

Coupling of Voltage-dependent Gating and Ba⁺⁺ Block in the High-Conductance, Ca⁺⁺-activated K⁺ Channel

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ABSTRACT Voltage-dependent Ca⁺⁺-activated K⁺ channels from rat skeletal muscle were reconstituted into planar lipid bilayers, and the kinetics of block of single channels by Ba⁺⁺ were studied. The Ba⁺⁺ association rate varies linearly with the probability of the channel being open, while the dissociation rate follows a rectangular hyperbolic relationship with open-state probability. Ba ions can be occluded within the channel by closing the channel with a strongly hyperpolarizing voltage applied during a Ba⁺⁺-blocked interval. Occluded Ba ions cannot dissociate from the blocking site until after the channel opens. The ability of the closed channel to occlude Ba⁺⁺ is used as an assay to study the channel's gating equilibrium in the blocked state. The blocked channel opens and closes in a voltage-dependent process similar to that of the unblocked channel. The presence of a Ba ion destabilizes the closed state of the blocked channel, however, by 1.5 kcal/mol. The results confirm that Ba ions block this channel by binding in the K⁺-conduction pathway. They further show that the blocking site is inaccessible to Ba⁺⁺ from both the cytoplasmic and external solutions when the channel is closed.

INTRODUCTION

Many K⁺-specific channels are inhibited by Ba ions. In the cases for which careful mechanistic studies have been carried out (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Armstrong et al., 1982; Vergara and Latorre, 1983), the evidence argues that the Ba ion acts via a simple blocking scheme; it enters the conduction pathway of the K⁺ channel, where it binds tightly and prevents the permeation of K⁺. In the case of the high-conductance, Ca⁺⁺-activated K⁺ channel from rat skeletal muscle plasma membranes, the effects of Ba⁺⁺ have been examined at the single-channel level (Vergara and Latorre, 1983). When added

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to the intracellular side of the membrane at concentrations >100 nM, Ba^{++} induces in this channel a nonconducting state of an average duration on the order of 5 s, much longer than the millisecond open and closed times. Vergara and Latorre (1983) showed that each of these long-lived, nonconducting intervals represents the binding of a single Ba ion to the channel under study, and that the association of Ba^{++} with the channel is a bimolecular process, with an association rate proportional to the Ba^{++} concentration and a Ba^{++} -independent dissociation rate.

The detailed characteristics of Ba^{++} interaction with the channel provide strong evidence that Ba^{++} literally blocks the channel's open pore (Vergara and Latorre, 1983). The block is voltage dependent, as though Ba^{++} bound at a site located $\sim 80\%$ of the way through the voltage drop, from the inside. The binding of Ba^{++} is competitive with K ions. Finally, the block by internal Ba^{++} is relieved by raising the external K^+ concentration. This last piece of evidence is particularly strong, since it suggests that K ions flowing through the channel can prevent Ba^{++} from reaching its binding site.

Vergara and Latorre (1983) showed that externally added Ba^{++} also blocks this channel, but at concentrations on the order of 10 mM, i.e., $\sim 10,000$ -fold higher than those required for internal block. The properties of external Ba^{++} -blocked states are identical to those induced by Ba^{++} from the internal solution. Therefore, Ba^{++} was envisioned as entering the channel's pore, attaining a well-defined binding site there, and residing on this blocking site on an average of 5 s before dissociating. Ba^{++} may reach this site from either side of the membrane, but the energy barrier for attaining the site is ~ 5.5 kcal/mol higher from the external solution than from the internal.

The recent finding (Miller, 1987) that Ba ions can be occluded inside this channel stimulates the present study. Here we investigate Ba^{++} block and Ba^{++} occlusion in more detail, with two purposes in mind: to test further whether Ba^{++} binds within the conduction pathway, and to use this blocker as a probe of the physical structure of the channel. We show that the kinetics of Ba^{++} block are strongly coupled to the gating of the channel: that the Ba ion can enter and leave only the open state of the channel, and that the channel can open and close in both the unblocked and blocked states. Our results demonstrate that Ba^{++} does indeed bind to the K^+ -conduction pathway. They also demonstrate the existence of a "gating region" located on the internally facing side of the pore, and suggest that a similar gating region may exist on the externally facing side as well. We also show that occupancy by Ba^{++} stabilizes the open conformation of the channel protein.

MATERIALS AND METHODS

Biochemical

Plasma membrane vesicles from rat skeletal muscle were prepared as described (Moczydlowski and Latorre, 1983a) and stored in 0.4 M sucrose at -70°C . The phospholipids used were 1-palmitoyl,2-oleoyl phosphatidylethanolamine (POPE) and the analogous phosphatidylcholine (POPC), obtained from Avanti Polar Lipids, Inc. (Birmingham, AL).

The lipids were stored in stock solutions in chloroform/methanol, 2:1, under N₂ at -70°C.

Planar Bilayers and Channel Incorporation

Ca⁺⁺-activated K⁺ channels were studied by fusing plasma membrane vesicles into planar lipid bilayers, as described (Latorre et al., 1982; Eisenman et al., 1986). Bilayers were formed by applying a drop of lipid solution (14 mM POPE plus 6 mM POPC in *n*-decane) to a 250- μ m-diam hole in a plastic septum separating the two aqueous solutions. Bilayer resistances were always >100 G Ω . Planar bilayers were initially formed with the "internal" solution containing 150 mM KCl, 10 mM HEPES, 20–40 μ M CaCl₂, and 5 mM KOH (pH 7.3), and the "external" solution containing 10 mM HEPES, 0.1 mM EGTA, and 5 mM KOH (pH 7.3). In the presence of this transbilayer salt gradient, channel insertion occurred spontaneously after addition of 1–5 μ g/ml of the plasma membrane vesicles. After the appearance of a single Ca⁺⁺-activated K⁺ channel (recognizable by its characteristic rapid fluctuations of ~15 pA at zero voltage), further channel insertion was suppressed by adding 160 mM NaCl to the external solution. In a few experiments, 150 mM KCl was added instead of NaCl. With these preparations of membrane vesicles, channel insertion always occurred with the cytoplasmic side of the channel facing the internal solution. The orientation of the channel could be unequivocally established by the polarity of voltage-dependent gating and by the sidedness of activation by Ca⁺⁺ (Latorre et al., 1982), of inhibition by charybdotoxin (Miller et al., 1985), and of blocking by tetraethylammonium (Vergara et al., 1984). Using this method, it was routinely possible to maintain a bilayer with a single channel for several hours. All experiments were carried out at 20–22°C.

The electronics and data acquisition system have been described (Eisenman et al., 1986). Briefly, the bilayer voltage was clamped and the transmembrane current was measured with a low-noise current-to-voltage converter circuit with frequency booster and a single time constant capacitance compensator. Current was filtered at 0.5–1 kHz using a eight-pole Bessel filter. The voltage command was applied and current was collected and analyzed by a laboratory computer (Indec, Sunnyvale, CA). The electrophysiological voltage convention is used here, with the external side of the channel defined as zero voltage.

Analysis of Block by Ba⁺⁺

In these experiments, we measured the rates of Ba ion binding to and dissociation from single Ca⁺⁺-activated K⁺ channels. This Ba⁺⁺ interaction occurs on a much slower time scale (1–10 s) than does channel opening and closing (1–10 ms). Consequently, as has been documented in detail (Vergara and Latorre, 1983), long-lived "blocking" events could be reliably distinguished from short-lived "closing" events by setting a cutoff time (100–300 ms) above which any nonconducting event is defined as a block. The rates of Ba⁺⁺ interaction were measured by analyzing the slow transitions of single channels between "burst" and "block" intervals (Vergara and Latorre, 1983). In a typical measurement, 40–100 burst and block events were collected under a given set of conditions, and the following parameters were calculated: mean burst time, mean block time, probability of being open within a burst, and the cumulative histograms of burst and block times. The usual corrections for missed events were applied (Sachs et al., 1982) and were never greater than 10% of the mean value. The time constants of the cumulative histograms always agreed within 15% with the corresponding mean times. We were forced to base these calculations on only 50–100 events because of the slowness of the Ba⁺⁺ blocking process and the finite lifetimes of single-channel planar bilayers (1–8 h).

