Effects of Barium on the Potassium Conductance of Squid Axon

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ABSTRACT Ba\textsuperscript{++} ion blocks K\textsuperscript{+} conductance at concentrations in the nanomolar range. This blockage is time and voltage dependent. From the time dependence it is possible to determine the forward and reverse rate constants for what appears to be an essentially first-order process of Ba\textsuperscript{++} interaction. The voltage dependence of the rate constants and the dissociation constants place the site of interaction near the middle of the membrane field. Comparison of the efficacy of Ba\textsuperscript{++} block at various internal K\textsuperscript{+} concentrations suggests that Ba\textsuperscript{++} is probably a simple competitive inhibitor of K\textsuperscript{+} interaction with the K\textsuperscript{+} conductance. The character of Ba\textsuperscript{++} block in high external K\textsuperscript{+} solutions suggests that Ba\textsuperscript{++} ion may be “knocked-off” the site by inward movement of external K\textsuperscript{+}. Examination of the effects of other divalent cations suggests that the channel may have a closed state with a divalent cation inside the channel. The relative blockage at different temperatures implies a strong interaction between Ba\textsuperscript{++} and the K\textsuperscript{+} conductance.

INTRODUCTION

The potassium conductances of several preparations can be blocked by organic and inorganic ions. The properties of the block suggest features of the structure of the potassium channels. Thus, tetraethylammonium (TEA) ions block the delayed rectifier of squid axon only when applied from the inside (Tasaki and Hagiwara, 1957; Armstrong and Binstock, 1965). In contrast, TEA blocks the potassium conductance of amphibian node of Ranvier from either side, the block from the inside resembling that for the squid (Armstrong and Hille, 1972). The dose-response relationship suggests that a single TEA molecule interacts with a single potassium channel (Armstrong, 1966). Longer chain analogs of TEA block with an exponential time-course. Because the TEA analogs do not appear to alter the development of the potassium activation, Armstrong (1971) suggests that these reagents interact with open potassium channels. That the TEA analogs interact with the conductance pathway and not the gating mechanism is also suggested by the observation that external potassium decreases the effective blockage (Armstrong, 1969). The rate constants calculated from the steady state block \((k_1/k_1 + k_{-1})\) and the time constant for the block \((1/k_1 + k_{-1})\) are both concentration and voltage
dependent. The voltage dependence may imply that the binding site is within the membrane field. Alternatively it may imply that the probability of finding the blocking ion in the channel is decreased by potassium ion entering the channel from the outside, with the potassium entry being voltage dependent. This latter mechanism is known as a "knock-off" model (Armstrong, 1975). The block by TEA and its analogs can be rationalized by a model where TEA and the hydrated potassium ion can interact with a superficial site on the inside. Only potassium ion can permeate the channel since only it can be dehydrated and so "fit" through the remainder of the channel. Inorganic ions can interact with potassium conductance as well (Armstrong and Bezanilla, 1972; French and Wells, 1977; Adelman and French, 1978). Internal cesium, lithium, and sodium can block the potassium conductance in a voltage-dependent manner, the block first increasing with depolarization, and then at large depolarizations the block actually decreases. These results suggest that the potassium channel may have at least two sites.

Barium ion is known to interact with the potassium conductances of several preparations (Werman and Grundfest, 1961; Hagiwara et al., 1974, 1978; Eaton and Brodwick, 1976; Stanfield and Standen, 1978; Taylor and Armstrong, 1978; Hermann and Gorman, 1979). In this paper we report the effects of internal barium and other alkaline earth cations on the delayed rectifier of squid axon. We find that barium blocks potassium channels with a one-to-one stoichiometry. The block is voltage and time dependent with an apparent dissociation constant between 10^{-8} and 10^{-9} M. The block can be removed by hyperpolarization especially in the presence of high external potassium. Strontium produces a similar effect with less potency except there appears to be a slower component of decreased blockage. Some of this material has been previously reported (Eaton and Brodwick, 1978).

