⁺ induces a polarity reversal of the voltage dependence of Ba²⁺ dissociation, indicating that Ba²⁺ leaves mainly to the external solution at low K⁺ ($\delta > 0$) and to the internal solution at high K⁺ ($\delta < 0$). In K⁺-free external solutions, k_{off} increased e-fold for 27 ± 2 mV depolarization ($z\delta = 0.9$). In 150 mM external K⁺, k_{off} decreased e-fold for 35 ± 2 mV depolarization ($z\delta = -0.71$).

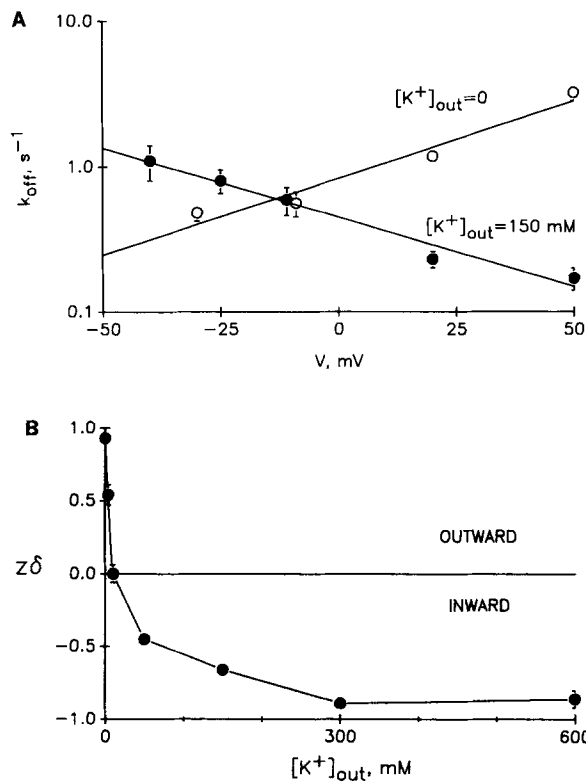


FIGURE 4. Influence of external K⁺ on the voltage dependence of Ba²⁺ dissociation. (A) Ba²⁺ off-rate vs. holding voltage was measured as described in Fig. 3 at two different external K⁺ concentrations. Solid lines are drawn according to Eq. 2, with effective valence measured from the slopes of the lines. Open circles: no added K⁺, $z\delta = 0.81$. Filled circles: 150 mM external K⁺, $z\delta = -0.64$. (B) Variation of effective valence ($z\delta$) with external K⁺. Ba²⁺ off-rate as a function of voltage was measured as in A, at the indicated external K⁺ concentrations. For each set of conditions, the effective valence was measured from three to six voltage values. Each point represents the mean \pm SE of three separate experiments.

The reversal of the voltage dependence of k_{off} by external K⁺ is a progressive effect. Fig. 4B shows the effective valence for Ba²⁺ dissociation as a function of external K⁺. This effective valence, initially positive at zero K⁺, rapidly approaches zero over the range 0–10 mM external K⁺, as the internal lock-in site becomes filled, and dissociation proceeds to both sides at roughly equal rates (Neyton and Miller, 1988). Only as K⁺ is raised above 50 mM does the effective valence become increasingly negative, reaching a saturation value at very high K⁺. It is precisely over this range of high K⁺ concentrations that the enhancement of k_{off} by K⁺ is observed. Thus, the effects of K⁺ on the effective valence of the Ba²⁺ off-rate are fully consistent with the picture that external K⁺ retards inward dissociation at low concentra-

tions (0–10 mM) and enhances outward dissociation at higher concentrations (>50 mM).

Voltage Dependence of the K^+ -enhancement Effect

The preceding section demonstrated that Ba^{2+} dissociation is voltage dependent. The efficacy of external K^+ in enhancing Ba^{2+} dissociation is also voltage dependent, as Fig. 5 demonstrates. Here, we show K^+ -enhancement curves at two values of applied voltage. It is clear that both the K_D and the maximum effect are voltage dependent. More negative voltages, which tend to draw K^+ into the channel from the external solution, and Ba^{2+} out of it into the internal solution, tend to favor K^+ binding at the enhancement site and to produce a larger maximum increase in Ba^{2+} off-rate. From a series of three experiments, K_D was found to increase with depolarization, with values of 300 ± 20 mM at -30 mV, and 550 ± 120 mM at 0 mV. The fact that the K^+ dissociation constant at the enhancement site is voltage dependent argues that this site lies within the transmembrane voltage drop, as will be discussed later.