A potential problem in experiments lasting many hours is that the gating of the channel is not always stationary, but shows spontaneous "shifts" of opening probability, on the time scale of a few minutes (Moczydlowski and Latorre, 1983*b*). Since, as we will show, the Ba⁺⁺ blocking and unblocking rates are tightly tied to the probability of channel opening, such shifts would be intolerable for the quantitative measurements intended here. We were therefore careful to test each channel continuously for stationarity in opening probability. Throughout each experiment, the opening probability and mean open and closed times were determined for each burst. If such shifts of gating activity were observed, the experiment was terminated. In practice, only ~20% of channels from our preparation of plasma membrane vesicles showed unacceptable nonstationarity, defined as channels showing persistent fluctuations in opening probability greater than ±30% of the mean value.

Another potential problem is that of Ca⁺⁺ block. Vergara and Latorre (1983) showed that internal Ca⁺⁺ at high concentrations (>50 μM) and depolarized potentials (>30 mV) blocks the channel in a way that mimics Ba⁺⁺ block. Since the block times for Ca⁺⁺ and Ba⁺⁺ are similar, it was necessary to ensure that the Ba⁺⁺-induced blockade under study was not contaminated by Ca⁺⁺ blocking events. Therefore, control experiments without added Ba⁺⁺ were carried out on each channel to find the range of Ca⁺⁺ concentration and voltage where Ca⁺⁺ blockade was negligible.

Procedure of "Ba⁺⁺-trapping" Experiments

Certain experiments were designed to demonstrate the occlusion of single Ba ions inside the closed channel (Miller, 1987). In such a "Ba⁺⁺-trapping" experiment, a channel was recorded in the presence of Ba⁺⁺ at a depolarized "holding potential," typically 40 mV. As soon as a Ba⁺⁺ blocking event was recognized, the voltage was shifted to a hyperpolarized "test potential" at which the channel's closed conformation should be favored (typically -70 mV). This voltage was maintained for a given time (usually chosen to be long compared with the average blocked time at the holding potential), and was then returned to the holding potential. Immediately after returning to the holding potential, the state of the channel was analyzed by measuring the time at which the first opening event occurred (first latency time). If the first latency was less than a given cutoff time (usually 150 ms), the channel was scored as unblocked, and otherwise as blocked. Care was taken to ensure a judicious choice of cutoff time, which should be at least 10-fold longer than the average closed time (typically 5 ms), and 20-fold shorter than the average blocked time (typically 5 s). In all experiments, we adjusted conditions to prevent the possibility of "reblock," i.e., a channel's becoming unblocked and then blocked again during the hyperpolarized test interval. This is a potential problem at test voltages less negative than -20 mV, at which the channel often becomes unblocked during the test interval.

These experiments were controlled automatically by an on-line pattern-recognition program that searched a channel record for blocking events, usually defined as nonconducting intervals longer than 300 ms. After recognizing such an event, the program applied the test voltage, maintained this voltage for the desired time, and then returned to the holding voltage to analyze the state of the channel. In a typical experiment, the above protocol was repeated automatically 20–100 times, and the first latency of each trial was recorded. Stored records immediately before and after the test pulses were always reviewed by one of the authors to correct any misjudgments made by the computer, a circumstance occurring in <5% of the trials. From the first latency times of a given set of trials, the probability of being in the blocked state at the end of the test pulse was calculated.

The large, voltage-dependent bilayer capacitance (200–300 pF) posed several problems that had to be overcome for this program to work faithfully. First, the bulk of the bilayer capacitance was canceled out by a single time-constant circuit. Second, a 2-s “baseline” record with no channel openings was always collected first and subtracted from the first 2 s after a trial pulse. Even with these precautions, it was necessary to “blank” the first 5–20 ms after returning to the holding potential; only after this time was the state of the channel analyzed. This was never a serious problem, since the average unblocked and blocked times were >100-fold longer than this blanked-out time.

RESULTS

Vergara and Latorre (1983) showed that Ba⁺⁺ added to the internal solution at low concentrations (~1 μM) or to the external solution at high concentrations (~10 mM) induces the appearance of nonconducting, or “blocked,” states lasting on the order of a few seconds, much longer than the 1–10-ms duration of the channel’s “closed” states. Each of these blocked intervals represents the binding of a single Ba ion with the single channel (Vergara and Latorre, 1983). The blocking and unblocking rates, k_{on} and k_{off} , can be directly measured from single-channel records, from the average times in the unblocked (τ_u) and blocked (τ_b) states, respectively:

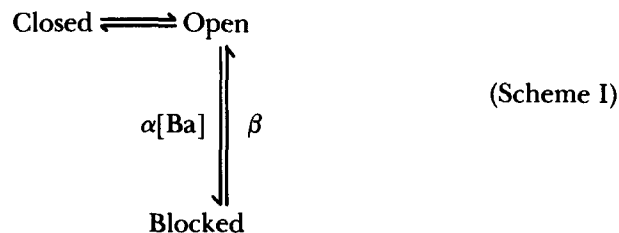
$$k_{\text{on}} = \tau_u^{-1}; \quad (1a)$$

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

These simple relations can be used because the blocking reactions are much slower than the gating reactions and because both the blocked and unblocked times are exponentially distributed (Vergara and Latorre, 1983).

Variation of Blocking Rate with Opening Probability

We embark upon this study with a preliminary model of Ba⁺⁺ block in mind, as proposed and tested by Vergara and Latorre (1983):



This model requires that the channel must open before it can be blocked by a Ba ion, and thus demands that the observed rate of blocking be directly proportional to the probability of opening, p_o , in the unblocked state:

$$k_{\text{on}} = \alpha[\text{Ba}]p_o. \quad (2)$$

In the original study, Vergara and Latorre (1983) demonstrated the above linear dependence on the Ba⁺⁺ concentration, under conditions in which the channel was almost all the time open ($p_o \approx 1$). We now systematically vary p_o , keeping other external variables constant, to see whether the apparent blocking rate

varies as expected. We do this by changing the internal Ca^{++} concentration at a fixed voltage, thus utilizing the Ca^{++} dependence of channel gating to control the opening probability (Methfessel and Boheim, 1982; Moczydlowski and Latorre, 1983; Magleby and Pallota, 1983). To avoid the problem of slow Ca^{++} blocking events (see Materials and Methods), in each experiment we determined the range of Ca^{++} concentrations in which Ca^{++} blocks did not occur; Ba^{++} was then added at the highest allowable Ca^{++} concentration, and Ca^{++} was subsequently varied by addition of EGTA.