METHODS

Living specimens of *Loligo pealei* were supplied by The Marine Biology Laboratory, Woods Hole, Mass. The axons (300–800 μm in diameter) were dissected and fine-cleaned to remove most of the adherent nerve fibers. Branches of the giant axon were kept as long as possible (~200 μm) to help prevent leakage. Axons were then transferred to a Plexiglas chamber with a volume of 0.5 ml. Axons were laid horizontally in the chamber and across posts on either side. The air gaps separating the post from the chamber proper were ~2 mm. Two incisions were made on each side of the axon in the vicinity of the post. An empty cannula was advanced from the right side through the slit into the axoplasm as far as the left side slit. The cannula was then retracted and a 0.5 M KF solution of perfusate containing 1 mg/ml Pronase (grade B, Calbiochem-Behring Corp., San Diego, Calif.) was left behind. When the cannula reached the right side, the axon was occasionally ligated to the cannula to prevent leakage. The Pronase solution was then perfused at ~50 μl/min for 3 min to remove the axoplasm. A small amount of Phenol red was added to visually monitor the quality of the perfusion. After the axoplasm was removed the axon was perfused with 0.5 M KF perfusate to wash out the Pronase. Through the left side slit an axial wire “piggyback” electrode (Fishman, 1970) was advanced into the length of the axon. The potential monitoring electrode consisted of a long capillary pulled from standard microelectrode glass. The capillary portion was ~75 μm in diameter and 1.8
cm long. A 25 μm platinized platinum wire was inserted to the tip inside the microelectrode and was fixed with wax to the wide portion of the electrode. The electrode was later filled with 0.5 M KCl. The floating wire served to lower the impedance of the electrode (Fishman, 1970). On top of the capillary another platinized-platinum wire, 75–125 μm (depending on axon diameter) was fixed with de Khotinsky cement. Bath potential was monitored with a sintered Ag-AgCl electrode. To measure current we used two sets of electrodes, one on either side of the axon with the following configuration. A central plate of platinized-platinum foil 4 mm wide was attached to a block of Plexiglas. On either side of the central plate, two similar guard plates were attached to the same block. Membrane current was measured in a virtual ground circuit. Only the current from the central electrode was monitored while the lateral guard electrodes were used to minimize current errors arising from lateral regions of the axon not under adequate voltage control. The control amplifier was an Analog Devices, Inc., model 48K (Norwood, Mass.). Fully tuned the clamp produced voltage steps with a rise time of better than 1 μs (to 90% of the voltage step magnitude). The resistance in series with the membrane was measured with current pulse whose rise time was ~0.5 μs. This series resistance was compensated with a feedback arrangement in the voltage control amplifier (see Hodgkin et al., 1952 a).

Current and voltage data were recorded on a digital oscilloscope (Nicolet Instrument Corp., model 1070A, Madison, Wis.) and then transferred to a digital tape recorder (Kennedy Co., Altadena, Calif., model 1090). The records on tape could then be displayed via a digital computer (DEC PDP 11/70, Digital Equipment Corp., Marlboro, Mass.). Leakage and capacitive currents were subtracted from the current responses by determining the leakage and capacitance currents for hyperpolarizing steps and then assuming a linear correlation between voltage and the leakage and capacitance currents.

Solutions

The axons were externally perfused with either filtered seawater or an artificial seawater containing 450 mM NaCl, 10 mM KCl, 50 mM CaCl₂, and 10 mM Tris or HEPES buffer. External pH was adjusted to between 8.0 and 8.3. To block sodium currents 10⁻⁷ M tetrodotoxin (Sigma Chemical Co., St. Louis, Mo.) was added to the external perfusate. Because fluoride, the internal anion preferred by squid axon (Tasaki et al., 1965; Adelman et al., 1966), complexes with divalent cations used in this study, we decided to use chloride as the internal anion. We found that the K⁺ currents in axons internally perfused with 0.5 M KCl were reduced to values only two to three times leakage levels after periods of 30–90 min. However, if the internal osmotic strength is increased from 950–1,000 mosM to 1,800 mosM with sucrose and the KCl concentration reduced from 500 to 100 mM, the axons lasted for considerably longer times. The increase of internal osmotic strength did not, itself, alter the ionic currents.

Many of the barium and strontium ion concentrations used in this study require buffering. To achieve this buffering we used mixtures of the divalent ions and ethylene-diamine tetraacetic acid (EDTA). EDTA-barium and EDTA-strontium stability constants in 0.1 M KCl are 1.74 × 10⁻⁸ M and 2.34 × 10⁻⁹ M respectively (Martell and Calvin, 1952). Buffer systems assuming one-to-one stoichiometry were made according to Eq. 1:

\[
A = \frac{C + X + \frac{1}{K} - \left[ \left( C + X + \frac{1}{K} \right)^2 - 4XC \right]^{1/2}}{2},
\]

(1)
where $A$ is the concentration of BaEDTA, $X$ is the original concentration of Ba added, $C$ is the original concentration of EDTA added, and $K$ is the dissociation constant. The free divalent cation can be calculated from Eq. 2:

$$B = X - A,$$

where $B$ is the concentration of free divalent ion. Eq. 3 gives the amount of barium required to produce a given free concentration of barium:

$$X = \frac{B + K (BC + B)^2}{1 + BK}.$$  

The internal pH was maintained at 7.3 with 10 or 20 mM HEPES. The preparation was cooled to within $0.2^\circ C$ of the desired experimental temperature with a Lauda circulating cooler (Lauda Div., Brinkmann Instruments, Inc., Westbury, N.Y.). The holding potential was $-60$ mV except where otherwise specified. Current-voltage families were generated with an interval of $\sim 3$ s between pulses. About 3-5 min were used between solution changes to guarantee complete exchange of internal perfusion solutions.

