Multi-Ion Occupancy Alters Gating in High-Conductance, Ca^{2+}-activated K$^+$ Channels

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ABSTRACT   In this study, single-channel recordings of high-conductance Ca^{2+}-activated K$^+$ channels from rat skeletal muscle inserted into planar lipid bilayer were used to analyze the effects of two ionic blockers, Ba^{2+} and Na$^+$, on the channel's gating reactions. The gating equilibrium of the Ba^{2+}-blocked channel was investigated through the kinetics of the discrete blockade induced by Ba^{2+} ions. Gating properties of Na$^+$-blocked channels could be directly characterized due to the very high rates of Na$^+$ blocking/unblocking reactions. While in the presence of K$^+$ (5 mM) in the external solution Ba^{2+} is known to stabilize the open state of the blocked channel (Miller, C., R. Latorre, and I. Reisin. 1987. *J. Gen. Physiol.* 90:427–449), we show that the divalent blocker stabilizes the closed-blocked state if permeant ions are removed from the external solution (K$^+$ < 10 μM). Ionic substitutions in the outer solution induce changes in the gating equilibrium of the Ba^{2+}-blocked channel that are tightly correlated to the inhibition of Ba^{2+} dissociation by external monovalent cations. In permeant ion–free external solutions, blockade of the channel by internal Na$^+$ induces a shift (around 15 mV) in the open probability–voltage curve toward more depolarized potentials, indicating that Na$^+$ induces a stabilization of the closed-blocked state, as does Ba^{2+} under the same conditions. A kinetic analysis of the Na$^+$-blocked channel indicates that the closed-blocked state is favored mainly by a decrease in opening rate. Addition of 1 mM external K$^+$ completely inhibits the shift in the activation curve without affecting the Na$^+$-induced reduction in the apparent single-channel amplitude. The results suggest that in the absence of external permeant ions internal blockers regulate the permeant ion occupancy of a site near the outer end of the channel. Occupancy of this site appears to modulate gating primarily by speeding the rate of channel opening.

INTRODUCTION

In ionic channels ions are translocated across lipid membranes through an aqueous pore via a diffusion mechanism along their electrochemical gradient. In most channels, at least some parts of the pore are so narrow that ions (and even water molecules) do not pass each other (Hille and Schwarz, 1978). In such a constricted environment, permeant ions may impede changes in channel conformation by steric hindrance. Moreover, their electrical charge is not screened by counter-ions and water molecule dipoles as in the bulk solution. During the permeation process ions may thus induce large electrostatic effects on the part of the channel protein surrounding the pore. This might be particularly relevant for voltage-activated channels where the voltage sensor, the part of the channel molecule sensitive to transmembrane voltage, could also be influenced by ions bound in the permeation pathway. Indeed, permeant ions do affect the gating process in K^+ inward rectifier channels (Hagiwara et al., 1976), K^+delayed rectifier channels (Dubois and Bergman, 1977; Århem, 1980; Swenson and Armstrong, 1981), Cl^- channels (Chesnoy-Marchais, 1983), and Ca^{2+} channels (Nelson et al., 1984; Chesnoy-Marchais, 1985). Permeant ion effects on gating were also observed in neurotransmitter-gated channels such as the ACh-activated cationic channels (Van Helden et al., 1977; Ascher et al., 1978).

Many channel blockers act by binding within the pore, and some of them were also shown to affect channel gating. In two classical examples—the effects of internal TEA on squid delayed rectifier (Armstrong , 1975) and of local anesthetics on the endplate channels (Neher and Steinbach, 1978)—the blockers were found to prevent channel closure.

In this paper we study the effects of two cationic blockers, Ba^{2+} and Na^+, on the gating properties of high-conductance, Ca^{2+}-activated K^+ channels (BK channels) from rat skeletal muscle. Both blockers enter deeply from the cytoplasmic compartment inside the permeation pathway of BK channels where they bind for much longer times than do permeant ions (for Ba^{2+}, see Vergara and Latorre, 1983 and Miller et al., 1987; for Na^+, see Marty, 1983 and Yellen, 1984a, b). Both blockers induce changes in BK channel gating equilibrium. However, whereas Na^+-blocking ions were shown to decrease the open probability of the BK channel under low external permeant concentrations (Marty, 1983), Ba^{2+} was found to stabilize the open-blocked channel (Miller et al., 1987). Ba^{2+}- and Na^+-unblocking kinetics are markedly affected by the presence of permeant cations in the external solution, but, again, in opposite directions. Ba^{2+} dissociation, which in permeant ion-free external solutions is oriented mainly toward the external compartment, is inhibited by external permeant ions at low millimolar concentrations (Neyton and Miller, 1988a). On the other hand, Na^+ seems to always dissociate toward the inner compartment and external permeant ions increase the Na^+-unblocking rate (Marty, 1983; Yellen, 1984b). This stimulated us to investigate a possible modulation of the effects of the blockers on the channel gating by permeant/blocker interactions inside the channel.

In this paper we first show that the effects of Ba^{2+} on the gating equilibrium depend critically on the presence of permeant ions in the external solution: Ba^{2+} stabilizes the open-blocked state in K^+-containing external solutions, whereas it

favors the closed-blocked state in permeant ion–free external solutions. We further show that, qualitatively, similar gating modulations can be observed in Na^+-blocked channels. We finally argue that occupancy of a K^+-binding site near the outer end of the channel, which was shown to control the Ba^{2+}-unblocking reaction (the external lock-in site described in Neyton and Miller, 1988a), may play an important role in the gating of BK channels.

MATERIALS AND METHODS

Biochemical

Plasma membrane vesicles were prepared from rat skeletal muscle as described (Moczydlowski and Latorre, 1983a) and stored in 0.4 M sucrose at $-70°C$. The lipids used were 1-palmitoyl,2-oleoyl phosphatidylethanolamine (POPE) and the analogous phosphatidylcholine (POPC), obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Lipids were stored in stock solutions in chloroform/methanol, 2:1, under N_2 at $-70°C$. N-Methyl-D-glucamine (NMDG) was obtained from Janssen Chemica (Beerse, Belgium). All other chemicals used were of analytical reagent grade purity.

Bilayer Formation and Channel Incorporation

BK channels were inserted into planar lipid bilayers by fusion of plasma membrane vesicles with the bilayers as described previously (Latorre et al., 1982; Miller et al., 1987). Bilayers were made using the painting method with a mixture of POPE and POPC, 7:3 ratio, solubilized at 20 mM in n-decane. As in Neyton and Miller (1988a), bilayers were formed in the absence of K^+ and NaCl agar bridges were used to keep contamination of external solutions by K^+ below 10 μM. This could be checked in each experiment by measuring the Ba^{2+} dissociation rate at a high unblocked channel open probability ($P_o > 0.9$) and comparing the $k_{off}(Ba)$ value obtained in that particular experiment with those previously measured, under the same conditions, in experiments where the effects of low external K^+ concentrations had been studied (Neyton and Miller, 1988a).

Recording and Data Aquisition System

The electronics followed the design of Hanke and Miller (1983). Briefly, the current flowing through the bilayer, whose voltage was clamped, was measured with a low-noise, current-to-voltage converter circuit. The current signal was stored on video tape for further off-line analysis, simultaneously filtered at 0.5–2 kHz using an eight-pole Bessel filter (Frequency Devices, Haverhill, MA), and then collected by a laboratory computer (MINC 1123; Digital Equipment Corp., Marlboro, MA). The electrophysiological voltage convention is always used here, with the external side of the channel defined as zero voltage. No corrections for liquid junction potentials were made in this paper, since these never exceeded 5 mV.

Data Analysis

In this study we analyzed the effects of permeant and blocking ions simultaneously bound in the pore on the gating of BK channels. Two kinds of blocking ions, Ba^{2+} and Na^+, were used. Ba^{2+}, applied at micromolar concentrations in the internal compartment introduces a "slow block" of the BK channel, which appears as discrete blocking events at the single-channel level (Vergara and Latorre, 1983). On the other hand, Na^+, when applied at concentrations larger than 10 mM in the internal compartment, induces a "flickery block" (Marty, 1983; Yellen, 1984a), with blocking events being too fast to be individually resolved under our experimental

conditions. The experiments described in this paper were aimed at allowing a comparison between channel open probabilities of both blocked and unblocked states under various ionic conditions. Following the work of Miller et al. (1987), we will draw inferences on Ba^{2+}-blocked channel open probability from Ba^{2+} block kinetics.