Fig. 1 shows that the effect expected is indeed observed: Ba^{++} block is relieved by closing the channel. In this experiment, we recorded a single channel in the presence of $4 \mu\text{M}$ internal Ba^{++} and we varied the open probability of the unblocked channel by changing the Ca^{++} concentration. Reducing Ca^{++} from 30 to $5 \mu\text{M}$ lowered the unblocked channel's open probability from 0.95 to 0.05

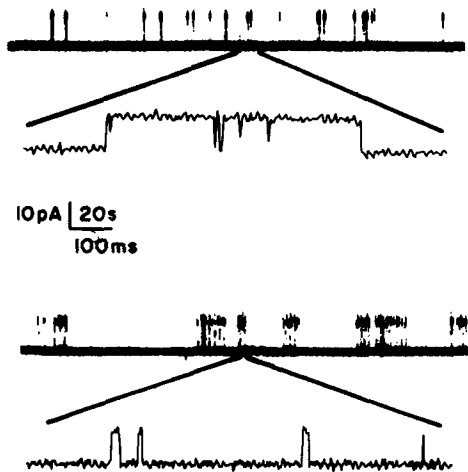


FIGURE 1. Ba^{++} block: dependence on probability of opening. A single Ca^{++} -activated K^{+} channel was incorporated into a planar bilayer as described in Materials and Methods, and records were collected at 45 mV, at two different internal Ca^{++} concentrations, 30 or $5 \mu\text{M}$. Ba^{++} ($4 \mu\text{M}$) was added to the internal solution to induce frequent blocking events. Upper trace: high- Ca^{++} record, where the probability of opening within a burst, p_o , was 0.95. Lower trace: low- Ca^{++} record, with $p_o = 0.05$. Expanded time-scale records illustrate the gating of the channel within a burst. Opening is always an upward deflection. The mean unblocked times were: high Ca^{++} , 0.4 s; low Ca^{++} , 8.7 s.

and led to a 22-fold increase in the average unblocked time. This was as anticipated if the channel becomes available for block by Ba^{++} in proportion to the fraction of time it spends in the open state.

Fig. 2 demonstrates that this coupling of the blocking rate to the open probability was quantitatively as expected from Eq. 2: the Ba^{++} association rate increased in proportion to the open probability. The figure also shows that the same result was obtained for Ba^{++} added on either side of the bilayer. Of course, the absolute rate constants from the two sides differed enormously, since $\sim 10,000$ times more Ba^{++} is required on the outside than on the inside; in both cases, however, the apparent blocking rate varied linearly with the opening probability. This result argues that the channel cannot become blocked while it is closed, as suggested in Scheme I.

Trapping of Ba⁺⁺ in the Closed Channel

Earlier work (Miller, 1987) suggested that closing the channel with a Ba ion on its blocking site leads to occlusion of the blocker, and that the Ba ion cannot escape its site until the channel opens. We wish to elaborate upon these initial results to demonstrate rigorously that Ba⁺⁺ can, in fact, be trapped within the closed channel. The protocol of the basic experiment is shown in Fig. 3. A channel was observed at a depolarized holding potential (40 mV in this example) in the presence of Ba ions. After a Ba⁺⁺ blocking event was recognized, the

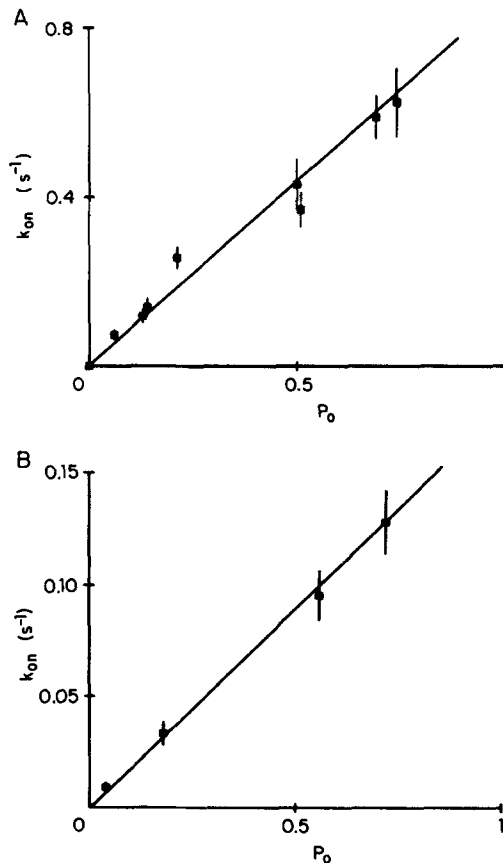


FIGURE 2. Rate constant of blocking vs. open-state probability. The apparent rate constant of Ba⁺⁺ blocking, k_{on} , was measured as a function of open probability, as in Fig. 1, by varying internal Ca⁺⁺ concentration in the range 15–105 μ M. (A) Internal Ba⁺⁺, 1 μ M; $V = 40$ mV. (B) External Ba⁺⁺, 100 mM; $V = 50$ mV.

membrane was strongly hyperpolarized to a test potential of -70 mV, to drive the channel rapidly into its closed conformation. After maintaining the test voltage for a given time, we returned the voltage to 40 mV and asked whether the channel was still in its blocked state.

The example in Fig. 3 shows the channel still blocked after a -70 -mV test pulse of 10 s duration. The channel is identified as blocked rather than closed after this pulse because the time to first opening was very long (720 ms) compared with the mean closed time (1–10 ms). Fig. 4A shows that the same result was

achieved every time the experiment was performed. In this example, we applied hyperpolarizing test pulses of 30 s duration, followed by a return to +40 mV. The figure illustrates seven consecutive trials of this kind, each trace showing the channel still blocked immediately after returning to the 40-mV holding potential.

We have carried experiments like this up to 10 min of hyperpolarization (Miller, 1987), with the same result: if the channel was hyperpolarized while blocked, then it was always blocked immediately after returning to the holding voltage. This result suggests that hyperpolarizing the membrane prevents Ba^{++} from escaping from its blocking site, and that the channel remains blocked throughout the hyperpolarizing pulse. To make this conclusion rigorous, however, it is necessary to demonstrate that during the long hyperpolarizing interval, the channel does not become unblocked and then reblocked by another Ba ion. We can rule out this possibility by applying the hyperpolarizing test pulse while the channel is unblocked (Fig. 4*B*). Now, after returning to 40 mV, the channel is always unblocked. This result, which is expected from our demonstration (Fig.

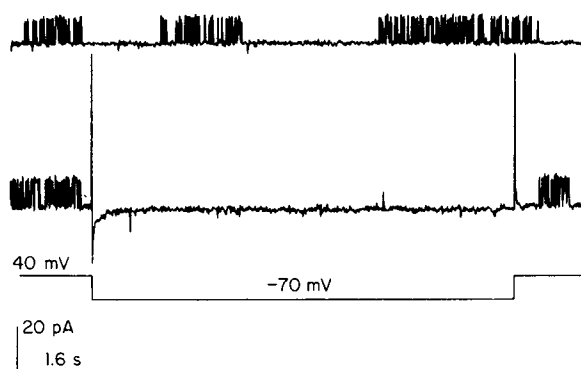


FIGURE 3. Ba^{++} -trapping experiment. A channel was inserted into a bilayer in the presence of $30 \mu M Ca^{++}$ and $0.2 \mu M$ internal Ba^{++} , and was observed at 40 mV. After a Ba^{++} blocking event was recognized, a 12-s test pulse to -70 mV was applied as shown in the voltage trace. After a return to 40 mV, the first latency to opening in this experiment was 0.72 s.

2) that the closed channel cannot be blocked, confirms that a Ba ion, once trapped in the closed channel at -70 mV, cannot escape until the channel is allowed to open again. The experiments above were performed with Ba^{++} added to the internal solution. Identical results were obtained with the blocker added (at 10,000-fold-higher concentrations) to the external solution (data not shown).

We performed a variation on the above experiment to show that Ba^{++} can be trapped within the channel even in the absence of Ba^{++} in the aqueous medium. Fig. 5 shows an experiment carried out on a bilayer containing three Ca^{++} -activated K^+ channels in the presence of internal Ba^{++} . At a time when all three of these channels were simultaneously blocked, a test potential of -75 mV was imposed (Fig. 5*A*). While maintaining the test voltage, we extensively perfused the internal chamber with EDTA-containing medium to remove all the Ba^{++} (and Ca^{++}) present. After 5 min under these Ba^{++} -free conditions, we reintroduced Ca^{++} and returned to the 50-mV holding voltage. The result is clear (Fig. 5*B*): even after 5 min of exposure to Ba^{++} -free medium at -75 mV, all three channels were still blocked. Over the next few seconds, the channels became

unblocked, as indicated in the figure; no subsequent Ba⁺⁺ blocking events were observed, since the aqueous medium at this time was virtually Ba⁺⁺ free. We repeated this perfusion experiment on eight channels, always with the same result.