RESULTS

Internal Ba$^{++}$ Blocks K$^+$ Currents

When Ba$^{++}$ ion is added to the internal perfusate containing 100 mM KCl so that the final concentration is in the range 0.1-50 nM, we observe a time-dependent blockage of K$^+$ currents. In Fig. 1 A, three current-time families are illustrated at successively higher barium concentrations. Note that the currents generated at higher potentials in the presence of barium actually cross over the lower. The rate of rise of the currents seems little affected at these barium concentrations. A gradual decrease of maximal potassium conductance may represent the effects of internal chloride perfusion or a much slower component of the barium block. The rapidity and the final steady-state magnitude of this blockage is a direct function of the internal Ba$^{++}$ concentration. That the blockage is also a function of potential can be seen by examining the current-voltage relationships for the currents at the end of a 100-ms voltage step (Fig. 1 B). For low concentrations of Ba$^{++}$ ($<2 \times 10^{-9}$ M), the blockage at potentials more negative than $+50$ mV is not significant ($<10$ percent). However, at more positive potentials, the block may be very large ($>70$ percent at the highest potentials tested). At higher concentrations of Ba$^{++}$, the blockage is significant at all potentials tested but again shows a marked increase for potentials more positive than $+50$ mV. These results are those that we have reported earlier (Eaton and Brodwick, 1978) and are similar to those reported by Taylor and Armstrong (1978).

The form of the blockage suggests an interaction between Ba$^{++}$ and some site in the K$^+$ channel which can be represented by the reaction sequence

$$\text{SITE} + n\text{Ba}^{++} \xrightleftharpoons{k_f}{k_{-1}} \text{SITE-Ba}_n,$$  

where $n$ is the number of barium ions reacting with the site, $k_f$ is the forward rate constant, and $k_{-1}$ is the reverse rate constant.
To estimate the order of the reaction, we calculated time constants from the slope of semilog plots and plotted the reciprocal time constant vs. the barium concentration. If the reaction of Ba$^{++}$ with a site in the K$^+$ channel is first order, the relationship should be linear with a slope equal to the forward rate constant ($k_1$ in Eq. 4) and the intercept equal to the reverse rate constant ($k_{-1}$ in Eq. 4).

![Figure 1](image-url)  
**Figure 1.** The effect of Ba$^+$ on K$^+$ currents. The top frame of A shows the normal K$^+$ current responses to voltage steps in increments of 10 mV from the holding potential of -60 mV. The internal potassium concentration is 100 mM KCl. The Na$^+$ currents are blocked by 10$^{-7}$ M tetrodotoxin and the records are corrected for leakage. In each of the lower two frames, the conditions are the same except that 5 x 10$^{-9}$ M and 10$^{-8}$ M Ba$^{++}$ has been added to the internal perfusate in the middle and bottom frames, respectively. In these records, the Ba$^{++}$ produces a time-dependent block that is particularly noticeable at the more depolarized potentials. (B) Effect of Ba$^{++}$ on the current-voltage relationships for late potassium currents. The response of the current at the end of a 100-ms voltage step was used to generate the current-voltage relationships shown. The control curve labeled 0 Ba (○) represents the current-voltage relationship with 100 mM KCl inside and artificial seawater with 10$^{-7}$ M tetrodotoxin outside the axon. The other relationships are obtained under the same conditions except that varying amounts of Ba$^{++}$ were added to the internal perfusate.

Fig. 2 shows data collected at 7°C for steps to +60 mV with the best fit linear regression line superimposed (regression coefficient = 0.976). This line gives a value for $k_1$ of 3.3 x 10$^9$ M$^{-1}$·s$^{-1}$ and for $k_{-1}$, a value of 3.5 s$^{-1}$. From the ratio of the forward and reverse rate constants, we can calculate a dissociation constant for the reaction at equilibrium of 1.06 x 10$^{-9}$ M$^{-1}$. 
After having determined the order of the reaction, we can obtain another estimate of the equilibrium constant from the dose-response curve. At any given potential, the steady-state blockage may be determined as a function of Ba\(^{++}\) concentration. Such a dose-response curve is shown in Fig. 3 for voltage steps to +60 mV. The solid curve is the best fit to a single site model using a nonlinear least square curve-fitting routine (Brown and Dennis, 1972). The dissociation constant, \(K_D\), calculated from this relationship is \(6.9 \times 10^{-9} \text{ M}^{-1}\). This value compares favorably with that obtained from the ratio of the forward and reverse rate constants.

The value of the dissociation constant varies with potential. If the site of the reaction was at a location where it was sensitive to the whole membrane field, then one would expect that, for a divalent cation, there would be an \(e\)-fold change in the dissociation constant for every 13-mV change in potential (Hille, 1975b). The amount of potential change necessary to produce an \(e\)-fold change will be increased if the site sees only some fraction of the membrane field. Therefore, the potential dependence of the dissociation constant can be used to estimate the position of the Ba\(^{++}\) interaction within the total membrane field. When the apparent association constants are plotted vs. the membrane potential as in Fig. 4 A, one finds that an \(e\)-fold change in \(K_D\), requires a 27 mV change in voltage. At 7\(^\circ\)C, this implies that divalent Ba\(^{++}\) at the blocking site must be sensitive to ~50 percent of the membrane field. We can obtain an additional estimate of the location of the site in the membrane field by examining the voltage dependence of the forward rate

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{The reciprocal time constant for Ba\(^{++}\) block vs. the internal Ba\(^{++}\) concentration. The reciprocal of the time constant for the development of the block induced by Ba\(^{++}\) (see Fig. 1) is plotted against the free Ba\(^{++}\) concentration in the internal perfusion solution (100 mM KCl). The time constant is obtained from the current response to a voltage step to +60 mV.}
\end{figure}
constant. In Fig. 4 B the pseudo-first-order forward rate constant is plotted vs. potential with the least-squares regression line drawn through the points. For an e-fold change in the rate constant, the potential changes 43 mV. Again for a divalent cation moving in the membrane field this implies a sensitivity to $\sim$56 percent of the transmembrane potential.