Ba^{2+} Block Kinetics Analysis

When K^{+} (≥ 5 mM) is present in the external solution, Ba^{2+}-block events are easily distinguishable from closure events due to the normal gating of the channel: their respective dwell times differ by more than two orders of magnitude (Vergara and Latorre, 1983; Miller et al., 1987; Neyton and Miller, 1988a). However, in the absence of permeant ions in the external compartment, Ba^{2+} blocks become much shorter-lived and closer to the duration of unblocked channel closures (Neyton and Miller, 1988a). Despite this difficulty, we showed in this previous paper that, under these conditions, it is still possible to measure the rates of Ba^{2+}-block reactions, because in "closed" time histograms Ba^{2+}-block events introduce a slow component which in all cases differs by more than one order of magnitude from the slowest component resulting from closures. The time constant of the Ba^{2+}-dependent slow component, τ_{b}, is related to the Ba^{2+} dissociation rate constant, $k_{off}(Ba)$:

$$k_{off}(Ba) = \tau_{b}^{-1} \tag{1}$$

As shown in Neyton and Miller (1988a), a cutoff time, t_{c}, used to define apparent bursts (periods of gating separated by nonconducting events longer than t_{c}), can be chosen from the double-exponential, nonconducting-time histogram. From the apparent mean burst time, $\tau_{u}(obs)$, and introducing the usual corrections due to missed block events (see, for example, Blatz and Magleby, 1986, Eq. 16), we calculate the true Ba^{2+} association rate constant:

$$[k_{on}(Ba)]^{-1} = \tau_{u}(obs) \exp\left(-t_{c}/\tau_{b}\right) - \tau_{b}\left[1-(1 + t_{c}/\tau_{b}) \exp\left(-t_{c}/\tau_{b}\right)\right] \tag{2}$$

Measurement of the Unblocked Channel Open Probability

The overlap between closures and block events observed in the absence of permeant ions in the external solution complicated not only the analysis of Ba^{2+}-block kinetics but also, and for the same reasons (missed block events included in apparent bursts), the measure of the unblocked channel open probability, P_{o}. Moreover, this difficulty was not limited to experiments where Ba^{2+} was applied, since we also observed slow blocking events *before* adding any Ba^{2+} ion when external solutions contained no permeant ions. As already discussed in a previous paper (Neyton and Miller, 1988a), we think that this "background" block was due mainly to contamination of the internal chamber by Ba^{2+} ions, rather than to a slow Ba^{2+}-like blockade induced by internal Ca^{2+}. Indeed, (a) the time characteristics of these blocking events were indistinguishable from those of Ba^{2+} blocks at all voltage and ionic conditions tested; (b)the frequency of background blocks sharply increased in the days following an experiment performed with high (≥ 1 mM) internal Ba^{2+} concentrations; and (c) background block occurrence was unrelated to Ca_{i}^{2+}, provided that P_{o} stayed above 0.9 (unpublished results). The origin of this Ba^{2+} contamination is still unclear. One possibility is a very slow desorbtion of Ba^{2+} ions from the polyvinyl chloride or plastic walls of the inner chamber.

We used two methods to estimate P_{o} in unblocked channels. In experiments performed to analyze Ba^{2+} block kinetics (in which added internal Ba^{2+} was such that $>75\%$ of the observed long-lasting, nonconducting intervals were actually due to added blocker ions), P_{o} was calculated as follows:

$$P_{o} = T_{o}/[T_{u}(obs) - T_{f}] \tag{3}$$

where T_o and $T_u(obs)$, the sum of open times and observed bursts times, respectively, were directly measured, and T_f, the sum of short blocks falsely counted as closures in observed bursts was calculated by:

$$T_f = \tau_b N_b (\exp (t_c/\tau_b) - 1) - N_b t_c \qquad (4)$$

where N_b was the number of observed blocks (this equation can be obtained by, for example, combining Eqs. 6, 7, and 11 of Blatz and Magleby, 1986).

In experiments performed without added Ba^{2+}, recordings were too short to gather enough background blocks and use the above method for P_o calculation. By visual inspection of the nonconducting-time cumulative histograms in semilogarithmic coordinates, a cutoff time, t'_c, was estimated at the intersection between the slowest component due to background blocks and the rest of the histogram. P_o was then calculated according to:

$$P_o = T_o/(T_o + T'_c) \qquad (5)$$

where T'_c is the sum of closed times shorter than t'_c. We applied this rough method of estimating P_o in Ba^{2+} experiments where both methods could be used successively and the two obtained values never differed by more than 5%.

Control of BK Channel Activation

In the present experiments the level of channel activation had to be varied over a broad range. BK channels are endowed with a double activation mechanism: they are opened by both internal Ca^{2+} increase and membrane depolarization. BK channels from rat skeletal muscle present a rather low sensitivity to Ca_i^{2+} compared with BK channels from other tissues. At +30 mV they are open 50% of the time at internal Ca^{2+} concentrations ~20–200 μM. At all voltages a low activation level of this channel is thus easily obtained by simply decreasing internal Ca^{2+}. The very high activation range, on the other hand, should be explorable using high Ca_i^{2+}. However, it was often difficult, in experiments with no external permeant ions, to raise the apparent P_o above 0.9, due to a complex blockade induced by high internal Ca^{2+} under these conditions. To overcome that problem we used the allosteric activator property of Mg^{2+}, which, when applied in the inner compartment at concentrations >5 mM, raises the affinity of the channel for activating Ca^{2+} ions (Golowasch et al., 1986). Therefore, many experiments reported here were performed with 10 mM internal Mg^{2+}. Under such conditions, the Ca_i^{2+} level required for channel activation is in the 0.5–50-μM range. These free Ca^{2+} concentrations were obtained by adding 0.5 mM EGTA and 0.5 mM HEDTA to the internal solution and varying Ca_i^{2+} between 0.4 and 0.6 mM (final total concentrations).

A second difficulty was occasionally met in the control of the channel activation level. BK channels can show spontaneous "shifts" in open probability which may or may not be reversible on a time scale of few minutes (Moczydlowski and Latorre, 1983b). Such shifts were not compatible with either steady-state analysis of Ba^{2+} block kinetics or with the measure and comparison of BK channel activation curves. In steady-state condition experiments, stationarity of P_o was simply checked visually on both an oscilloscope and a chart recorder. This allowed us to exclude long-lasting shifts in P_o larger than 30%. Moreover, if, in a measurement, an undetected shift in P_o had influenced Ba^{2+}-block kinetics, the block-time as well as the burst-time histograms should seriously deviate from a single exponential distribution. This happened in very few cases and the corresponding measurements were rejected.

In activation curve experiments, P_o stationarity between the beginning and end of a curve determination was systematically controlled. When P_o curves obtained in different ionic conditions had to be compared, we tried to come back to the initial control conditions after each ionic change.

RESULTS

External K^+ Ions Modulate the Interaction between Ba^{2+}-blocking Ions and Channel Gating

Fig. 1 illustrates the observation that initiated this study. It shows single-channel recordings obtained in the presence of Ba^{2+} ions and different Ca^{2+} concentrations in the inner compartment from two BK channels bathed by different external solutions.
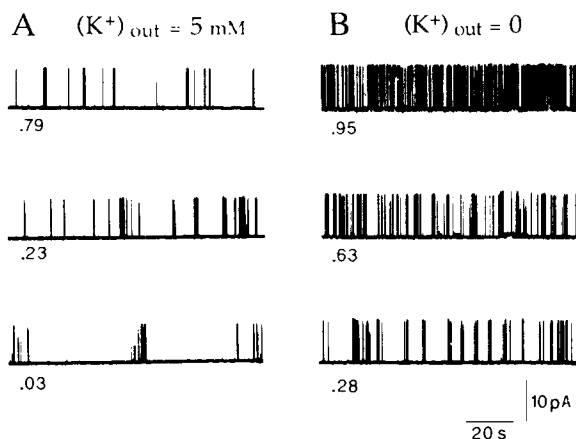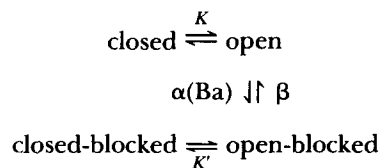
A $(K^+)_{out} = 5$ mM   B $(K^+)_{out} = 0$

.79   .95

.23   .63

.03   .28   10 pA / 20 s

FIGURE 1. Effects of external K^+ on Ba^{2+}-block kinetics at varied channel open probability. Single-channel recordings on the left column (*A*) were obtained in the presence of 5 mM KCl in the external solution and 5 μM $BaCl_2$ in the inner compartment. The recordings shown in the right column (*B*) are from a separate experiment performed with no permeant cation outside and 1 μM $BaCl_2$ in the inner chamber. Other conditions were the same: internal solution contained 150 mM KCl, 10 mM $MgCl_2$, 0.5 mM HEDTA, 0.5 mM EGTA, and 10 mM HEPES, and pH was adjusted to 7.4 with KOH; external solution contained 150 mM NaCl and 10 mM HEPES, and pH was adjusted to 7.4 with NaOH; transmembrane voltage was +30 mV. The level of channel activation was varied by changing the amount of Ca^{2+} added to the inner solution (range 400–500 μM, final total concentration). The numbers under each recording indicate the corresponding open probability of the unblocked channel measured during the bursts. Openings are upward deflections of the current trace. The internal Ba^{2+} concentrations were always higher in the experiments performed in the presence of external permeant ions. This was necessary to prevent the occurrence of too long lasting bursts at low P_o (external permeant ions strongly inhibit the Ba^{2+}-binding rate; Neyton and Miller, 1988a). However, this difference between experiments with or without external permeant is not relevant for the interpretation of the results since we are interested only by variations in the Ba^{2+} dissociation rate which do not depend on Ba_i^{2+} concentration (Vergara and Latorre, 1983; Neyton and Miller, 1988a). In the following figure legends we will indicate changes in the experimental conditions only.