The average blocked time of the open channel is ~5 s and is voltage independent, as will be shown below. If, in the above experiment, the channels had been in the open conformation at -70 mV, the probability of all three remaining

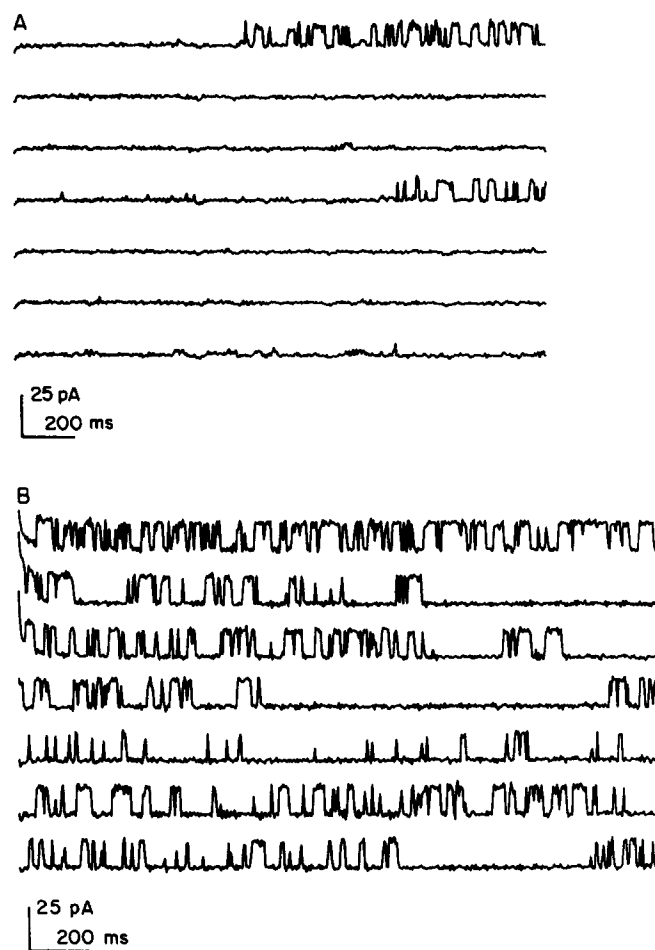


FIGURE 4. Ba⁺⁺ trapping: repetitive trials. (A) The Ba⁺⁺-trapping protocol, as in Fig. 3, was followed repetitively on a channel in the presence of 0.5 μ M internal Ba⁺⁺. Holding potential, 40 mV; test potential, -75 mV; pulse duration, 30 s. The figure illustrates the 2 s immediately following the return to the holding potential. The first seven trials are displayed. (B) All conditions were the same as in A, except that the test voltage was applied during a bursting interval.

blocked throughout the entire 5-min test pulse would be:

$$P = [\exp(-300/5)]^3 \approx 10^{-78}.$$

The fact that all three channels remained blocked during the test interval can only mean that these channels must not have been open during the test interval. Strong hyperpolarization, therefore, prevents the Ba ion from gaining access to the aqueous solution from its binding site. We conclude that it is the act of channel closure that traps the Ba ion on its blocking site. It is certainly not the highly negative potential itself that holds Ba⁺⁺ inside the channel; if anything, the negative test voltage would tend to draw Ba⁺⁺ out of the channel, not hold it in.

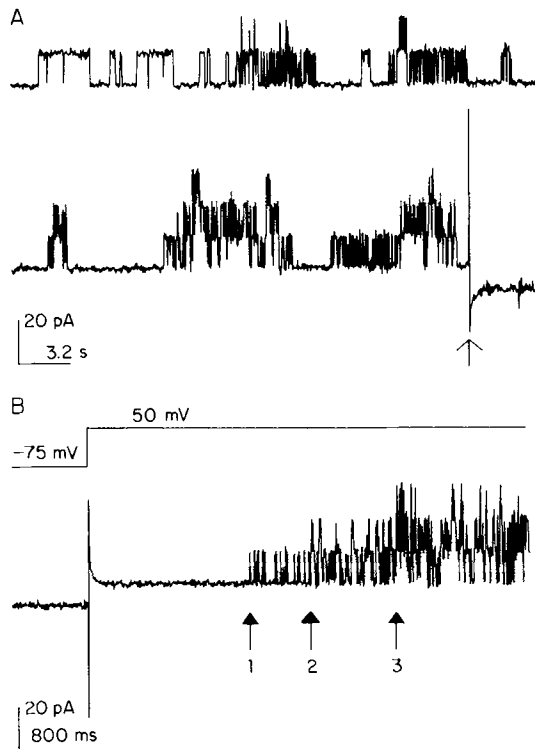


FIGURE 5. Occlusion of Ba⁺⁺: a Ba⁺⁺ perfusion experiment. A bilayer containing three identical channels was observed at 50 mV in the presence of 0.4 μ M internal Ba⁺⁺ and 30 μ M Ca⁺⁺. (A) A continuous record at 50 mV, showing steady state block of the three channels by Ba⁺⁺; at the arrow, when all three channels were simultaneously blocked, voltage was switched to -75 mV. The internal chamber was then perfused with 20 vol of medium containing 100 μ M EDTA. (B) After 5 min at -75 mV, 130 μ M Ca⁺⁺ was added to the internal medium, and at the arrow, voltage was returned to 50 mV to ascertain the state of the channels. The arrows mark the times at which the individual channels became unblocked.

The above experiments show that the same Ba ion remains bound to the channel throughout the hyperpolarized test interval. However, they do not demonstrate that the blocked state observed at the end of the test interval is the same blocked state as that originally present before the test pulse. We can gain insight into this question by trapping a Ba ion as above, and studying the kinetics of Ba⁺⁺ dissociation after returning to the holding voltage. These kinetics are observed by measuring the time at which the first opening occurs (first latency time). If the Ba⁺⁺-blocked states before and after the test pulse are identical, then the statistical distribution of latency times must agree with that of blocked

times under steady state conditions. Fig. 6 shows that this is the case. Both steady state block times and first latencies after a 10-s test pulse are exponentially distributed, with indistinguishable time constants of 5.2 s. Thus, the states of the system immediately before and immediately after a test pulse are identical.

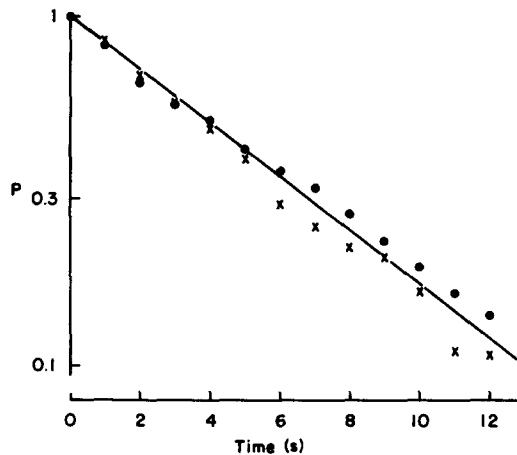
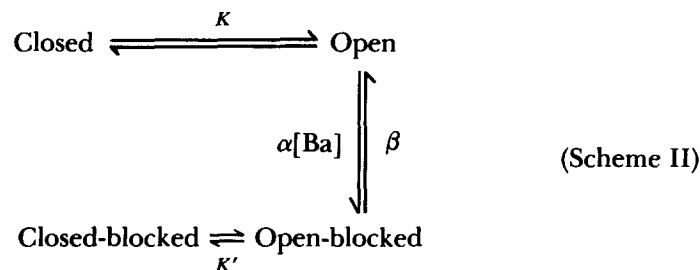


FIGURE 6. Distribution of block times and test pulse first latencies. A channel was incorporated in the presence of $0.5 \mu\text{M } Ba^{++}$, and a steady state record consisting of 111 blocking events was collected at a holding potential of 50 mV. The cumulative histogram of block times was calculated from these events (●). Then, a series of 137 Ba^{++} -trapping pulses (10 s duration) to -60 mV was applied, and the first latency to opening after returning to 50 mV was recorded for each. The cumulative histogram of first latencies was calculated from these pulses (x). The solid line is drawn according to a single exponential with a time constant of 5.2 s.