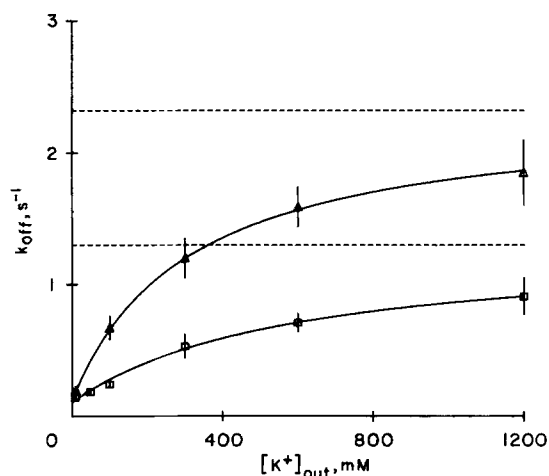


FIGURE 5. Voltage dependence of K^+ -enhancement effect. Ba^{2+} off-rate was measured, as in Fig. 2, as a function of external K^+ concentration, at two different holding potentials. Both curves shown were taken on the same channel. Triangles, -30 mV. Solid curve is drawn according to Eq. 2, with $k_1 = 0.1$ s^{-1} , $k_2 = 2.3$ s^{-1} , and $K_D = 580$ mM. Squares, zero voltage. Solid curve is drawn with $k_1 = 0.1$ s^{-1} , $k_2 = 1.3$ s^{-1} , and $K_D = 580$ mM.

Selectivity of the External Enhancement Site

Fig. 6 compares off-rate variations observed when raising external K^+ , Rb^+ , and Na^+ . As with K^+ , an external Rb^+ concentration above 50 mM increases k_{off} . The maximal amplitude of the Rb^+ enhancement effect, and also the value of K_D are smaller than for K^+ (for Rb^+ , $K_D = 205 \pm 45$ mM at zero voltage, less than half the value for K^+). Thus, Rb^+ appears to bind with a higher affinity to the enhancement site, but once bound it exerts less of a destabilizing force over Ba^{2+} than does K^+ . In contrast with the permeant ions, external Na^+ is unable to speed Ba^{2+} dissociation, even at concentrations as high as 1.2 M. The decrease in off-rate seen at high Na^+ in Fig. 6 is the tail of the Ba^{2+} lock-in effect by external Na^+ (Neyton and Miller, 1988). It is thus apparent that only permeant ions are able to exert an enhancement effect.

Internal K⁺ Locks Ba²⁺ into the Channel

The experiments above and in the preceding study focused on the interactions of Ba²⁺ with external K⁺. We now reverse the system's polarity and investigate interactions of internal K⁺ with Ba²⁺ (which is still added to the internal solution in all experiments). We maintained external K⁺ constant at 150 mM, and varied internal K⁺. Fig. 7 shows that the duration of Ba²⁺ blocks is strongly increased by internal K⁺. At 300 mM internal K⁺, the mean block time is over 10 times longer than K⁺-free conditions. Raising K⁺ also increases the duration of the bursts, but the effect is much weaker: a threefold increase in the mean burst time is observed when the internal concentration of K⁺ is changed from 0 to 300 mM. Thus, in analogy to the effect of external K⁺, internal K⁺ also induces a Ba²⁺ lock-in effect.

Fig. 8 illustrates quantitatively the effects of internal K⁺ on the Ba²⁺-blocking kinetics. In the complete absence of internal K⁺, the off-rate is rapid, and the addi-

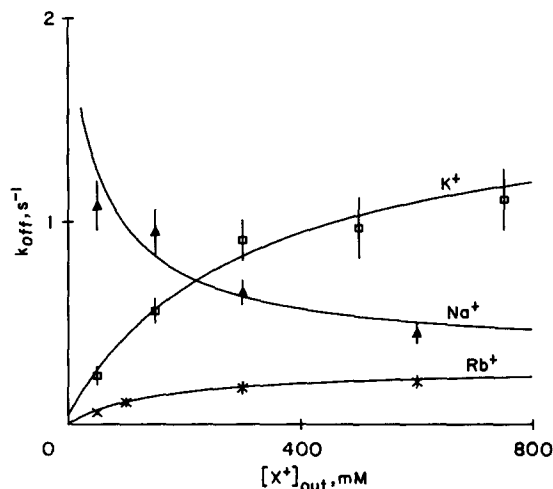


FIGURE 6. Ion selectivity of enhancement effect. Ba²⁺ off-rate was measured between -10 and 0 mV as a function of the concentration of the indicated cation added to the external medium. 150 mM NMDG⁺ was present in all external solutions. Curves for K⁺ and Rb⁺ are drawn according to Eq. 1, with a K_D of 330 mM for K⁺ and 160 mM for Rb⁺. Maximum values of off-rates are 1.7 and 0.2 s⁻¹ for K⁺ and Rb⁺, respectively. The decrease in off-rate with added Na⁺ is due to the very low-affinity "lock-in" effect seen for this cation.

tion of K⁺ reduces this rate. It is important to remember that these experiments were carried out in high external K⁺, where the external lock-in site is always occupied; therefore, nearly all the Ba²⁺ dissociation events proceed to the internal solution, as indicated by the voltage dependence above. As with the external lock-in effect, the inhibition of Ba²⁺ off-rate by internal K⁺ follows a rectangular hyperbolic function of the internal concentration of K⁺ (Fig. 8 A). The K⁺ concentration of half-inhibition (K_i) is in this case 8 mM. The Ba²⁺ association rate also decreases when the internal concentration of K⁺ is raised (Fig. 8 B). However, much higher internal K⁺ concentrations are required to obtain a significant reduction of the on-rate: K_i was ~200 mM.