The number below each trace indicates the corresponding open probability of the unblocked channel which was measured during the bursts (see Methods). In Fig. 1 *A* the external chamber contained 150 mM NaCl and 5 mM KCl. Under these conditions, as previously shown (Miller et al., 1987), the mean block times are long (> 3 s) and stay unchanged over a broad P_o range, becoming longer only when P_o is decreased below 0.2. On the contrary, in the absence of external K^+ (Fig. 1 *B*, 150 mM NaCl external solution) mean block times vary with the open probability over the

full P_o range. This novel effect appears superimposed with a shortening of mean block times, induced by removing permeant ions from the external chamber as previously described (Neyton and Miller, 1988a). The shortening of Ba^{2+} blocks was shown to result from a decrease in occupancy of a binding site very selective for permeant ions which is located in the pore, externally to the Ba^{2+}-blocking site, and is therefore called "external lock-in (ELI) site" (the adjective "external" was chosen not to mean that the site was outside of the pore, but rather in reference to the fact that there is another lock-in site located near the inner mouth of the channel).

The effect of removing external permeant ions on the variation of Ba^{2+} dissociation rate with P_o is shown in Fig. 2. In these two experiments, performed under conditions similar to those of Fig. 1, A and B, respectively, P_o was systematically altered by progressively increasing Ca_i^{2+}. As expected from Fig. 1 A, $k_{off}(Ba)$ clearly saturated for $P_o > 0.2$ when K^+ (5 mM) was present in the external solution (Fig. 2 A). On the contrary, in permeant ion–free external solution (Fig. 2 B), $k_{off}(Ba)$ continuously increased with P_o, and the rate of $k_{off}(Ba)$ increase was higher at high P_o values. Continuous theoretical curves fitting the data points in Fig. 2, A and B, were obtained using the model developed by Miller et al. (1987). In this paper, the authors first showed, by analyzing the phenomenon of Ba^{2+} trapping inside the channel, that a Ba^{2+}-blocked channel can still undergo closing and opening transitions and that blocking ions leave the channel only from its open-blocked state. The following scheme was thus proposed:

$$closed \overset{K}{\rightleftharpoons} open$$

$$\alpha(Ba) \updownarrow \beta$$

$$closed\text{-}blocked \underset{K'}{\rightleftharpoons} open\text{-}blocked$$

<div align="center">Scheme A</div>

where α and β are the rate constants for Ba^{2+} association with and dissociation from the open channel, and K and K' are the equilibrium constants for gating of the unblocked and blocked channel, respectively (K = open/closed and K' = [open-blocked]/[closed-blocked]). The authors then showed that, assuming that (*a*) the conformational "gating" reactions are much faster than Ba^{2+}-blocking kinetics, and (*b*) the equilibrium constants for opening in the unblocked and blocked states differ by a constant "stabilization factor" θ, i.e., $K' = \theta K$, $k_{off}(Ba)$ is related to the unblocked channel open probability P_o by the following equation:

$$k_{off}(Ba) = \beta\theta(\theta - 1 + 1/P_o)^{-1} \tag{6}$$

with β being the Ba^{2+} dissociation rate constant from the fully open channel, i.e., at $P_o = 1$. We will see below that the *constant* stabilization factor hypothesis is valid only at constant voltage and at constant concentration of permeant ions in the external solution.

Eq. 6 gave reasonable fits of the data in both the presence (Fig. 2 A) and absence (Fig. 2 B) of external K^+. These fits obtained with a least-squares fitting procedure gave the following estimates for θ: 9.5 ± 2.2 (3) (mean ± SD, n) with 5 mM external K^+ and 0.32 ± 0.03 (4) with K^+-free external solution. According to assumption *b*
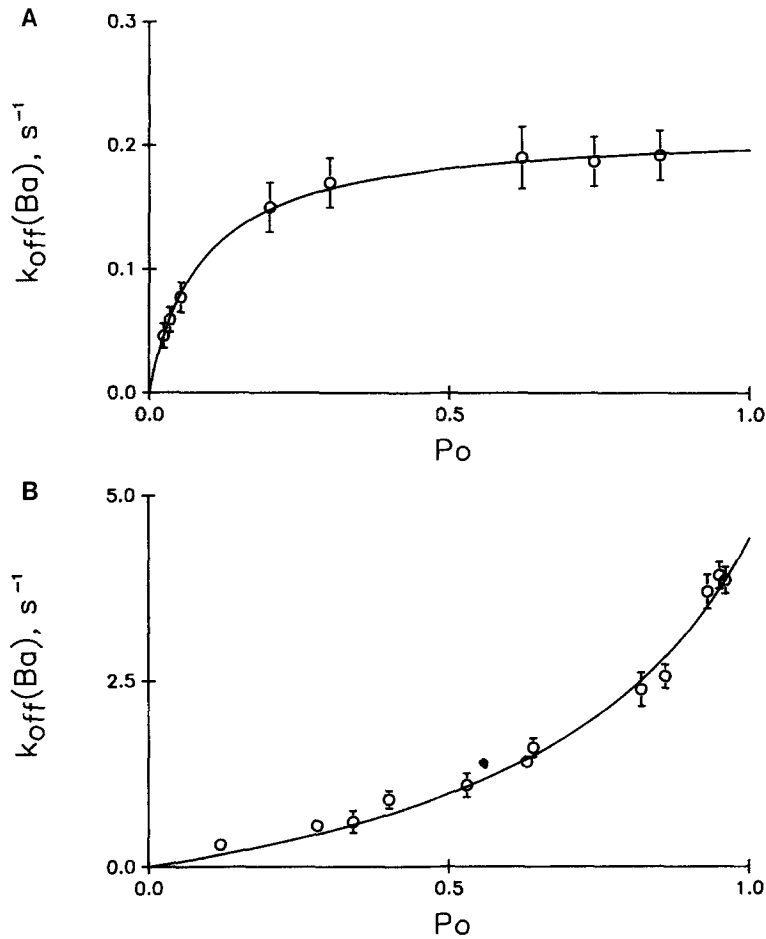
FIGURE 2. Variations of apparent Ba^{2+} dissociation rate with open probability depend on the presence of K^+ in the external solution. $k_{off}(Ba)$ was measured at varied open probabilities either in the presence (A; $(K^+)_{out} = 5$ mM) or the absence (B) of external permeant cations. Ba^{2+} was added at 5 μM in A and 1 μM in B. In both experiments the voltage was +30 mV and 10 mM Mg^{2+} were present in the inner compartment. Open probability was changed by varying the amount of free Ca^{2+} in the inner solution as indicated in Fig. 1. Data were fitted to rectangular hyperbolas (solid line) using Eq. 6 and an automatic least-square fitting program. In A, $\beta = 0.2$ s^{-1}, $\theta = 12.2$. In B, $\beta = 4.4$ s^{-1}, $\theta = 0.29$.

above, a θ value > 1 would mean a Ba^{2+}-induced stabilization of the open state over the unblocked channel, whereas a stabilization of the closed-blocked state would be manifested by a θ value in the range 0–1. Our results, then, strongly suggest that, depending on the presence or absence of K^+ in the external compartment, Ba^{2+} ions blocking the channel induce opposite shifts in the channel gating equilibrium.

The analysis of Ba^{2+} experiments relies on the assumption of a fast blocked channel gating equilibrium compared with β, the rate of unblocking reaction from the open-blocked channel. This requirement was shown to be fullfilled when the

external solution contained permeant ions (Miller et al., 1987). In permeant ion–free external solutions β becomes much faster, but Ba^{2+} blocks introduce a single exponential slow component in nonconducting dwell-time histograms. This was the case at all channel activation levels tested. We thus think that, in the absence of external permeants also, the gating reactions of the blocked channel could be considered to be at equilibrium in the measures of $k_{off}(Ba)$.

Voltage Activation Curve of a Ba^{2+}-blocked Channel in the Absence of External Permeant Ions

By studying the Ba^{2+} trapping in conditional voltage pulse experiments, Miller et al. (1987) were able to show that P'_o, the open probability of the blocked channel, increases with voltage following a sigmoid similar to the unblocked channel probability curve, but shifted, as expected for a stabilization of the open-blocked state, toward negative voltages. The magnitude of the shift (25 mV) was exactly that predicted by using a constant value of 12 for θ.

If, in the absence of external permeant ions, Ba^{2+} were to stabilize the closed-blocked state, a similar analysis should reveal a shift of P'_o vs. P_o curve toward more positive voltages. This is what we tried to show. Two difficulties prevented us from using the experimental approach of Miller et al. under these ionic conditions: (a) the brevity of Ba^{2+} blocks at high P_o and (b) the strong voltage dependence of β, the rate of Ba^{2+} dissociation from the open-blocked channel (see Neyton and Miller, 1988a, and below).

From Scheme A with the above assumption a (that conformational gating reactions are much faster than Ba^{2+}-blocking kinetics of the open channel), we have (Miller et al., 1987, Eq. 4):

$$P'_o = k_{off}(Ba)/\beta \qquad (7)$$

Thus, to be able to compare in a single experiment the two curves, $P_o = f(V)$ and $P'_o = f'(V)$, we first measured β at all tested voltages. This was done by measuring $k_{off}(Ba)$ under a saturating Ca_i^{2+} level. In such conditions, the blocked-channel gating equilibrium is assumed to be fully displaced toward the open state, i.e., $P_o = P'_o = 1$ and $k_{off}(Ba) = \beta$ even at negative voltages. Ca_i^{2+} was then lowered to allow an exploration of the full P_o range by varying the voltage between −50 and +80 mV. $k_{off}(Ba)$ and P_o were measured and P'_o could be calculated for the different potentials using Eq. 7 and β values determined previously in the same experiment. Note that, to be valid, such a protocol requires that β be independent of Ca_i^{2+}. This is questionable because the binding of Ca^{2+} involved in channel activation may affect Ba^{2+} dissociation from the open-blocked channel. We could not directly test this requirement. However, the fact that, in the presence of 5 mM external K^+, the rate of Ba^{2+} dissociation is constant over a wide range of internal Ca^{2+}, including Ca_i^{2+} levels which do not saturate channel activation (see Fig. 2 A), suggests that β is effectively independent of Ca_i^{2+}.