Ba^{++} Dissociation from the Blocked Channel

The results reported above require that Scheme I be modified to include both "open-blocked" and "closed-blocked" states of the channel; the closed-blocked state envisions the channel is in its closed conformation with a Ba ion trapped on its blocking site:



The results above show that Ba^{++} can escape from the closed-blocked state only by going through the open-blocked state, i.e., that transitions between the closed-blocked and closed states do not occur. As in Scheme I, α and β are the rate constants for Ba^{++} association with and dissociation from the open channel. The equilibrium constants for opening of the unblocked and blocked channels are denoted by K and K' , respectively. In this blocking scheme, the conformational "gating" reactions are assumed to be much faster than the Ba^{++} association and

dissociation rates. We can then write the expected behavior for the observed association and dissociation rates for Ba^{++} :

$$k_{on} = \alpha[Ba]p_o, \quad (3)$$

$$k_{off} = \beta p'_o, \quad (4)$$

where p_o and p'_o are the probabilities of the channel being in the open conformation when unblocked and when blocked, respectively. Only p_o is directly observable, since the open-blocked and closed-blocked states are both nonconducting.

We have seen that the rate of Ba^{++} blocking depends linearly on the probability of the channel being open (Fig. 2), as demanded by both blocking schemes above. How should the rate of unblocking, or dissociation of Ba^{++} , behave as open probability varies? Eq. 4 answers this question, but unfortunately it is unusable as it stands, since we cannot measure p'_o directly. But by making one assumption, we can express p'_o in terms of p_o . We assume that the equilibrium constants for opening in the unblocked and blocked states differ by a constant factor, Θ , which we call the "stabilization factor":

$$K' = \Theta K. \quad (5)$$

This is equivalent to saying that the presence of a Ba ion on its blocking site either stabilizes ($\Theta > 1$) or destabilizes ($\Theta < 1$) the open conformation with respect to the closed conformation by a fixed amount of free energy. It then follows that:

$$p'_o = \Theta p_o / [1 + (\Theta - 1)p_o]. \quad (6)$$

The expected variation of k_{off} with observed opening probability p_o is therefore a rectangular hyperbola:

$$k_{off} = \beta_{max} [1 + \rho/p_o]^{-1}, \quad (7)$$

$$\text{where } \beta_{max} \equiv \beta \Theta / (\Theta - 1) \quad (8)$$

$$\text{and } \rho \equiv (\Theta - 1)^{-1}. \quad (9)$$

Here, β is the dissociation rate constant from the fully open channel, i.e., at $p_o = 1$. Since p_o is defined only on the interval $[0, 1]$, the "maximum rate constant," β_{max} , is a mere mathematical construct with no physical meaning, but the "half-saturation probability," ρ , is a direct measure of the stabilization factor Θ (Eq. 9).

Fig. 7 shows that k_{off} does vary with opening probability according to a rectangular hyperbola. The stabilization factor, Θ , calculated from these data is 12 ± 1 (SE of 10 separate experiments). This means that a Ba ion in the channel shifts the gating equilibrium by ~ 1.5 kcal/mol toward channel opening. In other words, under a given set of conditions, the channel with a Ba ion inside is open more often than when it is unoccupied by the blocker. This difference of gating equilibria in the blocked and unblocked states explains the saturating shape of the k_{off} vs. p_o curve of Fig. 7. As Ca^{++} is raised and the unblocked channel's opening probability increases from a low value, the "hidden" opening probability

of the blocked channel increases more rapidly. When the value of p_o has reached only 0.1, for instance, p'_o has already reached 0.55. Thus, the maximal rate of Ba^{++} dissociation from the fully open channel is approached even when the unblocked channel is only rarely open.

As Fig. 7 shows, the same variation of k_{off} with opening probability is seen regardless of the side of the membrane to which Ba^{++} is added. Stabilization factors are identical for internal and external Ba^{++} . The stabilization factor does not vary with voltage in the range 0–50 mV (data not shown).

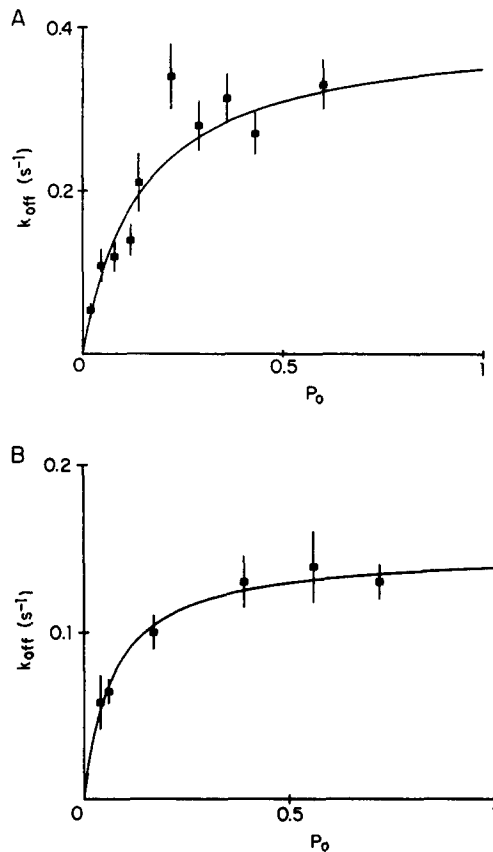


FIGURE 7. Ba^{++} dissociation rate vs. open-state probability. The apparent dissociation rate, k_{off} , was measured as a function of opening probability, as in Fig. 2. Opening probability was varied by varying Ca^{++} in the range 30–200 μ M. Data were fitted to rectangular hyperbolas (solid curves) as in Eq. 6, by double-reciprocal plots (not shown). (A) Internal Ba^{++} , 0.3 μ M; $V = 40$ mV; $\theta = 11$. (B) External Ba^{++} , 100 mM; $V = 50$ mV; $\theta = 15$.

Voltage Dependence of Gating in the Blocked State

The experiment above (Fig. 7) serves to validate the blocking model of Scheme II, but its interpretation relies on the assumption (Eq. 5) that the gating processes of the blocked and unblocked channel are fundamentally similar, with only a fixed free energy difference between them. If it were possible to measure directly the opening probability in the blocked channel, we would have a way of testing this assumption. Of course, we cannot do this, since both open-blocked and closed-blocked channels are nonconducting. However, we can measure p'_o indirectly by exploiting the Ba^{++} -trapping phenomenon documented above.

The experimental protocol is to trap Ba^{++} as above, but using test pulses to less hyperpolarized voltages. The idea is that with milder hyperpolarization, the blocked channel will not always be in the closed conformation. If the channel is sometimes open at the test voltage, then the Ba ion will have an opportunity to dissociate from the channel during the test interval. By performing the experiment repetitively, we can measure directly the probability of remaining blocked, P_b , during the entire test interval. This measurable quantity can be given in terms of the test pulse duration, Δt , and the Ba^{++} dissociation rates at the test voltage, k_{off} :

$$P_b = \exp(-k_{off}\Delta t). \quad (10)$$

By applying Eq. 4, we obtain an expression for p'_o , the probability of opening in the blocked channel, in terms of measurable quantities:

$$p'_o = -(\ln P_b)/\beta\Delta t. \quad (11)$$

In a typical experiment, β is measured at the holding voltage, via steady state blocking kinetics. Since the rate of Ba^{++} dissociation from the open channel is voltage independent, this is a valid measure of the dissociation rate at the test voltage as well.