The similarity between the Ba²⁺ lock-in effects induced by external and internal K⁺ argues for a similar underlying mechanism. In strict analogy to the effects of external K⁺ (Neyton and Miller, 1988), we propose that the conduction pathway

contains a K^+ -binding site located on the internal side of the Ba^{2+} -blocking site. This internal lock-in site must be unoccupied for a Ba^{2+} ion to enter the pore, or for a Ba^{2+} ion inside the pore to leave it to the internal solution.

Affinity and Selectivity of the Internal Lock-In Site

The external lock-in site was shown to be highly selective for permeant ions (Neyton and Miller, 1988). This is not the case for the internal lock-in site. Fig. 9 shows that both K^+ and Na^+ in the internal solution are able to lock Ba^{2+} in the channel. The affinity of the internal lock-in site is about fivefold lower for Na^+ than for K^+ . From a series of eight experiments carried out with 1–5 mM internal Ca^{2+} present, K_i values were found to be 7 ± 1 mM for K^+ and 36 ± 3 mM for Na^+ . Internal Rb^+ produced a lock-in effect as well ($K_i = 9$ mM), as did Ca^{2+} ($K_i = 27$ mM). Thus, the internal lock-in site appears poorly selective among cations, but definitely favors conducting cations.

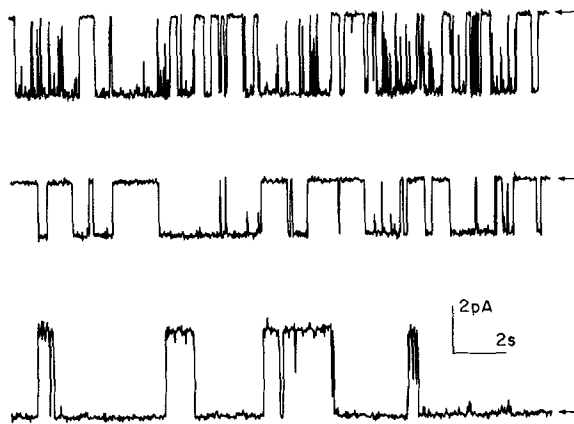


FIGURE 7. Effect of internal K^+ on Ba^{2+} dissociation. Single-channel recordings were collected with 150 mM external K^+ and 150 mM internal NMDG⁺ held fixed, while internal K^+ was added to the indicated concentrations. Ba^{2+} was 100 μ M and the voltage was 20 mV. Mean block times were 0.3, 1.2, and 3.0 s at 0, 10, and 300 mM internal K^+ , respectively.

DISCUSSION

In the preceding investigation (Neyton and Miller, 1988) we showed that two ions, Ba^{2+} and K^+ , can simultaneously bind with high affinity inside the pore of the large BK channel. By exploiting the interaction of Ba^{2+} with the channel as a probe, we have now identified two additional effects of K^+ on Ba^{2+} dissociation: an external enhancement effect and an internal lock-in effect. We therefore propose that the ion conduction pathway of the BK channel contains four ion-binding sites lined up in single file: two lock-in sites, an enhancement site, and the Ba^{2+} -blocking site. Several questions immediately arise. First, do the three distinct effects of K^+ on Ba^{2+} dissociation mean that there are three physically distinct K^+ -binding sites involved? How many of these sites may be simultaneously occupied? How are the observed affinities of these sites related to ionic conduction and selectivity of this channel? By examining these questions in light of our experimental results, we will see that a minimal model for the conduction pathway of this channel must contain four phys-

ically distinct K⁺-binding sites, all of which are simultaneously occupied most of the time during normal K⁺ conduction.

Are There Separate Sites?

Previous work has shown that the BK channel's conduction pathway contains a site that binds a single Ba²⁺ ion with high affinity (Vergara and Latorre, 1983; Miller, 1987; Miller et al., 1987). It is very likely, though not rigorously proven, that this site is designed for K⁺ conduction, and that Ba²⁺ binds as a "substrate analogue" because of its similarity in size to K⁺ (Latorre and Miller, 1983; Miller et al., 1987). We know that as long as Ba²⁺ ion resides on this blocking site, the channel cannot