Figs. 3 and 4 illustrate the two-step experiment proposed above. Fig. 3 shows the variation of β with V. Under the saturating activation conditions, the voltage dependence of $k_{off}(Ba)$ (e-fold change for ~25 mV at V higher than +30 mV) slightly decreases at low voltages. This may be due to the fact that when the voltage is
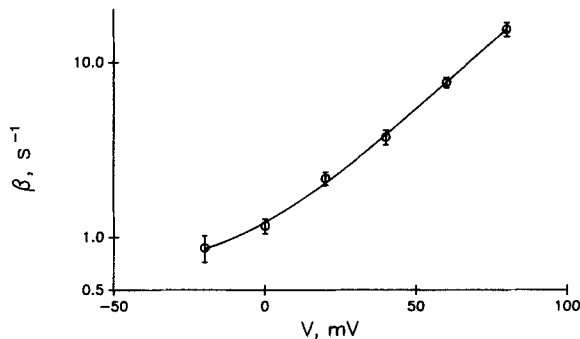
FIGURE 3. Ba^{2+} dissociation rate from an open-blocked channel vs. transmembrane voltage in the absence of external permeant cations. In this experiment 10 mM Mg^{2+} and 200 μM Ca^{2+} were added in the inner chamber to keep the channel open over the full voltage range studied ($P_o \geq 0.95$). $Ba_i^{2+} = 1$ μM. The solid line has no theoretical meaning.

decreased, occurrence of Ba^{2+} dissociation toward the inner compartment increases (see Neyton and Miller, 1988a). The final result of the experiment is shown in Fig. 4. As we expected, the probability–voltage curve for the blocked channel appears displaced toward the right relative to that for the unblocked channel. However, the effects of Ba^{2+} on the channel's voltage activation curves were themselves found to depend on the transmembrane voltage in a complicated way. They were systematically observed in three experiments where the half-activation voltage of the unblocked channel was above +40 mV, but were absent in two experiments performed with an unblocked channel half-activation voltage around 0 mV. Nevertheless, the Ba^{2+}-induced displacement of the voltage activation curve observed at depolarized
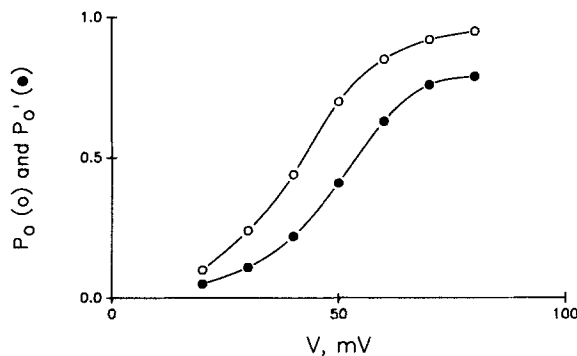

FIGURE 4. In the absence of external permeant cation, Ba^{2+} displaces the open probability–voltage curve toward more depolarized potentials. Same channel as in Fig. 3. In this experiment a constant low level of channel activation was achieved having a mixture of 10 mM Mg^{2+}, 0.5 mM HEDTA, 0.5 mM EGTA, and 440 μM Ca^{2+} present in the internal compartment. Ba^{2+} block kinetics were analyzed under steady-state conditions at different voltages. The open probability of the unblocked channel, P_o (open circles), could be directly measured from these records. The open probability of the blocked channel, P'_o (filled circles), was then calculated using Eq. 7 and the values of $k_{off}(Ba)$ and β measured here and in the previous part of the experiment shown in Fig. 3, respectively. We did not attempt to fit the data with Boltzmann distributions as the effect of Ba^{2+} on the channel activation curve appeared not to consist in a simple shift along the voltage axis. Indeed, in this particular experiment, P'_o values obtained at high voltages (+70 and +80 mV) were much lower than those expected in the case of a parallel shift of the P'_o vs. the P_o curve. This is probably not due to a lower level of saturation of the P'_o curve, since such an effect was not observed in other similar experiments. The solid lines connecting the data points thus have no theoretical meaning.

potential clearly indicates, as already suggested by the variation of $k_{off}(Ba)$ with P_o, that Ba^{2+}-blocking ions shift the channel gating equilibrium toward the closed state when permeant ions are excluded from the external solution.

The Modulation of Channel Gating by Ba^{2+}-blocking Ions Is Voltage Dependent

Miller et al. (1987) reported that, in the presence of K^+ in the external compartment, the stabilization factor θ did not depend on transmembrane voltage. The complexity of the Ba^{2+}-induced effects on the probability–voltage curve reported above suggested that the voltage independence of θ may not hold under zero external permeant conditions. This is effectively shown in Fig. 5. In this series of experiments, θ was measured as in Fig. 2, in different external ionic conditions and at varied voltages. θ appears to be only slightly voltage dependent when 5 mM KCl is present in the external solution (*e*-fold change for ~ 90 mV). In contrast, a marked voltage dependence (*e*-fold change for 30–35 mV) characterizes θ measured in the absence of
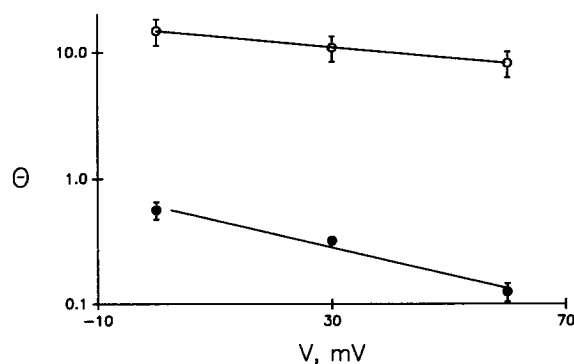
FIGURE 5. The effects of Ba^{2+} on the channel gating equilibrium become voltage dependent when permeant ions are excluded from the external solution. θ, the "stabilization factor" calculated as in Fig. 2, is plotted against voltage. In the presence of 5 mM external K^+, θ was only slightly voltage dependent (*e*-fold change for $\simeq 90$ mV; *open circles*). In zero external permeant solutions (*filled circles*), θ was significantly decreased by depolarization (*e*-fold change for $\simeq 30$ mV). Note that the y axis is logarithmic. Each point represents the mean of at least three separate experiments. Error bars are standard deviations.

external permeant ions (150 mM NaCl external solution). Such a voltage dependence of θ probably explains why an effect of Ba^{2+} on probability–voltage curves was barely detected in the experiments performed with an unblocked channel half-activation voltage around 0 mV: at low voltages, θ is close to 1 and thus the activation curves of the blocked and unblocked channels become very similar. A voltage-dependent θ should also induce a "bend" of the probability–voltage curve of the Ba^{2+}-blocked channel (P'_o) toward the voltage axis, an effect that may be present in Fig. 4, but which was more clearly observed in other similar experiments (not shown).

Thus, θ, the ratio between the gating equilibrium constants of Ba^{2+}-blocked and unblocked channels is not independent of experimental conditions. It clearly depends on the ionic composition of the external solution and on transmembrane voltage in the absence of external permeant ions. However, the good fit of the data obtained in Fig. 2 B using Eq. 6 suggests that, at a fixed external solution composition and constant voltage, θ does not depend on the channel's activation

level. We thus used Eq. 6, based on the *constant* stabilization factor hypothesis, in these restricted conditions only.

Stabilization of the Open Ba^{2+}-blocked Channel by Different External Monovalent Cations

So far, we have analyzed the variation of Ba^{2+} dissociation rate with channel open probability in only two sets of external ionic conditions, 150 mM NaCl and 150 mM NaCl + 5 mM KCl, respectively. In an attempt to quantitate the effects of external K^+ on θ, we explored two other external K^+ concentrations, 1 and 150 mM. In the latter case, NaCl was omitted from the external chamber to keep the ionic strength of the external solution constant. Fig. 6*A* shows the variation of the inverse of θ with external K^+ concentration (such a plot was chosen to facilitate comparison with previous work; see below). This graph indicates that the change in the nature of the Ba^{2+}-induced stabilization (closed-blocked vs. open-blocked state) takes place at very low (< 1 mM) external K^+ concentrations.