To obtain an estimate of the reverse rate constant, a stimulus of sufficient duration to saturate the response was delivered as a conditioning stimulus. This was followed at varying times by test stimuli to the same potential as the conditioning stimuli. The recovery of the test response from its depressed level (80% control) to the original level is shown in Fig. 5 for a barium concentration of $5 \times 10^{-9}$ M and a temperature of 10°C. The best fit regression line for the data gives a time constant of 0.71 s which implies a reverse rate constant of 1.4 s$^{-1}$. Considering the difference in temperature of the two experiments, this value is in good agreement with the results obtained from the data of Fig. 3.

**Instantaneous Current-Voltage Relationship**

To determine if there are any rapid components of the "off" process, the instantaneous current-voltage relationship was determined from responses to voltage steps of various magnitudes immediately after a conditioning voltage step to $+100$ mV. The conditioning step was of sufficient duration to allow complete development of the Ba$^{++}$ block. Comparison of the tails in the control and the Ba$^{++}$-blocked case shows that there are no time-dependent components of the current that can be attributed to Ba$^{++}$. Fig. 6 shows the
current-voltage relationship. Both the control (with 100 mM KCl internally and 10 mM externally) and the Ba\(^{++}\)-blocked axon (with the same KCl concentrations plus \(10^{-8}\) M Ba\(^{++}\) added to the internal perfusate) have linear instantaneous \(I\)/\(V\) relationships, but the control case has approximately four times the conductance of the Ba\(^{++}\) blocked axon. Under these conditions no unusual features of the tails are apparent. If "knock-off" (Armstrong, 1975) of the Ba\(^{++}\) is being induced by entry of external K\(^+\), the rate is significantly slower than the normal turn-off kinetics of the potassium channel because the current tails in both the control fiber and the Ba\(^{++}\)-blocked fiber have similar time constants. This observation is consistent with the time constant for the off-response being of the order of seconds.

Figure 4. The voltage dependence of the Ba\(^{++}\) dissociation constant and the forward rate constant. In A, the dissociation constant obtained from plots similar to that shown in Fig. 3 is plotted vs. the potential of the stimulus associated with that \(K_{Ba}\). The line drawn through the points is the least-squares linear regression line. For an \(e\)-fold change of \(K_{Ba}\), there is a 27 mV change in potential. In part B the pseudo-first-order forward rate constant (obtained from plots similar to that shown in Fig. 2 at a variety of potentials and a concentration \(5 \times 10^{-9}\) M Ba) is plotted vs. potential. The least-squares linear regression line drawn through the points has a slope of 43 mV for an \(e\)-fold change in the rate constant.
Effects of Ba$^{++}$ in High External K$^+$

To discriminate between current- and voltage-dependent effects and also to examine the possibility that inward movement of K$^+$ could enhance the removal of Ba$^{++}$ from its blocking position in the channel, we examined the effects of high external K$^+$ concentrations. Fig. 7, in which the tail currents at various potentials after a conditioning step to +100 mV are depicted, and Fig. 8, which shows the conductance-voltage relationship for single voltage steps, demonstrate that there is some blockage of conductance for inward current in the presence of 10$^{-8}$ M Ba when 460 mM K$^+$ is in the external solution. The voltage at which the block of conductance for outward currents becomes substantial is similar to the potential at which block occurs in normal external K$^+$. This suggests that at least in part the Ba$^{++}$ block is voltage dependent. But even though there appears to be a voltage-dependent component to the block, there must also be current-dependent components as well, because examination of the inward tail currents shows a significant “hook” in the presence of Ba$^{++}$ (cf. Yeh and Narahashi, 1977); that is, the initial inward current starts at a low level, rapidly increases, and then decreases with a time-course that is approximately exponential (see Fig. 7). This result implies that, although internal Ba$^{++}$ may block inward K$^+$ currents to some
extent and outward $K^+$ currents substantially, there is a substantial knock-off of $Ba^{++}$ ions from conductance sites by external $K^+$ (Armstrong, 1975). The removal of $Ba^{++}$ ions from the channel follows an exponential time-course. When the reciprocal of the time constant for this process is plotted vs. potential, there is very little voltage dependence at very negative potentials with increasing voltage dependence at more positive potentials (Fig. 9). Whether or not the large change in the reciprocal time constant is a true voltage-dependence of the reverse rate constant is problematic. Two alternative explanations are: (a) the time constant also contains contributions from the forward rate constant. At high potentials, these contributions may be substantial. (b) Since the voltage dependence of the reverse rate constant in normal $K^+$ was relatively linear and small compared to the steep voltage dependence observed here, the apparent voltage dependence may be due to a current-dependent effect. At very negative potentials, knock-off of $Ba^{++}$ by external $K^+$ may be significant thus producing a large off rate constant for $Ba^{++}$. As the inward driving force for the current is reduced, the knock-off effects become less important with a concomitant reduction in reverse reaction rate. A comparison of the off rate-constant under these conditions for large negative potentials with that for normal external $K^+$ suggests that the increase
in external $K^+$ by a factor of 46 times increases the off rate-constant by close to 2.5 orders of magnitude. Examination of the instantaneous current-voltage relationship under high $K^+$ conditions shows a change in the $I-V$ relationship that is similar to that observed in normal external $K^+$ (Fig. 7).