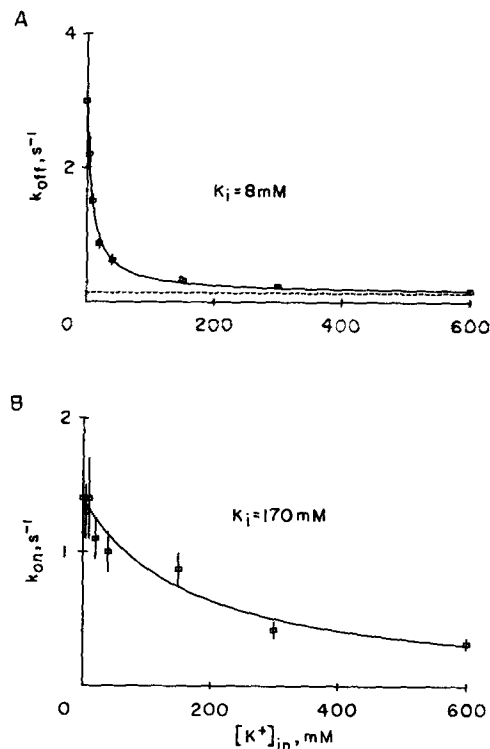


FIGURE 8. Lock-in effect induced by internal K⁺. Ba²⁺ off-rate (A) and on-rate (B) were measured as a function of internal K⁺, under conditions similar to those found of Fig. 7. Solid curves were fit by rectangular hyperbolas, with half-inhibition concentrations of 8 mM for the off-rate and 160 mM for the on-rate.

conduct K⁺ ions, since, as with many K⁺ channels (Hille and Schwarz, 1978), the pore is so narrow that ions cannot pass one another.

The evidence for three additional ion-binding sites comes from the present studies on K⁺ interactions in the Ba²⁺-blocked channel. We found that low concentrations of external K⁺ greatly reduce the rate of Ba²⁺ dissociation. The very high affinity of K⁺ in producing this lock-in effect, and the high ion selectivity for conducting ions demonstrate that a specific site exists at which K⁺ "blocks" the dissociation of Ba²⁺ (Neyton and Miller, 1988). An identical argument can be made for the internal lock-in site documented in this work; here, neither the affinity nor the selectivity of the binding is as high as with the external site, but still they are high enough to rule

out nonspecific effects, such as the screening of local surface potentials. It is also clear from these results that the two lock-in sites are physically distinct, since they are located on different sides of the blocking Ba^{2+} ion.

Our results further demonstrate the existence of a third K^+ -binding site located on the external side of the blocking Ba^{2+} ion, the enhancement site. This site, when occupied by a K^+ ion, destabilizes Ba^{2+} on its binding site, and thus speeds its dissociation to the internal solution; this is analogous to the conventional "knock-off" effects of permeant ions on blockers, long known in a variety of K^+ channels (Bezanilla and Armstrong, 1972; Adelman and French, 1978; Yellen, 1984; Cecchi et al., 1987). This enhancement site, requiring K^+ at concentrations >50 mM, is of very low affinity, but it maintains high selectivity; only channel-permeant ions produce the effect. This selectivity allows us to conclude that the enhancement effect is the result of specific occupancy of a site, and, together with the voltage dependence of the enhancement site affinity, that this site is most likely located within the chan-

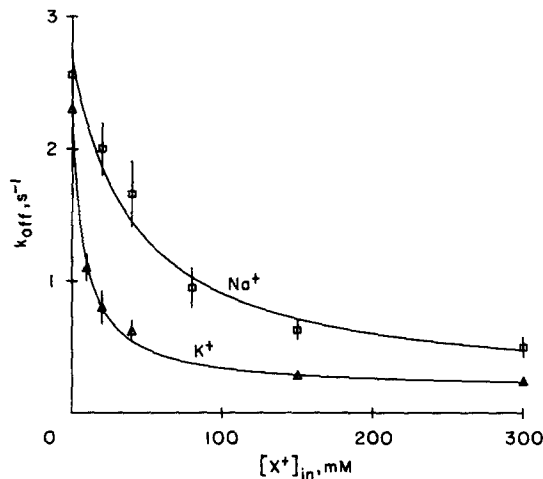


FIGURE 9. Ion selectivity of internal lock-in effect. The Ba^{2+} off-rate was measured against internal concentrations of either K^+ or Na^+ , under conditions similar to those found in Fig. 7. Half-inhibition constants of solid curves under 8 mM for K^+ (triangles) and 41 mM for Na^+ (squares).

nel's conduction pathway. Moreover, it is clear that the enhancement site is distinct from either of the lock-in sites. Concentrations of K^+ producing the enhancement effect are orders of magnitude higher than those required to saturate the lock-in sites.

In studying ion channels, or any other types of enzymes, it is usually improper to identify a saturating process with occupancy of a binding site. The reason that we can make this identification here without apology is that all our experiments are designed so that the K^+ interactions under scrutiny are at true thermodynamic equilibrium. The reason for this is that these experiments focus on the dissociation of Ba^{2+} from a blocked, nonconducting channel, i.e., a channel which K^+ cannot permeate. Because the Ba^{2+} ion prevents K^+ movement across the pore, any K^+ ion inside the channel must leave it to the same solution from which it had entered. This means that as long as Ba^{2+} remains bound, K^+ occupying a site within the channel is in equilibrium with the bulk solution on the same side of the Ba^{2+} . By focusing on the Ba^{2+} dissociation process, we are necessarily studying the channel while it is

blocked in this way. We assume, of course, that K⁺ enters and leaves the channel on the conduction time-scale (nanoseconds to microseconds) many orders of magnitude more rapidly than Ba²⁺ dissociates from its blocking site. It is for these reasons that we can validly identify the saturating effects of K⁺ on Ba²⁺ dissociation kinetics with occupancy of sites within the conduction pathway.