This result was reminiscent of the Ba^{2+} lock-in phenomenon induced by external K^+ (see Neyton and Miller, 1988*a*). In that case, external K^+ at low concentration was shown to inhibit Ba^{2+} dissociation from the channel by binding to a site located in the
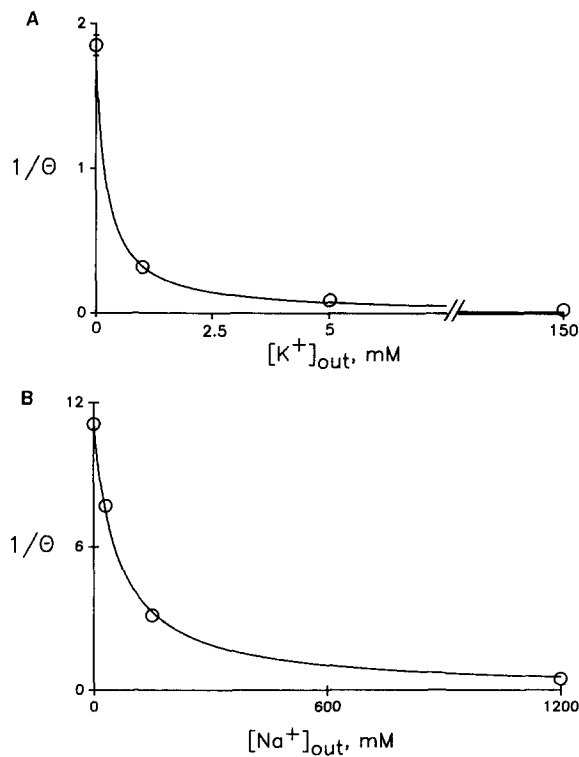

FIGURE 6. The composition of the external solution interferes with the Ba^{2+}-induced effects on channel gating. (*A*) Variation of $1/\theta$ with external K^+ concentration. Each point is the mean of at least three independent determinations. Data were fitted to a rectangular hyperbola consistent with the decrease in $1/\theta$ due to the saturation of a K^+-binding site having an apparent K_d of 0.21 mM. Experimental conditions: +30 mV, 150 mM NaCl external solution, except for the experiments with 150 mM K^+. (*B*) Same plot obtained with increasing external Na^+ concentration. Each point was obtained in a single experiment (except at 150 mM, four experiments). In the experiments with 0 or 30 mM Na^+, the external solution also contained 150 mM NMDG. The fit of the data is consistent with an external binding site having an apparent K_d of 62 mM for Na^+.

pore on the Ba^{2+} exit pathway. The Ba^{2+} dissociation inhibition by external K^+ followed a classical Michaelis inhibition curve from which the apparent affinity of the external lock-in site (ELI) for K^+ could be measured. The apparent K_d for K^+ was found to be in the range 200–300 μM (150 mM NaCl external solution).

The data points in Fig. 6 *A* are reasonably well fitted by a Michaelis rectangular hyperbola (continuous line) with a half-inhibition K^+ concentration of 215 μM. This result indicated the existence of an intimate relation between the amplitude of the stabilization factor θ and the degree of occupancy of the ELI site. Such a relation was confirmed by other external substitution experiments. As shown in Fig. 6 *B*, θ was measured in external solutions containing varying Na^+ concentrations but no permeant ions. The variation of $1/\theta$ with $(Na^+)_{out}$ again follows a rectangular hyperbola, but in this case the half-inhibition concentration is ~ 60 mM. This is in the same range as the apparent affinity of the ELI site for Na^+ measured under the same conditions (apparent $K_d \sim 30$ mM; see Neyton and Miller, 1988a). θ was also measured in the presence of 5 mM Rb^+ in the external compartment. Rb^+ ions permeate BK channels (Blatz and Magleby, 1984; Yellen, 1984a; Eisenman et al., 1986) and were shown to bind to the ELI site even more avidly than K^+ (apparent $K_d \sim 100$ μM in 150 mM NaCl external solution; see Neyton and Miller, 1988a). As expected from a degree of ELI site occupancy slightly higher in 5 mM Rb^+ than in 5 mM K^+, we obtained θ values higher in Rb^+ experiments (15.1 $\pm$ 1.6 [3]) than in K^+ ones (10.9 $\pm$ 2.5 [3]) otherwise performed under similar conditions (+30 mV, no Mg_i^{2+}).

Stabilization of the Open State in a Channel Occupied by Two Ba^{2+} Ions

As indicated by the voltage dependence of the dissociation rate under certain sets of internal/external ionic conditions, Ba^{2+} ions actually permeate BK channels (Neyton and Miller, 1988a, b, and above). Like all monovalent permeant ions, external Ba^{2+} is able to exert lock-in effects on Ba^{2+} ions blocking the channel from the internal solution. This is shown in Fig. 7. In this experiment the initial external solution contained no permeant ions (150 mM NaCl external solution). Ba^{2+} (2 μM) was added to the 150 mM KCl internal solution, and Ba^{2+} block kinetics was analyzed. Fast rate constants were measured for Ba^{2+} dissociation (4.2 s^{-1}) and association (8 × 10^7 M^{-1} s^{-1}). $BaCl_2$ was then progressively added in the *external* chamber, and Ba^{2+}-block kinetics were analyzed at different external Ba^{2+} concentrations. Fig. 7 shows that the variation of Ba^{2+} dissociation rate with external Ba^{2+} concentration follows a classical Michaelis inhibition curve. Thus, external Ba^{2+}, by presumably binding to the ELI site, is able to inhibit Ba^{2+} dissociation from the blocking site, as do monovalent permeants.

How, in these experiments, can we restrict the effects of external Ba^{2+} to an occupancy of the ELI site since Ba^{2+} is also known to block BK channels from the external compartment? This conclusion was based on two observations indicating that the Ba^{2+} ions that blocked the channel under our experimental conditions were coming mainly from the internal compartment: first, the blocking rate was increased by depolarization, and second, Ba^{2+} association to the blocking site was inhibited by external Ba^{2+} (results not shown). From a series of three similar experiments we found the apparent K_d of the ELI site for Ba^{2+} to be 340 $\pm$ 40 μM.
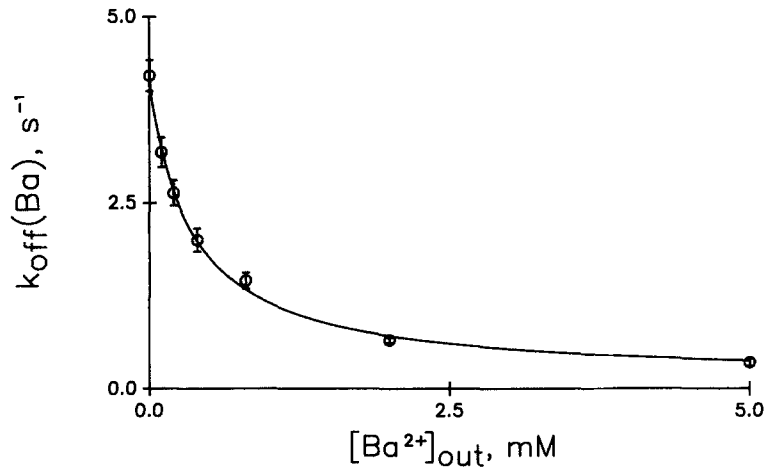
FIGURE 7. Ba^{2+} lock-in by external Ba^{2+} ions. This plot shows the inhibition of Ba^{2+} dissociation rate by external Ba^{2+}. The Ba^{2+}-blocking ions were mainly coming from the inner solution which contained 2 µM $BaCl_2$ (see text). The fit to a rectangular hyperbola indicates that external Ba^{2+} binds to the ELI site with an apparent K_d of 0.35 mM (see Neyton and Miller, 1988a). Experimental conditions: +50 mV, 150 mM NaCl external solution. Open probability was kept high throughout the experiment with 200 µM internal Ca^{2+}.

The phenomenon of Ba^{2+} lock-in by external Ba^{2+} demonstrates that BK channels can be simultaneously occupied by two Ba^{2+} ions. We were curious to measure θ, the Ba^{2+}-induced stabilization factor, in a doubly divalent occupied channel. Fig. 8 shows the variation of $k_{off}(Ba)$ with P_o in the presence of 10 mM $BaCl_2$ in the external solution. From this and another similar experiment, θ was found to be 4.2 ± 0.2. This result shows that, despite the presence of a divalent cation bound near the external end of the pore, the blocked-open conformation is stabilized.
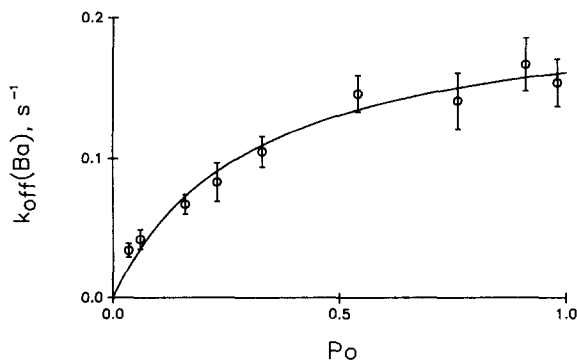

FIGURE 8. Stabilization of the open state in a doubly Ba^{2+}-occupied channel. We measured the variation of the apparent Ba^{2+} dissociation rate with channel open probability in the presence of Ba^{2+} in both the inner (5–50 µM) and the external (10 mM) compartments. The superlinearity of the curve indicates, as in Fig. 2 A, a stabilization of the open-blocked state (θ = 4.3). Experimental conditions: +30 mV, 150 mM NaCl external solution.