**$K^+-Ba^{++}$ Competition**

Our experimental data suggested that for constant external $K^+$, the membrane potassium conductance saturates as the internal $K^+$ concentration is increased.

![Graph showing effect of Ba++ on tail currents in high K+](image)

**Figure 7.** Effect of Ba++ on tail currents in high K+. When axons are bathed in an external solution containing 460 mM K+, the tail currents in Ba++ display a substantial time-dependent component. The conditioning step was to +100 mV and the tail currents were in response to voltage steps immediately afterward in 10-mV increments from −100 to +100 mV.

For sequential reactions that display saturation, double reciprocal plots are often useful in providing information about the system (Ainsworth, 1977).

To determine that there was significant competition between $K^+$ and Ba++, axons were perfused with 50, 100, and 200 mM KCl at a constant Ba++ concentration of $10^{-8}$ M. In Fig. 10 the reciprocal of the conductance at the end of a 100 ms pulse to +80 mV is plotted vs. the reciprocal of the $K^+$ concentration. The difference in the y-axis intercepts of the two lines is not
significantly different suggesting that Ba\(^{++}\) is a simple competitive inhibitor. This idea is also lent support by the large difference in the x-axis intercepts of the lines. From Fig. 11 we can also gain some additional information about the equilibrium blockage of Ba\(^{++}\) ion. The two lines in Fig. 10 may be described by the relationship (White et al., 1959; Ainsworth, 1977; Dubois and Bergman, 1977).

\[
\frac{1}{G_K} = \frac{K_K}{G_{max}} \left( 1 + \frac{[Ba^{++}]}{K_{Ba}} \right) \frac{1}{[K^+] + \frac{1}{G_{max}}}
\]  

(5)

where \(G\) is the conductance through the potassium channels, \(K_K\) is the equilibrium concentration of K\(^+\) that produces half-maximal current, \(K_{Ba}\) is Ba\(^{++}\) concentration at which half of the K\(^+\) channels are blocked, and \(G_{max}\) is

![Figure 8. Effect of Ba\(^{++}\) on K\(^+\) conductance in high external K\(^+\). The chord conductance for an axon bathed in 460 mM KCl externally and 100 mM KCl internally is plotted (○). The conductance under the same conditions but with 10\(^{-8}\) M Ba\(^{++}\) added to the internal perfusate is represented by (●).](image)

Effects of Temperature on Ba\(^{++}\) Blockage

We hoped to gain some information about the characteristics of the energy barriers and interaction sites by examination of the temperature dependence of the Ba\(^{++}\) blockage. We varied the temperature between 6 and 16°C and monitored the currents produced by steps to several voltages in both the Ba\(^{++}\)-
Reciprocal time constant for removal of Ba\(^{++}\) from the K\(^+\) conductance pathway in the presence of high external K\(^+\). The time constant at different potentials for removal of Ba\(^{++}\) from the K\(^+\) channel was determined from the slope of a semilogarithmic plot of the tail currents during the “hook” (Fig. 10). Internal Ba\(^{++}\) concentration was 10\(^{-8}\) M with 100 mM KCl internally and 460 mM KCl externally.

Double reciprocal plot of Ba\(^{++}\) interaction with the K\(^+\) conductance. The reciprocal of the conductance produced by a voltage step to +80 mV is plotted vs. the reciprocal of the internal K\(^+\) concentrations for three different concentrations (50, 100, and 200 mM) in the presence and absence of 10\(^{-8}\) M internal Ba\(^{++}\). The difference in intercept on the vertical axis is probably not significant while the difference in the intercept on the horizontal axis is highly significant suggesting that Ba\(^{++}\) is a competitive inhibitor of the interaction of K\(^+\) with the K\(^+\) conductance mechanism.
blocked state and the control. Fig. 11 shows the superimposed response of a single axon treated internally with 10^{-8} M Ba^{++} to voltage steps of +30, +60, and +80 mV at 6 and 16°C. The Ba^{++} blockage at the higher temperature is significantly more rapid and steady-state blockage is more complete. The equilibrium block can be determined from steady-state levels of current at the end of long voltage steps and the rate constants can be determined from the time-course of the block and the value of the steady-state response as described previously. When these calculations are made the equilibrium blockade for a step to +60 mV has a Q_{10} of 2.2 whereas the Q_{10} for the forward rate constant is 8.5. Even the large Q_{10} for the forward rate constant is not sufficient to explain the acceleration in the onset of block that is observed in Fig. 11 when the axon is warmed. Appreciable block has occurred in the warm axon before much current has even been activated. But addition of Ba^{++} in either the warm or cold does not significantly change the initial rate of development of K^{+} current from that observed in the absence of Ba^{++}. This implies that, at least, one mode of Ba^{++} blockade is the interaction with open K^{+} channels.