How Many Sites Can Be Simultaneously Occupied?

The arguments above identify the three K⁺-binding sites as distinct, but they do not tell us whether these sites can be occupied simultaneously. The apparent dissociation constants of both the external and internal lock-in sites are low: ~20 μM and 8 mM, respectively, when measured in 150 mM *N*-methyl-D-glucamine (NMDG⁺)-containing solutions. Each of these dissociation constants was measured in the presence of 150 mM K⁺ in the opposite compartment, i.e., with the opposite lock-in site almost saturated. Thus, in 150 mM K⁺ on both sides, both lock-in sites are nearly always occupied simultaneously, with Ba²⁺ on its blocking site between them.

Is the enhancement site occupied simultaneously with the lock-in sites? The enhancement site, located on the external side of the blocking Ba²⁺, starts to be significantly occupied only above 50 mM K⁺, many orders of magnitude above the dissociation constant of the external lock-in site. The externally located lock-in and enhancement sites can therefore be occupied at the same time. Moreover, enhancement of Ba²⁺ dissociation by external K⁺ was analyzed in 150 mM internal K⁺, i.e., with the internal lock-in site highly occupied. It might be argued that under the very high external concentration of K⁺ required to fill the enhancement site, the affinity of the internal lock-in site for K⁺ might be lower. This is not the case, however, since identical lock-in effects are seen at all external K⁺ concentrations tested; at 150, 300, and 500 mM external K⁺, *K_D* values for the lock-in effect were 10, 11, and 8 mM, respectively. In conclusion, in a Ba²⁺-blocked channel, the external lock-in and enhancement sites as well as the internal lock-in site can be occupied at the same time. Moreover, the simultaneous occupancy of these three K⁺-binding sites occurs along with a Ba²⁺ ion residing inside the conduction pathway in addition. These four cation-binding sites under study must therefore be separate entities.

A Physical Picture of K⁺-Ba²⁺ Interactions Inside the Pore

The lock-in effects observed at low K⁺ concentrations are easily rationalized in terms of the single-filing behavior of this K⁺ channel (Neyton and Miller, 1988). How can we understand the ability of external K⁺ at high concentrations to speed Ba²⁺ dissociation? Our results show that this effect is due to the low-affinity binding, in the external part of the channel's pore already occupied by the lock-in K⁺, of a second K⁺ ion. Given that this is a single-filing channel, we would like to know which of these sites is located more externally, and which is deeper inside, closer to the Ba²⁺ ion.

We can distinguish the two possible arrangements of sites by taking advantage of the fact that K⁺ binds to these sites at true thermodynamic equilibrium, as discussed above. Because of this, the observed "half-saturation concentration" for each effect rigorously represents the dissociation constant of the K⁺ ion on the relevant site. In such a case, the voltage dependence of the *K_D* provides an unequivocal indicator of

the position of the site in the applied voltage drop within the channel, δ (Eq. 2). We therefore measured the voltage dependence of K_D for all three K^+ -binding sites (Fig. 10). The binding of external K^+ to the external lock-in and enhancement sites weakens with depolarization, while binding of internal K^+ to the corresponding lock-in site strengthens. From a series of five experiments, the values of δ for the externally facing site were 0.18 ± 0.02 for the external lock-in site, and 0.57 ± 0.13 for the enhancement site. The external lock-in site is therefore located in the most external part of the channel's conduction pathway. The enhancement of Ba^{2+} dissociation by external K^+ arises from the occupancy of a site located more deeply in the pore, closer to the Ba^{2+} -blocking site. The most natural explanation of this effect is simple electrostatic destabilization of Ba^{2+} on its site by the K^+ ion nearby. Fig. 10 further shows that the internal lock-in site experiences about one-third of the voltage drop, as measured from the inside (δ , -0.36 ± 0.04).

An immediate objection may arise to the picture of the enhancement effect just offered. The very high affinity of the lock-in site ensures that this externally located site is virtually always occupied at the high K^+ concentrations required to load the

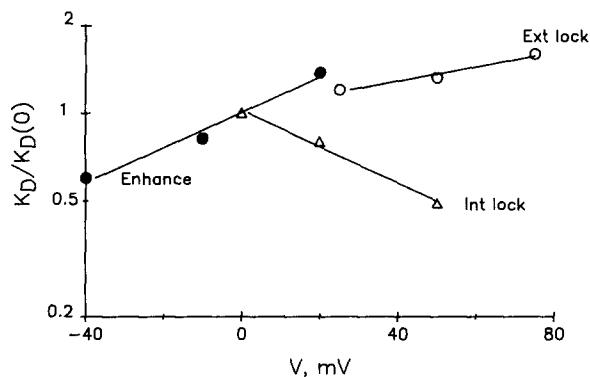


FIGURE 10. Voltage dependence of the three K^+ binding effects. Variation of the apparent dissociation constants for the three effects of K^+ on Ba^{2+} off-rate, measured in three separate experiments. Open circles: external lock-in site, $z\delta = 0.18$. Filled circles: enhancement site, $z\delta = 0.42$. Open triangles: internal lock-in site, $z\delta = -0.45$.