Block by Internal Na⁺ in the Absence of External Permeant Ions

The experiments with doubly divalent occupied channels showed that the electrical charge of the ion binding the ELI site had no major impact on the channel gating modulation induced by Ba^{2+}-blocking ions. To test if the charge of the blocking ion itself was important in this phenomenon, we decided to analyze the effects on gating of a monovalent blocking ion, Na^+. This cation, when applied at a rather high concentration (≥ 10 mM) in the internal compartment, is known to induce a "flickery" block of the BK channel (Marty, 1983; Yellen, 1984a). Because of the high voltage dependence of this blocking phenomenon, it was proposed that Na^+ blocks the channel by entering deeply inside the pore (Yellen, 1984b) like Ba^{2+}-blocking ions do. Our guess was that during a Na^+-blocking event (time scale of several tens of microseconds; Yellen, 1984a, b), as in a Ba^{2+}-blocked channel, the ELI site will be at equilibrium with the external solution (we were assuming that external K^+ binds to
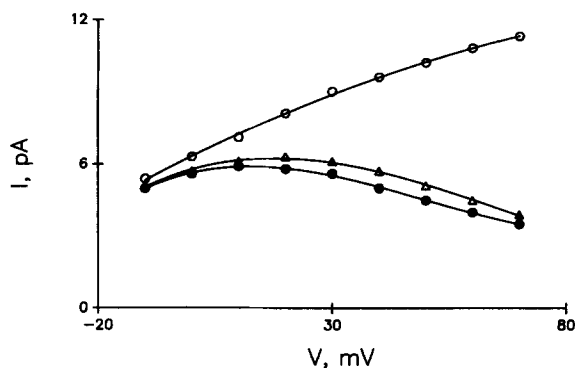

FIGURE 9. Block of a BK channel by internal Na^+. *I–V* curves of a single BK channel measured in the absence of external permeant cations before adding internal Na^+ (*open circles*), in the presence of the blocker (30 mM internal Na^+, *filled circles*), and in the presence of the blocker in the internal solution and 1 mM K^+ in the external solution (*open triangles*). Under initial conditions, the *I–V* curve showed unusual slight inward rectification and small unitary conductance, which both resulted from the presence of 10 mM Mg^{2+} in the inner compartment. The strongly voltage-dependent block induced by internal Na^+ was almost unaffected by the low external K^+ concentration. External solution contained 150 mM NaCl. The solid lines through data points have no theoretical meaning.

and leaves the ELI site on the conduction time scale of nanoseconds to microseconds). Thus, changes in the activation level of the Na^+-blocked channel should be observed depending on the presence of permeant ions in the external solution.

In the experiment shown in Figs. 9 and 10, a BK channel was incorporated in the bilayer in the absence of permeant ions in the external compartment. We measured, under these conditions (150 mM NaCl external solution), both an *I-V* curve and a voltage–activation curve. We then added, initially, 30 mM NaCl (final concentration) in the *inner* chamber and then, for an additional observation, 1 mM KCl in the *outer* compartment. In the two situations, both *I-V* and P_o–voltage curves were measured again. Due to the fast time scale of Na^+-blocking reactions, changes induced by Na^+ in the amplitude of the single-channel current measure the time-averaged amount of Na^+ blockade (see Marty, 1983; Yellen, 1984a).

Fig. 9 shows the *I-V* curves obtained under the three ionic conditions. The strong voltage-dependent block induced above +20 mV by internal Na^+ was practically unaffected by the low external K^+ concentration tested in that experiment. Fig. 10 shows the corresponding P_o–voltatge curves. In the absence of external permeant ions, internal Na^+ provoked a marked shift of the activation curve toward more depolarized potentials. In two series of experiments performed either with or without 10 mM Mg_i^{2+}, the shifts were +15.7 ± 0.7 mV (3) and +15.3 ± 2.1 mV (5), respectively. In all these experiments, the half-activation voltage under control conditions was between +20 and +50 mV, and a complete recovery of the Na^+ effects could be obtained by perfusing the inner chamber with the control solution.
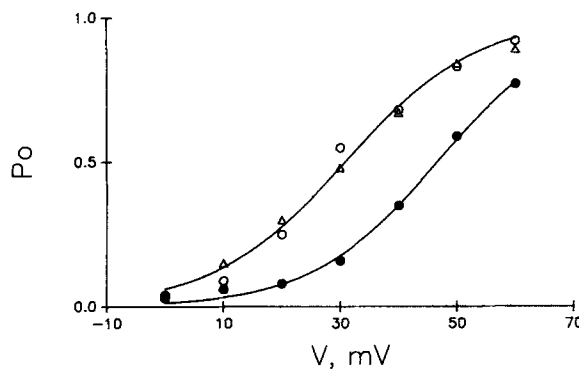

FIGURE 10. Effects of internal Na^+ on open probability–voltage curves. Same experiment as in Fig. 9. Steady-state records were obtained at varied voltages under the three sets of experimental conditions described in Fig. 9. Open probabilities directly measured from these records are plotted against voltage. In the absence of external permeants, internal Na^+ (30 mM; *filled circles*) induced a shift of the activation curve along the voltage axis toward more depolarized potential (+15 mV). 1 mM external K^+ completely reversed the Na^+-induced effects (*open triangles:* 30 mM internal Na^+ and 1 mM external K^+; *open circles:* initial conditions, no internal Na^+ and no external K^+). Data points were fitted to Boltzmann distributions using the following equation (see Moczydlowski and Latorre, 1983):

$$P_o = [1 + \exp[-zF(V - V_0)/RT]]^{-1} \qquad (8)$$

The parameters were: $z = 2.2$ and $V_o = 30.8$ mV for open circles and triangles; $z = 2.3$ and $V_o = 46.6$ mV for the filled circles.

Fig. 10 also shows that addition of 1 mM KCl in the external compartment entirely reversed the effects of internal Na^+ on the voltage–activation curve. A similar reversal was observed in two other experiments, one with 1 mM and the other with 5 mM external KCl. Moreover, we observed no effects of internal Na^+ on the channel activation level in two other experiments performed in the presence of 5 mM KCl in the outer solution. The differential modulation by low external K^+ concentration of the two effects of internal Na^+, decrease in P_o and decrease in apparent single-channel conductance, was particularly interesting. Indeed, it was important for the interpretation of the effect of Na^+ on P_o to exclude the possibility of an artefact in the measure of P_o in the presence of internal Na^+. Some of the Na^+-block events may have been long enough to be falsely counted as closures, thus contaminating P_o measurements. But the reversal by external K^+ of the P_o curve shift induced by Na_i^+

with, simultaneously, almost no change in the amount of Na^+ block, as indicated by the *I-V* curves, provides an excellent control showing that such an error cannot account for the Na^+-induced P_o change.

Another objection could be raised against our interpretation of the Na^+ experiments. How do we know that Na^+-blocked channels undergo gating? If Na^+ binding were to prevent channel closure, we should observe an increase in apparent mean open times, no change in mean closed times, and, as a consequence, an increase in apparent P_o. Since, in fact, a decrease in apparent P_o was observed, it appears that Na^+-bound channels can close.

We next compared gating kinetics of Na^+-blocked and unblocked channels recorded in permeant ion–free external solution (Fig. 11). With the filtering conditions used in these experiments, three major components could usually be distinguished on cumulative closed-time histograms. The slowest one (not visible in Fig. 11) was due to blockade presumably by contaminating Ba^{2+} ions and was excluded from the analysis of gating kinetics (see Materials and Methods). The two other components were fitted by the sum of two exponentials. Open times were usually distributed monoexponentially. In the experiment shown in Fig. 11, Na_i^+ induced a marked increase (up to near 300% of control value) in both time constants of closed-time distribution (compare Fig. 11 *C* with Fig. 11, *A* and *E*), with practically no change in their respective amplitudes. The time constant of the open-time histogram was only slightly decreased (down to near 70% of control value; compare Fig. 11 *D* with Fig. 11, *B* and *F*).

These kinetic data first confirm that Na^+-blocked channels do actually gate, since Na_i^+ affects closed times and does not lengthen, but rather shortens, open times. However, the apparent simplicity of the effects of Na_i^+ on dwell-time histograms was surprising. Indeed, due to the fast kinetics of blocking/unblocking reactions compared with the gating kinetics that can be measured under our recording conditions, dwell-time histograms obtained in the presence of the blocker correspond to a mixture of events arising from both blocked and unblocked channels. Nevertheless, a slow Na^+ blockade as the origin of the Na^+-induced slowing of the closed-time histogram slow component can be reasonably excluded: in the presence of Na_i^+ this slow component was becoming faster with depolarization (not shown), as is expected for an opening process (the opposite should be observed in the case of an unblocking reaction if the blocker has to dissociate back to the inner chamber, which is generally agreed for Na^+; Marty, 1983; Yellen, 1984*a*, *b*).

The kinetic data shown in Fig. 11 indicate that the decrease in P_o induced by internal Na^+ results mainly from a slowing of opening kinetics. Similar results were observed in a series of eight experiments: at +40 mV, Na^+-induced changes in mean dwell times (% of control) were 240 ± 70% and 77 ± 11% for closed times and open times, respectively.