Fig. 8 illustrates the results of Ba^{++} -trapping experiments performed at several different test voltages. As before, when the test pulse was made highly negative, the channel was always blocked upon return to the holding voltage (Fig. 8, top). At less negative test voltages, however, we see (Fig. 8, middle and bottom) that the channel sometimes became unblocked during the test interval, since the blocked channel, now open some of the time, permitted the dissociation of Ba^{++} .

By analyzing experiments as those of Fig. 8 quantitatively according to Eq. 11, we can measure p'_o as a function of voltage. What do we expect for this variation? The unblocked channel open probability follows a Boltzmann curve (Moczydlowski and Latorre, 1983):

$$p_o = \{1 + \exp[-zF(V - V_o)/RT]\}^{-1}, \quad (12)$$

where z is the effective gating charge and V_o is the voltage at which the channels are open half of the time. It then follows from Eq. 6 that the blocked state opening probability, p'_o , should follow a similar curve, with the only difference being that the voltage at half-opening, V'_o , is more negative than V_o . That is, the probability-voltage curve for the blocked channel should be shifted to the left along the voltage axis by an amount ΔV_o :

$$\Delta V_o = V_o - V'_o = (RT/zF) \ln \Theta. \quad (13)$$

The equilibrium constant for opening of the unblocked channel increases e -fold for each 10 mV of depolarization ($z = 2.6$; Fig. 9). Thus, from Eq. 13, a stabilization factor of 12 corresponds to a shift of 25 mV to more negative potentials.

When we carry out the measurement, determining p'_o as a function of voltage via the test pulse method, we find quantitative agreement with our expectations

(Fig. 9). The activation curve for the blocked channel parallels that for the unblocked channel, but is shifted to the left by 24 mV (± 1 mV, SE of five determinations, each in a separate bilayer). This agreement is a powerful confirmation of the blocking model of Scheme II in general and of the validity of defining the stabilization factor as in Eq. 5. The experiment clearly shows that the "hidden" opening and closing equilibria for the blocked channel are similar to the directly observable reactions for the unblocked channel, with the single difference that under a given set of conditions the blocked channel prefers to be in the open conformation, by 1.5 kcal/mol, with respect to the unblocked channel.

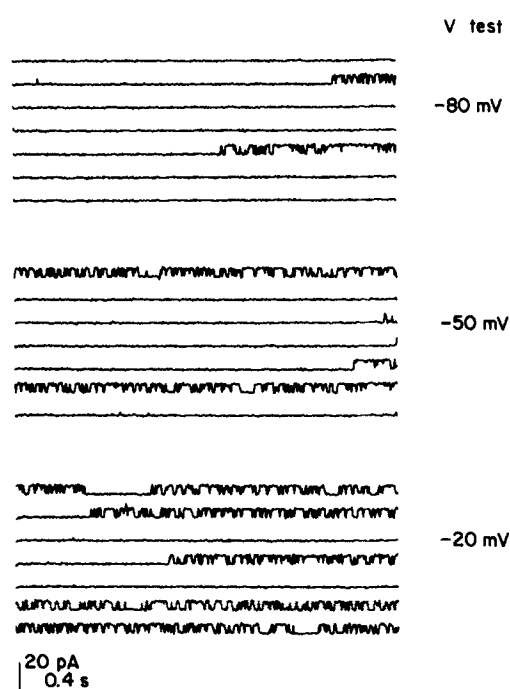


FIGURE 8. Ba⁺⁺-trapping experiments: effect of test voltage. The effect of test voltage is illustrated for Ba⁺⁺-trapping experiments carried out as in Fig. 4A. Holding potential, 40 mV; internal Ba⁺⁺, 0.5 μ M. Each panel displays the responses after seven consecutive 10-s test pulses at the indicated test potential.

Voltage Dependence of Open-Channel Blocking Reactions

Vergara and Latorre (1983) originally found that Ba⁺⁺ block is voltage dependent, with association displaying much more voltage dependence than dissociation. Since we now know that Ba⁺⁺ binds to and dissociates only from the open channel, it is necessary to reexamine the voltage dependence of the Ba⁺⁺ blocking kinetics, to take into account the fact that the probability of the channel's being open itself depends upon voltage. We therefore measured the steady state blocking and unblocking rates, k_{on} and k_{off} , as a function of applied voltage, and corrected these for opening probability to ascertain the true voltage dependence of the open-channel blocking kinetics:

$$\alpha[Ba] = k_{on}/p_o, \quad (14)$$

$$\beta = k_{\text{off}}/p'_o, \quad (15)$$

where p_o is measured directly and p'_o is calculated from p_o and Eq. 6.

We found (Fig. 10), as did Vergara and Latorre (1983), that only the association rate constant was voltage dependent; no variation in the dissociation rate was discernible under conditions where the association rate varied over 20-fold. This conclusion applies to Ba^{++} added to either side of the membrane, although the voltage dependences from the two sides are of opposite polarity. Since the opening probability varies from 0.1 to 0.95 over the voltage range studied here, substantial corrections must be applied to the "raw" values of k_{on} to obtain the

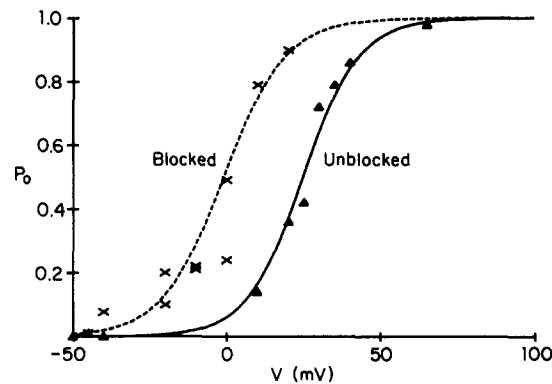


FIGURE 9. Voltage-dependent gating of the blocked and unblocked channel. Experiments as in Fig. 8 were performed to calculate the probability of opening, p'_o , in the blocked channel, and to compare this to the directly measured probability of opening, p_o . Internal Ba^{++} , $0.5 \mu\text{M}$. First, steady state records were collected to determine the mean blocked time at the holding potential, 40 mV ($6.5 \pm 0.9 \text{ s}$). This value can be used to calculate β , the dissociation rate from the fully open channel, since p_o is adjusted to be >0.8 . Repetitive pulses of 5 and 10 s durations were then applied at the test potentials indicated (20–95 trials at each test potential), and p'_o was calculated as in Eq. 11, using the value of β determined from the steady state data above. The open probability of the unblocked channel, p_o , was measured as a function of voltage before and after the series of test pulses. Voltage activation curves were fitted by eye, using Eq. 12 (solid and dashed curves). Unblocked channel (Δ): $z = 2.6$; $V_o = 26 \text{ mV}$. Blocked channel (\times): $z = 2.5$; $V_o = 1 \text{ mV}$.

open-channel blocking rates. These corrections tend to lower the voltage dependence of block from the internal side and raise it from the external side. The true "effective valences" of block, defined as the equivalent charge moved across the membrane in the blocking process (Vergara and Latorre, 1983), are different from those measured previously without correction for open probability.

The results of Fig. 10 show that the true effective valence of internal Ba^{++} block is $1.6 (\pm 0.1, \text{ SE of 10 membranes})$. If this channel could permit only a single ion to occupy the conduction pathway, we would conclude that the binding site for Ba^{++} is located $\sim 80\%$ of the way down the voltage drop, as measured

from the internal side. However, it is clear that this channel allows simultaneous occupancy by several ions (Yellen, 1984; Eisenman et al., 1986), and so we cannot interpret the voltage dependence of block in this simple way. The voltage dependence of the external Ba^{++} blocking rate gives an effective valence of -1.5 , as measured from the external side.