enhancement site. How, then, can K^+ ions ever gain access to the more internally located site if the single-filing rule applies? There are two answers to this objection. First, we may remind ourselves that in a true binding equilibrium, the kinetic path for going from an initial state to a final state need not be specified. The enhancement effect simply represents a change from the initial state with only the externally facing lock-in site occupied, to a final state with both K^+ -binding sites occupied. The voltage dependence of the K_D tells us that regardless of how this reaction occurs, a net charge moves about half-way down the voltage gradient.

Despite the thermodynamic purity of this argument, it might be worthwhile to suggest a concrete way for the enhancement site to become occupied even though the more externally located lock-in site is of much higher apparent affinity. When only one of these K^+ -binding sites is occupied, there will be a dynamic equilibrium of the single K^+ ion between the two sites. Since the external site is of much higher affinity, the K^+ ion resides on this site nearly all the time. Very rarely but inevitably, however, the K^+ ion will occupy the lower affinity enhancement site, and during such moments the externally located site will become available for attack by a K^+ ion

in bulk solution. The net result of this reaction will be to fill the enhancement site by a conventional vacancy mechanism. There is really no theoretical problem at all, as long as the rates of ion movement among the K⁺-binding sites are fast compared with the Ba²⁺ dissociation rate.

Ironically, the site about which we have the least information is the Ba²⁺-blocking site itself. We know neither its affinity for K⁺ nor its ion selectivity. Since we rely upon the Ba²⁺-blocked channel as the object of all our measurements here, we cannot manipulate the blocking site. We should ask if this even is a single, well-defined site, or whether the Ba²⁺ ion can exchange among the other K⁺-binding sites, as apparently occurs in the squid axon delayed rectifier (Armstrong et al., 1982). The present model asserts that when either lock-in site is empty, the Ba²⁺ moves into this site just before dissociation; the high Ba²⁺ off-rate at zero K⁺ on either side ($>4 \text{ s}^{-1}$) would represent the rate of movement from the high-affinity blocking site into the lock-in site from which Ba²⁺ rapidly exits. This would be possible with both lock-in sites filled (K⁺ > 1 mM external and 100 mM internal), but when the enhancement site is empty, Ba²⁺ would be able to exchange between the blocking site and the enhancement site. Thus while Ba²⁺ may not be physically confined to a single site, it must remain in the region between the two lock-in sites for times on the order of hundreds of milliseconds.

One puzzle for which we do not have a satisfactory explanation is the fact that as K⁺ on both sides raised, the Ba²⁺ off-rate approaches a nonzero value of $\sim 0.2 \text{ s}^{-1}$. A model with only a lock-in site on the internal side would predict that at fixed high external K⁺, as internal K⁺ is increased without limit, the Ba²⁺ off-rate should approach zero. A possible explanation for this is that an additional enhancement site exists on the inner side of the Ba²⁺-blocking site, and that as this site becomes filled at very high internal K⁺, Ba²⁺ is pushed out to the external solution. We have no direct evidence for such a fifth binding site, and are presently searching for experimental conditions suitable for observing internal enhancement effects.

An apparent oddity about the phenomena reported here is the different effects of K⁺ on the association and dissociation rates for Ba²⁺. K⁺ decreases both these rates, but over different concentration ranges. How can this be, if K⁺ exerts its effect at the same site in both places? The resolution of this paradox lies in the fundamental difference in K⁺ interaction with the channel in its conducting and blocked states. As pointed out above, in the blocked channel, K⁺ binds to its site in a true equilibrium reaction; the blocking Ba²⁺ ion effectively isolates one side of the channel from K⁺ ions on the other side. In contrast, the conducting channel, which allows K⁺ to enter from both sides, is a much more complicated system. Here, the effect of external K⁺, for instance, of reducing the Ba²⁺ on-rate does not measure the occupancy of the lock-in site, but is the consequence of a large number of rate processes in this multi-ion pore. We have no inclination to model such a complicated process with so many free parameters.

Multi-Ion Conduction by the BK Channel

Our picture of K⁺-binding sites within this channel's conduction pathway has emerged from experiments carried out on the nonconducting, Ba²⁺-blocked channel. Nonetheless, these results inform us about the mechanism of K⁺ permeation

through the conducting channel. Assuming that the Ba^{2+} -blocking site is designed to interact with K^+ under normal conducting conditions, we conclude that this channel contains at least four sites that may all be simultaneously occupied during K^+ conduction. We further propose that these four K^+ -binding sites are of inherently very high affinity for K^+ , and selective for K^+ over Na^+ , although with different degrees of selectivity. We will argue that this proposal leads to a plausible picture of ion conduction through this channel.