In conclusion, Na^+-block experiments show that a monovalent (Na^+), as well as a divalent (Ba^{2+}) ion blocking the BK channel induces a shift of the gating equilibrium toward the closed-blocked state when permeant ions are excluded from the external compartment.
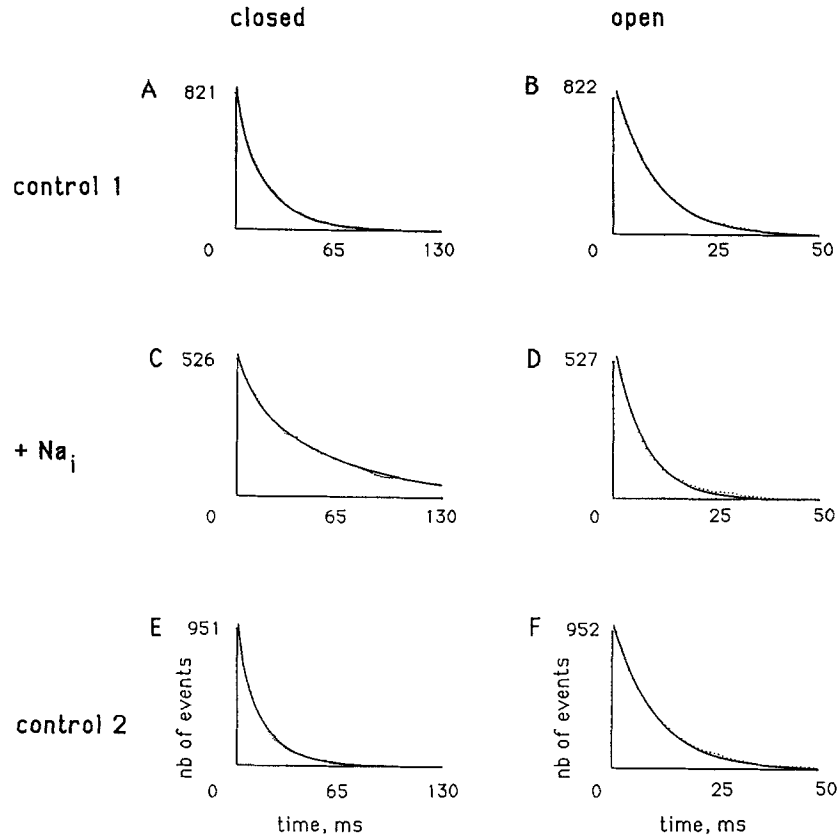
closed   open

FIGURE 11.   In the absence of external permeant cations, internal Na⁺ affects the gating kinetics of the BK channel. Cumulative dwell-time histograms obtained from a single BK channel bathed successively by a control solution (*A* and *B*), a 30 mM Na⁺-containing solution (*C* and *D*), and again a control internal solution (*E* and *F*). The control internal solution contained 100 μM Ca^{2+} as divalent only, the external solution contained 150 mM NaCl and the voltage was +40 mV. Records were filtered at 500 Hz before acquisition (1-ms sampling interval). The closed-time histograms (*A*, *C*, and *E*) were fitted by two exponentials, whereas only one exponential seemed to satisfactorily fit open-time distributions (*B*, *D*, and *F*). The time constants and their respective amplitudes were 3.1 ms, 23% and 20.4 ms, 77% in *A*; 9.8 ms in *B*; 10.4 ms, 26% and 61.3 ms, 74% in *C*; 7.4 ms in *D*; 3.2 ms, 27% and 17.6 ms, 73% in *E*; 10.6 ms in *F*. In this experiment P_o was 0.37 in control 1, 0.15 in the presence of Na_i^+ and 0.43 in control 2; mean closed times were 15 ± 1, 42 ± 3, and 12 ± 1 ms, respectively, and mean open times were 9.4 ± 0.3, 7.4 ± 0.4, and 10.0 ± 0.4 ms, respectively (mean ± SE).

Open Probability of Unblocked Channels Is Unaffected by Low External K⁺

The results described so far favor a mechanism of gating modulation controlled by the occupancy of the ELI site. We looked for changes in the channel activation level of unblocked channels by just introducing permeant ions in the external solution. In a series of seven experiments performed without added blocking ions, low concentrations (1–10 mM, final concentrations) of KCl were added in the external chamber.

We observed no significant shift in probability–voltage curves (1.5 ± 2.0 mV, mean ± SD).

DISCUSSION

In this article we demonstrate that multiple ion occupancy in the permeation pathway of BK channels is involved in the control of channel gating. This conclusion was reached by comparing the gating properties of Ba^{2+}- or Na^+-blocked channels with those of unblocked channels. We show that, in a blocked channel, the gating equilibrium is displaced toward the closed state if permeant ions are carefully excluded from the solution bathing the extracellular face of the channel. Admission of permeant ions in the external compartment at low millimolar concentration either reverses (Na^+-blocked channel) or even inverts (Ba^{2+}-blocked channel) blocker effects on gating.

External Permeant Ions, by Binding inside the Permeation Pathway, Profoundly Affect the Gating Properties of Ba^{2+}-blocked Channels

Following the work of Miller et al. (1987), we took advantage of the fact that Ba^{2+} ions get trapped in blocked-closed channels to study the gating equilibrium in Ba^{2+}-blocked channels. We first showed that the variations of the apparent Ba^{2+} dissociation rate with the channel activation level differ strikingly depending on the presence or absence of permeant cations in the external solution (Fig. 2). $k_{off}(Ba)$ follows a saturating rectangular hyperbolic function of P_o in K^+-containing external solutions, and this behavior was shown to result from a Ba^{2+}-induced stabilization of the open-blocked state over the unblocked channel (Miller et al., 1987). On the contrary, in permeant ion–free external solutions, $k_{off}(Ba)$ sharply increases when P_o is raised in the range 0.8–1.0. This rapid variation of $k_{off}(Ba)$ at high P_o suggested that Ba^{2+} was stabilizing the closed-blocked state under these external ionic conditions. This hypothesis was confirmed by analyzing the variations of $k_{off}(Ba)$ with voltage in the absence of external permeant ions. This indeed showed a Ba^{2+}-induced displacement of the voltage–activation curve along the voltage axis toward more depolarized potentials (Fig. 4).

We used the "stabilization factor" hypothesis of Miller et al. (1987) to quantitatively characterize the effects of Ba^{2+} on the gating equilibrium. This hypothesis was found to allow a satisfying description of the variation of $k_{off}(Ba)$ with the open probability of the unblocked channel (see Fig.2), provided that voltage and external solution composition are kept constant. At +30 mV and in the presence of 5 mM external K^+, Ba^{2+} was found to stabilize the open state in the blocked channel gating equilibrium by 1.3 kcal/mol (θ = 9.5) over the unblocked channel, whereas with no permeant ion in the external solution, the closed-blocked state was stabilized by 1.3 kcal/mol (θ = 0.11, 150 mM NMDG external solution). Since low external K^+ does not affect the gating equilibrium of the unblocked channel, we can thus conclude that the addition of 5 mM K^+ to an ideal external solution containing only perfectly inert cations will induce a displacement of at least 2.6 kcal/mol of the Ba^{2+}-blocked channel gating equilibrium toward the open state.

We then showed that external K^+ as well as external Na^+ ions do modulate the

effects of Ba^{2+} on the gating in a way closely related to their potency to inhibit Ba^{2+} dissociation from the blocked channel (Fig.6). This inhibition phenomenon has been interpreted as resulting from the binding of monovalent cations coming from the outer compartment to a site located near the external mouth of the channel, called the ELI site (Neyton and Miller, 1988a). We observed an excellent correlation between the affinities of K^+ and Na^+ for the ELI site on one hand, and the concentration dependence of the effect of these two ion species on the gating of the blocked channel on the other hand. These results clearly indicate that it is the combination of both the presence of a Ba^{2+}-blocking ion inside the pore *and* variations in the level of occupancy of the ELI site which are responsible for the observed shifts in the channel gating equilibrium. This conclusion is supported by the results of other external substitution experiments with Rb^+ and Ba^{2+} ions, which both bind to the ELI site with affinities higher and lower than K^+, respectively, and which affect the Ba^{2+}-blocked channel gating equilibrium in the same order of potency.

Thus, Ba^{2+} alone, by its presence in the pore, does not induce one-way effects on BK channel gating. Simple models where the blocker will prevent either channel closure by the so called "foot in the door" mechanism (Armstrong, 1975; Neher and Steinbach, 1978), or channel opening (case for Ba^{2+} in the squid delayed rectifier; Armstrong and Taylor, 1980; Armstrong et al., 1982) do not account for the complex interactions between Ba^{2+} and BK channel gating. Since both the polarity and the amplitude of the gating modulation of Ba^{2+}- blocked BK channels were shown, in this study, to be closely related to the binding of external cations to the ELI site, we propose that occupancy of the ELI site is the effector of the gating modulation described in this paper. In this model, Ba^{2+}-blocking ions will be involved in the gating modulation just as a tool used to induce changes in the ELI site occupancy level. This proposal may sound unreasonable in the view of the absence of effect of low external K^+ on the unblocked channel's gating equilibrium (see last paragraph in Results). We will return later to this apparent paradox.

An additional argument for a major role of ELI site occupancy in the modulation of BK channel gating comes from the Na^+-block experiments. In the absence of permanent ions in the external solution, internal Na^+ ions were found to induce, in addition to a fast blockade, a shift in the channel gating equilibrium which was qualitatively similar to the effects on gating observed in Ba^{2+} experiments (Fig. 10). Like those of Ba^{2+}, the effects of Na_i^+ on gating were found to be relieved by external K^+ concentrations as low as 1 mM (Fig. 10). Note that the interpretation of these experiments does not rely on approximations of a particular kinetic model, as in the case of the Ba^{2+}-block analysis. In Figs. 9–11 directly measurable quantities are plotted. Thus, not only do the basic effects of occupancy of the ELI site (or at least of a K^+ binding site accessible from the external compartment) on the gating process occur when Na^+ is the blocker, but, in addition, the mechanistic conclusions can be dissociated from the approximations in the model used for the analysis of the Ba^{2+} effects. A Na^+-induced decrease in the activation level of BK channels at very low external permeant ion concentrations had been observed by Marty (1983) on BK channels from bovine chromaffin cells. In that work, however, Na^+ effects on gating were still observed at an external K^+ concentration ($\simeq 3$ mM) that abolishes Na^+ effects in our hands. We have no explanation to account for these discrepancies.