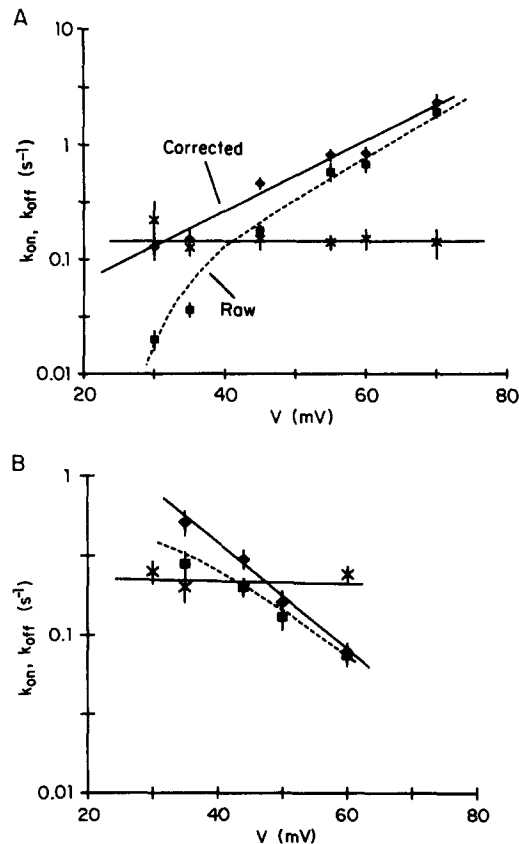


FIGURE 10. Voltage dependence of open-channel blocking kinetics. Steady state records of single channels in the presence of Ba^{++} were collected as a function of voltage, and apparent association (\blacksquare , \blacklozenge) and dissociation (\times) rates were measured. At each voltage, the open probability of the unblocked channel was also measured, and Eq. 14 was used to correct the raw values of k_{on} (\blacksquare) to obtain the open-channel association rates (\blacklozenge). For clarity, only the corrected values (Eq. 15) of the dissociation rates are shown and they are negligibly different from the raw values. (A) Internal Ba^{++} , 3 μM ; effective valence, 1.7. (B) External Ba^{++} , 30 mM; effective valence, -1.5 . These experiments were carried out with 150 mM KCl in both solutions; similar results (not shown) were obtained with 150 mM internal K^+ and 170 mM external Na^+ .

DISCUSSION

The new information arising from this work addresses two classes of questions about the high-conductance, Ca^{++} -activated K^+ channel. First, we have obtained a clearer picture of the mechanism by which Ba^{++} inhibits the channel. Second, we can begin to draw inferences about the physical structure of this channel's conduction pore. The phenomenon of Ba^{++} occlusion not only leads to strong conclusions about the mechanism of Ba^{++} action, but also permits us to characterize the channel's transitions among several nonconducting states.

Mechanism of Ba^{++} Block

This study continues a series of investigations (Vergara and Latorre, 1983; Miller, 1987) into the physical mechanism by which Ba^{++} inhibits the high-

conductance, Ca^{++} -activated K^+ channel. In these studies, we have endeavored to subject this mechanism to as many experimental attacks as possible, and thus far all these lines of evidence point to the same conclusion: that the site of Ba^{++} blockade is located within this channel's K^+ -conduction pore. Considering the already powerful evidence pointing to this picture, as summarized in the Introduction, this conclusion seems inescapable in the face of the occlusion of Ba^{++} under conditions leading to channel closing (Figs. 4 and 5), and the voltage-dependent relief of this occlusion, paralleling the channel's normal voltage-dependent opening (Fig. 9).

Ba^{++} is a potent blocker because it is readily accessible to the channel from the internal solution, and because it binds tightly to its blocking site. Since association is voltage dependent, while dissociation is not, the transition state for Ba^{++} entry must be located well into the conduction pore, possibly just proximal to the binding site. The voltage dependence of Ba^{++} block, equally strong from both sides of the channel, suggests that the binding site is located well inside the pore, although the multi-ion nature of the channel does not allow us to determine the precise location of this binding site.

Sidedness of Ba^{++} Action

External Ba^{++} also blocks the channel, but at concentrations 10,000-fold higher than those needed for internal block. We worried that external Ba^{++} block might be an artifact caused by leakage across the membrane and subsequent block from the inside. This artifact is ruled out, however, by the fact that external Ba^{++} block is identical with 150 mM KCl or 75 mM K_2SO_4 in the internal solution (data not shown). The solubility product of BaSO_4 is so low that the free Ba^{++} concentration in SO_4^{--} medium could never exceed 1 nM, at which concentration internal block would almost never be observed. External Ba^{++} block, therefore, is a real phenomenon.

The reason that external Ba^{++} block is so weak is that the rate constant for association is sluggish, $\sim 30 \text{ M}^{-1} \text{ s}^{-1}$ at zero voltage, 3,000-fold slower than that from the inside. This difference corresponds to an energy barrier to entry that is 4.8 kcal/mol higher from the external solution than from the internal (Vergara and Latorre, 1983). The dissociation rate constant is identical for Ba^{++} added to either side. This is to be expected if the same binding site is attained from either side of the membrane. The large difference in energy barriers on the two sides means that Ba^{++} dissociation takes place almost exclusively to the internal solution, regardless of the side from which it entered. (Given this difference in the association rate constant, only 1 out of 3,000 dissociation events would be to the external solution.) This evidence compels us to the conclusion that the same site is reached by Ba^{++} from both sides of the membrane. All of the characteristics of the blocked state are the same from the two sides: the absolute rate of dissociation, the voltage independence of this rate, the occlusion of Ba^{++} by negative voltage, and the value of the stabilization factor, θ .

Voltage Dependence of Ba^{++} Block

While all of the results above, as well as those of Vergara and Latorre (1983), fit naturally into the simple picture of a single Ba^{++} blocking site located within the

conduction pore, the voltage dependence of the blocking reaction appears to contradict this view. We originally interpreted the effective valence of internal block, 1.6, to mean that the blocking site is located ~80% of the way across the voltage drop within the channel, as measured from the internal side. We therefore expected that the voltage dependence of external block would be much weaker (with an effective valence of -0.4) than was actually observed (-1.5). These two values of effective valence are flatly inconsistent with the simplest picture: a single-ion pore in which Ba⁺⁺ blocks at a single, well-defined site. In trying to find a way out of our dilemma, we have two choices: to reject either the idea of a single Ba⁺⁺-binding site within the pore, or to abandon the effective valence as a valid measure of the position of the blocking site.

The results here argue strongly that the same Ba⁺⁺ blocking site is attained from both sides of the membrane, as discussed above, and so we are unwilling to reject this idea. However, since the high-conductance, Ca⁺⁺-activated K⁺ channel is known to operate by a multi-ion mechanism (Yellen, 1984; Eisenman et al., 1986; Cecchi et al., 1987), we can easily abandon the effective valence of block as an indicator of the physical position of the blocking site. In multi-ion channels, where the movement of conducting and blocking ions are coupled, the measured effective valence of blocking can be higher than that expected from the physical position of the site (Hille and Schwarz, 1978; Adelman and French, 1978; Cecchi et al., 1987). The actual value of effective valence is dependent on the particular kinetic conduction model employed, and so we are unwilling to "locate" the Ba⁺⁺ blocking site from the effective valence of block. We do not consider that the channel ever becomes multiply occupied by Ba⁺⁺, since the blocking reaction follows strictly bimolecular kinetics as the Ba⁺⁺ concentration is varied (Vergara and Latorre, 1983). Multi-ion effects could easily arise from simultaneous occupancy of the conduction pore by Ba⁺⁺ and K⁺, a possibility we are currently investigating experimentally.