How can we reasonably argue that all the sites are of inherently high affinity for K^+ , when we observe an enormous range of affinities for these sites? The external lock-in site shows a K_D of $20 \mu\text{M}$, while the K_D s of the internal lock-in and enhancement sites are on the order of 10 and 300 mM, respectively. To answer this objection, we must distinguish between the *inherent* affinity of a site and its *observed* affinity. By the inherent affinity of a site in this channel, we mean the affinity of the site

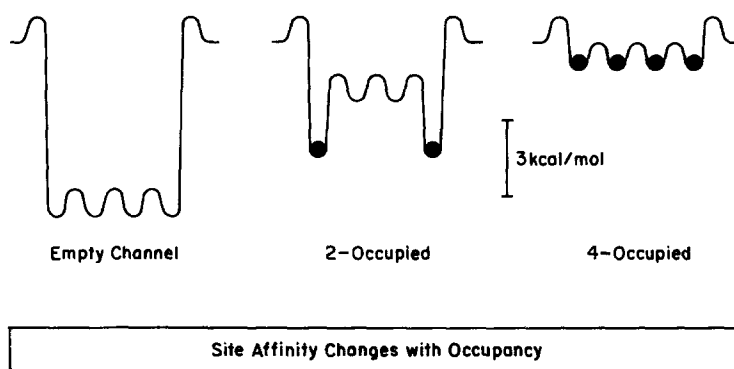


FIGURE 11. Destabilization of ion binding by multiple occupancy. This cartoon qualitatively represents the effect of ion occupancy on the free energy profile of a multiply occupied channel. The channel has four identical sites of high affinity. The free energy profile for the unoccupied channel is drawn ~ 8 kcal/mol below the level of the external solution (1 molal standard state), which is equivalent to $K_D = 1 \mu\text{M}$ for the first K^+ -binding step. Also drawn are the doubly occupied channel (free energy profile seen by the third ion binding step) and the fully occupied channel. Free energy scale is drawn for heuristic purposes only, and to correspond roughly to the range of K_D values seen in this study.

for a K^+ ion, given that all the other sites are unoccupied. In other words, the inherent affinity refers to the binding of an ion to the empty channel. In general, the observed affinity of sites in a multi-ion channel will be lower than the inherent affinity because of electrostatic repulsion between ions (Levitt, 1978).

The point is illustrated in Fig. 11, which shows the free energy profiles we propose for this channel at various degrees of occupancy by K^+ . The empty channel contains four identical sites of very high affinity, equivalent to a K_D in the submicromolar range. A K^+ ion alone inside this channel would be stuck in a very deep energy well; it would be able to move rapidly among the four sites, but not to leave the channel easily. As K^+ concentration is raised, additional sites begin to become significantly occupied. A K^+ ion about to enter the doubly occupied channel would see a much higher energy profile, equivalent to an observed K_D of 1 mM, for exam-

ple. The last K⁺ ion would bind with an even lower affinity to the triply occupied channel. In this way, all the ions in the fully occupied channel would mutually destabilize each other, so that the rates of escape from the pore would be high enough to account for the high conductance actually observed in this channel. This argument must remain qualitative, however, since we do not know the physical separation between the sites, and thus cannot calculate the energies of mutual destabilization. Experimental and theoretical studies on gramicidin A, (Levitt, 1978), however, show that identical sites separated by 1 nm can easily produce K_D values differing by factors of 100–1,000. Since these are the sorts of destabilization factors we observe here, it is reasonable to propose that these four sites span a distance of ~3 nm. This estimate is in harmony with the length of the channel measured by bis-quaternary ammonium blockers, 3.5 nm (Villaruel et al., 1988).

We find a very low K_D of 20 μM for K⁺ binding to the external lock-in site. This is the highest affinity yet reported for any K⁺-specific protein binding site. Since this measurement was made with a divalent Ba²⁺ ion in the blocking site and a K⁺ in the

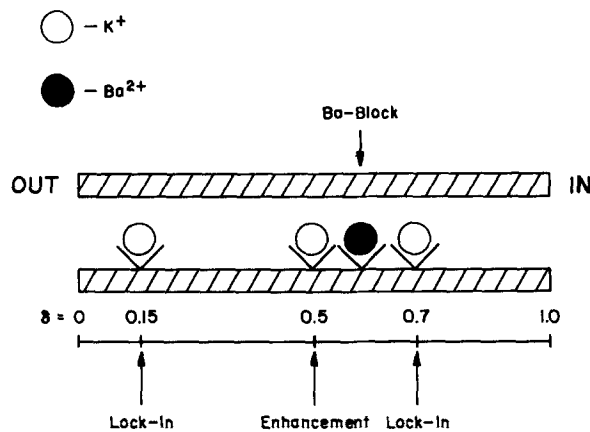


FIGURE 12. A model of the pore of the BK channel. This cartoon summarizes our conclusions from this and the companion study. The channel is pictured as containing four K⁺-binding sites located at the indicated electrical distances, measured from a series of 12 experiments as in Fig. 10. The Ba²⁺-blocking site is constrained to be located between the internal lock-in and enhancement sites.

internal lock-in site, the inherent affinity of this site must be even higher than this. Likewise, the observed K_D (8 mM) of the internal lock-in site was measured in the presence of Ba²⁺ and a single K⁺ already in the channel. The enhancement site begins to fill only above 50 mM K⁺, but this occurs in a channel already occupied by a Ba²⁺ and two K⁺ ions. Moreover, the enhancement site is located closer to the Ba²⁺ site, as judged by the voltage dependence of its binding (Fig. 10), so ionic repulsion here is expected to be particularly strong. For these reasons, we consider it plausible to propose the simple picture here of four structurally similar, high-affinity K⁺-binding sites.