How May Occupancy of the ELI Site Alter BK Channel Gating Equilibrium?

Effects of external permeant ions on K^+ channel gating have been known for a long time (Hagiwara et al., 1976; Dubois and Bergman, 1977; Hagiwara and Yoshii, 1979; Århem, 1980; Hestrin, 1981; Swenson and Armstrong, 1981). In delayed rectifier K^+ channels, external K^+ or Rb^+ keep channels open longer (Århem, 1980; Swenson and Armstrong, 1981; Cahalan and Pappone, 1983; Cahalan et al., 1985; Matteson and Swenson, 1986). A simple model accounting for such an effect had been proposed earlier in the case of ACh-activated channels, which are similarly stabilized in their open state by permeant ions (Van Helden et al., 1977; Ascher et al., 1978; Marchais and Marty, 1979; Gage and Van Helden, 1979; but see also Adams et al., 1982). In this model, as with the "foot in the door" mechanism postulated for the effects of certain blockers cited above, the binding of a permeant ion inside the pore was supposed to prevent channel closure. As a consequence, closure, and thus open dwell-time distribution, should be affected by permeants, whereas the opening reaction (and the closed-time distribution) should not. This simple model is certainly not correct in the case of BK channels, as it fails to explain the effects of Na_i^+ on gating (see Fig. 11).

Another model, based on a closed-state stabilization by external divalents (Gilly and Armstrong, 1982) and the relief of this effect by competing external monovalents (Armstrong and Matteson, 1986) had been proposed to explain external permeant effects on delayed rectifier K^+ channel gating. This second model, which attributes a fundamental role to external divalent ions in channel closure, is not relevant to the case of the BK channel: in doubly Ba^{2+}-occupied channels, where one of the Ba^{2+} ions is bound near the external mouth of the channel, the open state was stabilized; moreover, we never saw any effect on BK channel gating of the total removal of Ca^{2+} from the external solution with EGTA (unpublished results).

The kinetic analysis of Na^+-blocked channel gating equilibrium indicates that the stabilization of the Na^+-blocked close state observed in the absence of external permeant ions results mainly from a decrease in opening rate with a much weaker increase in closing rate (see Fig. 11). Since there is no effect of Na_i^+ on gating in the presence of permeant ions in the external compartment, we can conclude that external permeant ions strongly facilitate opening of the Na^+-blocked channel without greatly affecting its closure. Binding of a permeant ion in the outer part of the BK channel thus appears to exert effects on channel gating that are the opposite of those induced by external divalents on the squid K^+ channel. We speculatively propose that, in BK channel, the opening of the channel may involve the movement of a positively charged group away from the vicinity of the ELI site, as shown in Fig. 12. The presence of a cation on the ELI site in a closed channel will markedly destabilize the closed state (speeding up opening), whereas the same cation bound in an open channel may have much less influence on the closing rate if, in the limiting step of this reaction, the positive moving group is far enough from the ELI site (compare energy diagrams *A* and *B* in Fig. 12).

In this model, the energy level of the ELI site in a closed channel is expected to be raised, compared with an open channel, by the amount by which occupancy of the ELI site destabilizes the closed state (at least 2.6 kcal/mol; see above). However, in
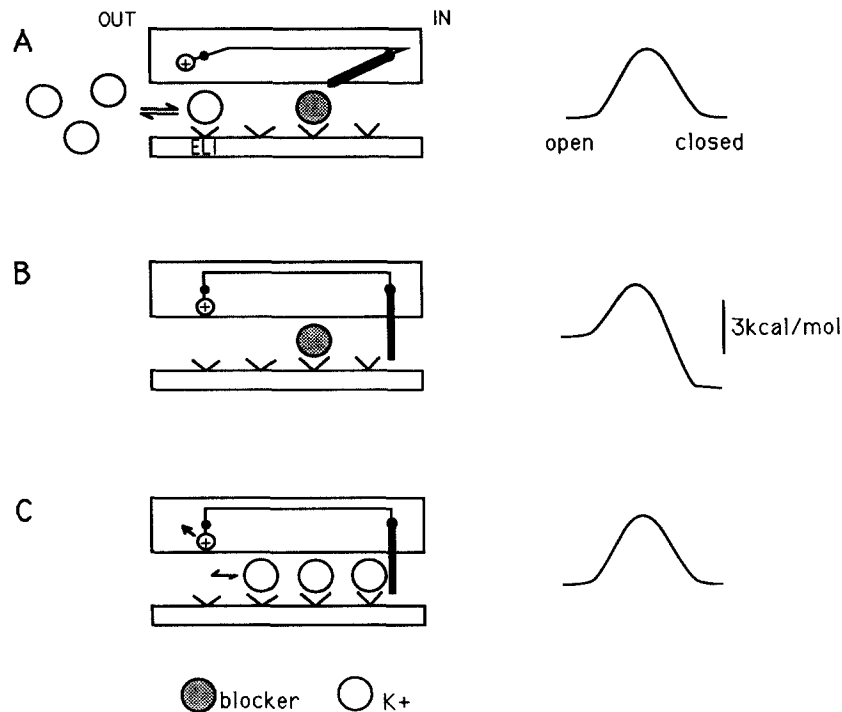
FIGURE 12. Model for the link between ELI site occupancy and BK channel gating. As in Neyton and Miller (1988b), the channel has four cation binding sites. In the external part of the pore wall, there is a positive charge near the ELI site in the closed channel which moves away (in this scheme, toward the external chamber) from this site when the channel opens. A physical gate coupled to this positive charge is sketched near the inner mouth of the channel (*thick bar*). Occupancy of the ELI site by a cation destabilizes the closed state by electrostatic interaction. In a blocked channel and in the presence of external permeant ions (A), the ELI site is occupied most of the time. On the right is drawn the energy profile of the gating reactions under experimental conditions (V, Ca_i^{2+}) giving a blocked channel P_o of 0.5 (the height of the energy barrier has been arbitrarily chosen). In a blocked channel, the ELI site is vacated when permeant ions are excluded from the external chamber (B), and the closed-block state is stabilized over the blocked-open state. In a closed-unblocked channel (C), the K^+ ions trapped in the closed channel can move back and forth on the different sites. This markedly decreases the apparent affinity of the ELI site for external cations and maintains a high occupancy of ELI site, even in the absence of external permeant ions.

view of the astonishingly high affinity of the ELI site for permeant ions in Ba^{2+}-blocked channels (apparent K_d's in the 5–20-μM range when measured in 150 mM NMDG external solution; see Neyton and Miller, 1988a), such a rise in the energy level may not prevent access of external cations to this site in the closed state. Thus, the positive charge whose movement away from the ELI site is postulated to be part of the opening mechanism may not necessarily constitute, in a closed channel, a physical gate by itself. This speculation would help to explain how, mechanistically, external K^+ does facilitate opening of Na^+-blocked channels.

The present model is certainly not unique. Among others, the following mechanism may be proposed: ELI site occupancy may force Ba^{2+}- as well as Na^+-blocking ions to stay in a position closer to the inner mouth of the channel. This, in turn, could change the occupancy of a more internal site, which would ultimately control channel gating. This latter proposal, which receives support from recent experiments on external Cs^+ block performed under varied voltage and internal ionic conditions (Demo and Yellen, 1990), is not exclusive of the model developed here.

External Permeant Ions and the Gating of the Unblocked Channel

Addition of low (< 10 mM) K^+ concentrations to 150 mM NaCl external solution affects neither the gating equilibrium nor the gating kinetics of the unblocked channel. This observation may appear at first sight to contradict a gating mechanism controlled, among other factors, by occupancy of the ELI site, especially if, as we suggested above, the ELI site remains accessible to ions in the external solution when the channel is closed. Below, we propose a speculative explanation that reconciles these results with our ELI site occupancy model. The explanation relies on the fact that, when the channel closes, K^+ ions become trapped inside the pore. This statement is supported by the phenomenon of Ba^{2+} trapping in closed channels. It would be difficult to explain how a channel conformation that can hold a divalent in a very stable way could also be stable with no positive charge in it. Moreover, the open channel can simultaneously bind two Ba^{2+} ions with high affinity (apparent $K_d < 500$ μM; see Fig. 7) together with a K^+ ion (bound on an internal lock-in site under our experimental conditions; see Neyton and Miller, 1988b). Such a high density of positive changes in a pore estimated to be 3.5 nm long (Villarroel et al., 1988; Alcayaga et al., 1989) strongly suggests the presence of fixed negative charges or a high density of electrical dipoles on the pore wall. For energetic reasons, these fixed charges or dipoles require the presence of counter-ions inside the pore independent of whether the state is open or closed. Note that, in our view, the trapped K^+ ions cannot exit the closed channel due to the depth of the energy wells rather than to the closure of physical gates (high energy barriers), although we do not exclude the possibility that such a gate may be present at the inner end of the channel. We further speculate that electrostatic repulsion will keep the trapped ions maximally spaced in the pore. The K^+-binding sites near both ends of the channel may thus be occupied most of the time in an unblocked, closed channel. This will significantly decrease the availability of the ELI site for external cations, and thus its apparent affinity, compared with the case of a Ba^{2+}- or Na^+-blocked channel. The absence of effect of low external K^+ on the gating of unblocked channels thus does not necessarily invalidate our conclusion that BK channel gating is controlled by ELI site occupancy. Rather, it may simply result from a persistent high level of ELI site occupancy in the unblocked channel, regardless of whether the channel state is open or closed.

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