**Effect of Internal BaF_{2} and External Ba^{++}**

Because of the relatively high solubility product of BaF_{2} and the relative potency of Ba^{++} blockage, we thought that solutions containing F^{-} might
allow enough Ba to remain as an uncomplexed ion to produce significant blockages. A simple calculation supports this view. The solubility product of BaF$_2$ at 9.5°C is $1.6 \times 10^{-6}$ M$^2$. Even at fluoride concentrations of 500 mM, this implies free Ba concentrations of $6.4 \times 10^{-6}$ M which should, if present in the ionized form, be more than sufficient to produce blockage. When an axon was perfused with a 500 mM KF solution saturated with Ba$^{++}$, there was significant blockage but much less than one would anticipate if all the Ba were in the ionized form. The blockage appeared similar to buffered Ba$^{++}$ concentrations between $5 \times 10^{-9}$ and $10^{-8}$ M. This suggests that a vast majority of the Ba in solution is present as dissolved BaF$_2$ or BaF$^-$ complexes.

Also, we examined the effect on the K$^+$ conductance of externally applied Ba$^{++}$. For external Ba$^{++}$ concentrations greater than 100 mM, a slight time- and voltage-dependent blockage of the K$^+$ conductance was present. The blockage was approximately equal to $10^{-8}$ M Ba$^{++}$ applied internally. The blockage was only evident in intact axons and was not observed in perfused fibers. Consequently, we have tentatively concluded that the blockage may represent Ba$^{++}$ entry into the fiber via divalent permeability pathways (Baker et al., 1971; Meves and Vogel, 1973), and that the site of action of externally applied Ba$^{++}$ may actually be from the inside of the membrane. These results are somewhat at variance with the findings of Taylor and Armstrong (1978), who felt that under conditions of repetitive activity external Ba$^{++}$ was an effective blocker of K$^+$ conductance.

**Effects of Other Divalent Cations**

Besides Ba$^{++}$, Mg$^{++}$, Ca$^{++}$, and Sr$^{++}$ were also tested for their ability to block K$^+$ conductance. Ca$^{++}$ and Mg$^{++}$ had little if any effect below internal concentrations of 1 mM. Sr$^{++}$ was a more effective blocker but not as potent as Ba$^{++}$. In Fig. 12, the dose-response curves for the blockage of Ba$^{++}$, Sr$^{++}$, and Mg$^{++}$ are compared. The dissociation constant for Ba$^{++}$ that produces

![Figure 12](image-url)
the theoretical curve in the figure is $7 \times 10^{-9}$ M, for Sr$^{++}$ $10^{-6}$ M, and for
Mg$^{++}$ $10^{-3}$ M. The dose-response curve for Ca$^{++}$ was similar to that for Mg$^{++}$.
The Ca$^{++}$ and Mg$^{++}$ blocks were only observable at relatively high potentials
($>100$ mV) and at high concentrations. For Sr$^{++}$ the block was qualitatively
similar in some ways to the Ba$^{++}$ blockage of K$^{+}$ currents. In the case of both
Sr$^{++}$ and Ba$^{++}$, examination of the current-voltage relationship after perfusion
with the divalent shows close to normal conductance until large positive
potentials are reached, when there is a sharp reduction in conductance for all
more positive potentials. The difference between the blockage by the various
divalent ions was apparent on examination of the current-time records. The
block of K$^{+}$ by Mg$^{++}$ and Ca$^{++}$ showed no noticeable time dependence while
that by Sr$^{++}$ was very unusual in time-course. For certain Sr$^{++}$ concentrations
and potentials steps, the currents displayed multiple time constants suggesting
multiple reaction pathways to the steady-state current levels at the end of a
long pulse (Fig. 13).

![Figure 13. Interaction of Sr$^{++}$ with the K$^{+}$ conductance. Current-time records
for the response to a step to +60 mV in the presence and absence of Sr$^{++}$ are
superimposed to demonstrate the multiple component nature of Sr$^{++}$ blockage
of the K$^{+}$ current.]

**Ba$^{++}$ Effects on Leakage Current**

When all voltage-dependent currents were blocked with $10^{-7}$ M externally
applied tetrodotoxin and 20 mM internally applied tetraethylammonium
chloride, the remaining currents were not sensitive to internal application of
Ba$^{++}$ even in relatively high concentrations. This suggests to us that the
character of the leakage pathway is significantly different than the Ba$^{++}$-
blockable voltage-dependent K$^{+}$ channel.