According to this view, significant K⁺ conduction occurs only when the channel is occupied by at least three K⁺ ions. For lower occupancies, K⁺ conduction is retarded because the resident ions bind too tightly to the pore. Only at higher ion occupancies do the K⁺ ions mutually destabilize each other and rapidly permeate the channel; this mutual destabilization raises the K⁺ dissociation constants by factors on the order of 10⁵, thus lifting the free energy profile up by ~7 kcal/mol (Fig.

11). Since this channel allows K^+ conduction at rates approaching diffusion limitation (Latorre and Miller, 1983), there cannot be significant barriers to K^+ movement within the pore itself. Instead, we picture the pore as a structure that allows thermodynamically favorable specific liganding of K^+ over an extended region of ~ 3 nm, as pictured in Fig. 12. As a speculative interpretation of our results, this cartoon cannot be taken seriously in detail; but the essential point expressed in this proposed structure, the existence of a long pore containing numerous sites suited to interact strongly with the K^+ ion, follows inescapably from our results. This model is consistent with this channel's unusual relation between single-channel conductance and symmetrical K^+ concentration (Moczydlowski et al., 1985), which can be fit in the high concentration range (where local surface potentials are largely screened) by a rectangular hyperbola of $K_m = 130$ mM. While this relation cannot be expected to correspond precisely to the binding steps described here, the increase in conductance in the range of 100–1,000 mM probably represents the filling of the fourth site identified in this study.

The kind of picture offered here is a wholly conventional view of K^+ channel structure. Hodgkin and Keynes (1955) originally proposed a "long pore" model for the squid axon delayed rectifier, and Hille and Schwarz (1978) pointed out that multi-ion, single-file conduction applies in many K^+ channels. Estimates of pore occupancies of two or three K^+ ions have been made from flux ratio measurements on squid axon–delayed rectifier K^+ channel (Hodgkin and Keynes, 1955; Begenisich and deWeer, 1980), the inward rectifier of skeletal muscle (Spalding et al., 1981), and a Ca^{2+} -activated K^+ channel from human erythrocytes (Vestergaard-Bogind et al., 1985). The only novel aspect of our structure is the enhanced detail that we can plausibly propose. Our data force us to two unexpected conclusions: (a) that a large number of ion-binding sites, at least four, can be simultaneously occupied inside the confines of this highly selective pore, and (b) that the inherent affinity of this pore for K^+ is extremely high, many orders of magnitude higher than the affinity deduced by conduction properties alone. Of course, the high inherent binding affinity is required for multiple occupancy here; it is the large favorable free energy of binding that allows several ions to overcome their mutual repulsion and cohabit the pore.

These results lead us to a less conventional view of the mechanism of K^+ selectivity. Traditionally, K^+ -selective channels have been considered to select against Na^+ , not by favoring the binding of K^+ , but by placing large barriers to Na^+ movement at the channel's "selectivity filter" (Armstrong, 1975; Coronado et al., 1980), although Hille and Schwarz (1978) have pointed out that multi-ion channels are so complicated that their ionic conduction properties tell us almost nothing about the underlying energy profile experienced by a permeating ion. Our results show that the channel contains binding sites that interact strongly with K^+ and much less strongly with Na^+ . For example, the K^+/Na^+ binding affinity ratio is $>1,000$ for the external lock-in site, and at least 10 for the enhancement site. (The internal lock-in site is less selective, with K^+ favored over Na^+ only by a factor of 5.) We imagine that Na^+ fails to permeate the channel simply because most of the cation-liganding region has a low inherent affinity for this ion. The presence of even a minute concentration of K^+ will effectively exclude Na^+ from the channel, exactly as Ca^{2+}

blocks monovalent cation conduction through the "L-type" Ca²⁺ channel (Almers and McCleskey, 1984; Hess and Tsien, 1984). The fact that the internal lock-in site can accommodate a Na⁺ ion with a Ba²⁺ and a K⁺ in the channel may mean that the inherent affinity of this particular site is actually quite high for Na⁺. Indeed, this may be the site of internal Na⁺ block of this channel (Marty, 1983; Yellen, 1984). A combination of a single high-affinity Na⁺ site in series with several other low-affinity Na⁺ sites would explain this channel's failure to conduct Na⁺ appreciably in the total absence of K⁺.

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