Voltage- and Ca⁺⁺-dependent Gating of the Blocked Channel

The model used here (Scheme II) identifies three nonconducting states of the channel: closed, open-blocked, and closed-blocked. Although all three of these states are electrically silent, they can be experimentally distinguished. The closed state is short-lived (1–10 ms) and is directly observed in the unblocked channel. The two blocked states are both long-lived, but the open-blocked state is able to release the Ba ion, while the closed-blocked state occludes Ba⁺⁺. By establishing the Ba⁺⁺ occlusion phenomenon, we have designed two kinds of experiments to observe voltage- and Ca⁺⁺-dependent gating in the blocked channel.

First, we studied the variation of the Ba⁺⁺ dissociation rate with the Ca⁺⁺ concentration (Fig. 7). We found that at high Ca⁺⁺, leading to a high opening probability of the unblocked channel, Ba⁺⁺ dissociation proceeded at a maximum rate characteristic of escape from the fully open channel. Only when Ca⁺⁺ was lowered such that the unblocked open probability decreased below 0.1 did we observe a decrease in the Ba⁺⁺ dissociation rate, since only then did the blocked channel begin to spend a significant fraction of time in the closed conformation. Quantitatively, we concluded that Ba⁺⁺ stabilized the open conformation by 1.5 kcal/mol ($\theta = 12$) over the unblocked channel. Ba⁺⁺ tends to "hold the channel

open." Certain blockers of the nicotinic acetylcholine receptor (Neher and Steinbach, 1978) and of the sarcoplasmic reticulum K^+ channel (Coronado and Miller, 1982) behave as though they absolutely prevent these channels from closing ($\theta = \infty$). The Ba^{++} stabilization phenomenon we observed is merely a more general case of such blocker-gating interaction.

The stabilization of the open conformation is strikingly and quantitatively confirmed in an entirely different kind of experiment using voltage activation (Fig. 9). Here, we used the Ba^{++} -trapping method to examine the probability of escape of trapped Ba^{++} as a function of test voltage. We showed (Eq. 11) that this technique measures the open/closed equilibrium in the blocked channel. We found again that Ba^{++} stabilized the channel's open conformation; the voltage activation curve of the blocked channel was shifted 24 mV negative to the curve for the unblocked channel. This is in excellent agreement with the shift of 25 mV predicted on the basis of a stabilization factor of 12 (Eq. 13). This experiment also shows that the voltage dependence of gating is the same whether or not the channel is blocked.

The analysis of these experiments assumes that the gating reactions are rapid with respect to the blocking reactions. This assumption is palpably valid for the unblocked channel. The gating kinetics of the blocked channel, however, cannot be observed directly, and we need to resort to an indirect argument to validate the assumption in this case. First, the agreement of the stabilization factor measured by two different methods argues that the blocked-state gating kinetics do not contaminate the measurements of Ba^{++} dissociation kinetics. Second, and more directly, the time distribution of the first latencies after a hyperpolarizing test pulse agrees exactly with the steady state blocked-time distribution (Fig. 5). This means that the transition from the closed-blocked to the open-blocked channel is much faster than the Ba^{++} dissociation rate.

Conduction of Ba^{++} through the Ca^{++} -activated K^+ Channel

Ba^{++} is a conducting ion in this channel. A Ba ion in the external solution (at, say, 30 mM concentration) must wait for the channel to open in response to either voltage or Ca^{++} before it can enter the pore. The ion then lingers on its binding site for several seconds and dissociates to the internal solution. It is instructive to compare Ba^{++} conduction with that of the physiological substrate, K^+ . The single-channel current carried by Ba^{++} considered above, corresponding to one ion per 10 s, comes out to $\sim 3 \times 10^{-20}$ A, nine orders of magnitude lower than K^+ current under similar conditions. This represents a very impressive selectivity between two conducting ions of exactly equal crystal radii.

What is the basis for this selectivity? The second-order entry rate for K^+ is $>10^9 M^{-1} s^{-1}$, approaching diffusion limitation (Latorre and Miller, 1983; Yellen, 1984), and four to five orders of magnitude greater than the internal Ba^{++} entry rate. The dissociation rate for K^+ is $\sim 10^8 s^{-1}$ (Moczydlowski et al., 1985; Eisenman et al., 1986), nine orders of magnitude faster than Ba^{++} dissociation. Thus, Ba^{++} is much more reluctant than K^+ to enter the channel, and, once inside, is much more reluctant to leave.

We cannot offer any physical picture for this selectivity, other than to say that there is site within the channel that binds Ba ions very well, with a dissociation

constant of $\sim 1 \mu\text{M}$, an affinity at least five orders of magnitude higher than for K^+ and its monovalent analogues (Moczydlowski et al., 1985; Eisenman et al., 1986). We suggest that Ba^{++} binds to a structure normally used for binding K^+ , a structure intimately involved in establishing the high K^+/Na^+ selectivity of this channel. The large Ca^{++} -activated K^+ channel permits conduction only of ions close in physical size to K^+ : Rb^+ , NH_4^+ , and Tl^+ , and now Ba^{++} (Blatz and Magleby, 1984; Yellen, 1984; Eisenman et al., 1986). This fact argues (Hille, 1975; Latorre and Miller, 1983) that the channel contains a narrow and rigid "selectivity region" through which permeant ions must squeeze as they traverse the pore. We therefore favor the suggestion (Latorre and Miller, 1983) that Ba^{++} is a "transition-state analogue" for K^+ conduction, i.e., an ion similar to K^+ , which binds tightly to a structure governing a rate-determining step for K^+ permeation. It is the Ba ion's divalent charge, we propose, that forces a highly favorable interaction with this structure, which is designed to interact only weakly with K^+ .

A Physical Picture of Ba⁺⁺ Block

While it is always risky to draw structural inferences from purely functional studies, the unique characteristics of ion channels permit us to do this with less trepidation than we might feel in the case of other classes of proteins. We know that channel proteins conduct ions by forming an aqueous pore spanning the membrane. Similarly, the closing of a channel represents a change in conformation such that this pore is obstructed to the passage of ions. Only through true structural studies can we hope to understand the physical mechanism by which the pore is closed, whether this is a result of a mechanical, steric constriction of the pore, or a more subtle conformational change leading to increased electrostatic barriers for permeating ions. Nevertheless, we do know that very large barriers to ion movement through the conduction pathway are created upon channel closure. Given this basic picture of ion channel structure, we can use our results on Ba^{++} block to envision several structural features of this Ca^{++} -activated K^+ channel.

The evidence implicating Ba^{++} as an open-channel blocker allows us to exploit this ion as a probe of the conduction pathway. Since Ba^{++} dissociates from the blocked channel only to the internal solution, the Ba^{++} -trapping phenomenon shows that a large barrier to ion permeation is established on the cytoplasmic side of the conduction pathway when the channel closes. Historically, this would be termed a "gating region" of the channel. That the channel pore becomes obstructed to the escape of Ba^{++} is directly evident from the trapping experiments; we propose that this represents the same barrier that cuts off the flow of K^+ through the normal, conducting channel.

The result (Fig. 2) that external Ba^{++} cannot gain access to the closed channel suggests further that some sort of gate exists on the externally facing side as well. The channel acts as though, upon closing, both sides of the pore become obstructed, leaving a "cavern" with enough room to accommodate a Ba ion quite comfortably and destabilizing the closed state by a mere 1.5 kcal/mol. This two-sided channel closure contrasts sharply with the behavior of Ba^{++} in the squid axon delayed rectifier K^+ channel (Armstrong et al., 1982); for this channel,

Ba⁺⁺ can enter from the internal solution only when the channel is open, but can block both open and closed states from the external medium.

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