**DISCUSSION**

**Internal Ba$^{++}$ Concentrations**

Because the alkaline earth cations are very poorly soluble or form strong
complexes with many anions typically used to perfuse squid, we performed
our experiments in Cl$^{-}$-containing solutions in which the alkaline earths are
very soluble and in which no significant ion pair formation takes place except at high concentrations (>10^{-2} M). In addition, we buffered the divalent ion concentration with EDTA so that concentration changes due to interaction of the divalents with axoplasm or internal membrane components would be minimized. Buffering with EDTA also had some additional advantages. Firstly, because of the use of EDTA for a variety of purposes, the stability constants for EDTA-divalent complexes have been determined under a wide variety of conditions. The values for the constants we used in our work were obtained in 0.1 M KCl at neutral pH. All of our experiments with the exception of the Ba-K competition experiments were performed in 0.1 M KCl at pH 7.3. Secondly, the stability constants of EDTA are remarkably temperature insensitive (Martell and Calvin, 1952), thus making experiments possible in which temperature is an experimental parameter (Fig. 11). Because of these considerations, we feel that the internal Ba^{++} concentrations reported in this paper are an accurate representation of the true Ba^{++} concentrations to which the K^{+} channels were exposed.

**Mechanism of Action**

If K^{+} ion interacts with the sites in the K^{+} channel in its unhydrated state, then it is not very surprising that Ba^{++} should also interact with this site inasmuch as the crystal diameters of the two ions differ only by 0.004 nm. That it should block so strongly once it interacts may be somewhat surprising. However, examination of the permeability properties of the K^{+} channel may give us some insight into the potent blocking ability of Ba^{++}. If the suggestion that the K^{+} channel selectivity site is ~0.3 nm in diameter is correct (Hille, 1975 b), then ions larger than 0.3 nm will be impermeable, although they may display some blocking ability if the channel mouth is wide (Armstrong and Bezanilla, 1972). Examples of such ions are Cs^{+}, tetraethyl ammonium, and hydrazinium. Ions smaller than 0.3 nm would be expected to be permeable if they gained enough energy from interaction with channel sites to compensate for the energy loss associated with loss of their waters of hydration (Szabo et al., 1973). These very small ions, such as Na^{+}, Li^{+}, and Ca^{++}, which, because of their small size cannot come in close proximity to polar groups in the channel, should be relatively impermeable although they may at high concentrations produce some blockage of the channel. Ions close to the 0.266 nm size of unhydrated K^{+} should be permeable and, in fact, Tl^{+} with a diameter of 0.295 nm, NH_{4} at 0.286 nm, and Rb^{+} at 0.295 nm are all appreciably permeable. But Ba^{++} with a diameter of 0.270 nm and strontium at 0.224 nm are not permeable but rather block strongly. The primary difference may be the divalent character of the ions. The extremely strong binding that might be associated with divalent interactions with channel sites could lead to extremely rapid interaction at low concentrations with very low permeability. If the strong interaction is present then it should be reflected in the heights of the energy barriers and the depths of the energy wells from movement of Ba^{++} into the channel. In Fig. 14 A and B we have plotted the temperature dependence of the forward rate constant and of the equilibrium constant. The
The slopes of these lines yield the energy of activation for the forward reaction of Ba\(^{++}\) with the K\(^{+}\) channel, i.e., blockage, and the change of enthalpy of the reaction. From these values and the values of the forward rate constant and equilibrium constant, we can construct a description of the energy profile across the membrane. The various thermodynamic parameters are summarized in Fig. 15. As we anticipated, the barrier to go from the site back to the external solution is very high (26.1 kcal/mol) implying a strong tendency for a slow removal of Ba\(^{++}\) from a blocked channel. The barrier for entry is also high (15.4 kcal/mol) compared to the barriers proposed by others for permeable ions (Hille, 1975a). However, other types of reactions often have energies
of activation comparable with that calculated for Ba\(^{++}\). The hydrolysis of sucrose and \(\beta\)-methyl glucoside has activation energies of, respectively, 25.6 and 32.6 kcal/mol (White et al., 1959), whereas the energy of activation for one step in the interaction of TTX with the Na\(^+\) channel is greater than 21 kcal/mol (Ulbricht and Wagner, 1975).

**Unit Conductance of K\(^+\) Channel**

The rate at which Ba\(^{++}\) is capable of blocking open potassium channels can be used to estimate the unit conductance of the channel. To do this, we must assume that Ba\(^{++}\) enters and interacts with the K\(^+\) channel at the same rate as K\(^+\) ions do. If this assumption is correct we can proceed in a manner similar to Armstrong (1975). We find that for a step to +140 mV we have 1.26 mA·cm\(^{-2}\) or 7.86 \(\times\) 10\(^{10}\) ions·sec\(^{-1}\)·cm\(^{-2}\). This gives a channel density of 2.35 \(\times\) 10\(^7\) channels/cm\(^2\). The channel density gives a value of 5.35 \(\times\) 10\(^{-11}\) A/channel. With \(E_K\) at –55 mV, the unit conductance is 2.7 \(\times\) 10\(^{-10}\) S per channel.

This value seems somewhat high, being ~10 times larger than the value.
obtained for the gramicidin A channel (Hladky and Haydon, 1970) and two orders of magnitude larger than the conductance estimated by Armstrong (1975) using a TEA analogue. This value suggests that even though Ba²⁺ and K⁺ are of similar size, Ba²⁺ may reach the channel site significantly faster than K⁺. Alternatively, the concentration of Ba²⁺ near the surface of the membrane may be higher than in the bulk solution. Either of these effects might be due to the divalent charge on the Ba²⁺ ion. If either of these considerations were correct, then this value for the unit conductance would represent an upper limit for the conductance in Cl⁻-containing solutions.

**Nature of the Reaction of Sr²⁺ and Ba²⁺ with K⁺ Channel**

Initially in our study, we felt that a simple model of Ba²⁺ blockade would suffice to explain the experimental results. The model was to have been similar to the model suggested by Armstrong (1966, 1969) for K⁺ channel blockage by tetraethylammonium ion (TEA). The reaction sequence for such a model of the channel is

\[
\text{CLOSED} \xrightleftharpoons{voltage} \text{OPEN} \xrightleftharpoons{TEA} \text{OPEN BLOCKED.} \quad (A)
\]

But our observations of the effect of Sr²⁺ blockage suggested that a somewhat more complicated process was occurring. The reaction sequence above would predict a simple reduction in peak potassium current if the forward rate constant from OPEN to OPEN BLOCKED was much faster than the rate of going from CLOSED to OPEN. If the rate from CLOSED to OPEN was faster than the rate from OPEN to OPEN BLOCKED, then one would expect to see an exponential reduction in the potassium current as the open, conducting channels were slowly blocked. For TEA itself the former case is true whereas for the Ca-substituted derivative of TEA the latter observation is correct. The experimental results with Ba²⁺ would seem to put it in the Ca-derivative category, but the multiphasic response of K⁺ currents in the presence of Sr²⁺ (Fig. 13) cannot be the result of a reaction sequence like that above. Because of the unique size of Sr²⁺ and Ba²⁺ with respect to K⁺, it seemed that the alternative possibility suggested by Armstrong (1971) might be correct. The K⁺ channel might close with one of the blocking divalent cations in place. The complete sequences would then be

\[
\text{CLOSED} \xrightleftharpoons{voltage} \text{OPEN} \xrightleftharpoons{Ba²⁺, Sr²⁺} \text{OPEN BLOCKED.} \quad (B)
\]

The course of events that produces the characteristic strontium blockade would require previous depolarization to force some channels into the CLOSED BLOCKED state. Alternatively, Sr²⁺ could enter potassium channels open at rest. Presumably some equilibrium between the open and closed states is attained at the resting potential driven by thermal fluctuations. (Some of these channels could then be available for blockade and a new equilibrium between closed, open, and blocked states could be established.) Then, as the
voltage was depolarized, CLOSED channels would become OPEN producing an initial increase in $K^+$ current. These OPEN channels could be entered by free Sr$^{2+}$ and become OPEN BLOCKED channels with a concomitant exponential decrease in current. If left otherwise undisturbed this forward reaction would finally reach some equilibrium state with a fixed ratio of OPEN channels to OPEN BLOCKED channels, but the CLOSED BLOCKED channels would, with a slow time course, contribute additional OPEN BLOCKED sites. This increase in OPEN BLOCKED sites would force the equilibrium towards more OPEN channels with the incident increase in $K^+$ current.

To decide that a model that allowed closed channels with Ba$^{2+}$ or Sr$^{2+}$ remaining in the channel was reasonable, we numerically solved the rate equations that represented the reaction sequence B. For the voltage-sensitive transitions between closed and open states (whether blocked or unblocked) we used fourth-order kinetics like that described by Hodgkin and Huxley (1952 b). We let the block of open channels be represented by a single reaction whose rate constant from OPEN to OPEN BLOCKED was $k$ and in the reverse direction was $l$. We calculated the rate constants for the reaction going from CLOSED to OPEN from fits to the control data in the absence of Sr$^{2+}$. We also determined from the control data, the maximum conductance due to open channels available at each of the potential steps examined. The values determined were somewhat smaller than those obtained by Armstrong (1969). The smaller currents are attributable to the perfusion with internal Cl$^-$. We then assumed that the rate constants for the reaction sequence from CLOSED BLOCKED to OPEN BLOCKED were the same as the rate constants for the CLOSED to OPEN transition except that both the forward and reverse rate constants were slowed by a multiplicative factor. We also assume that the sum of OPEN plus OPEN BLOCKED channels was the same as the number of OPEN channels in the control. With these assumptions we found that, with a time constant for the reaction from OPEN to OPEN BLOCKED that was 10–12 times slower than the time constant for the reaction CLOSED to OPEN and a time constant for the reaction CLOSED BLOCKED to OPEN BLOCKED that was 40–45 times slower than the CLOSED to OPEN time constant, we could qualitatively reproduce all of time-dependent current changes for Sr$^{2+}$ as well as the responses for Ba$^{2+}$. The best fit to the Sr$^{2+}$ data was obtained when 15–20 percent of the $K^+$ conductance was in the CLOSED BLOCKED state prior to the voltage step. Occasionally, the quantitative fit to the data was in error by as much as 20 percent, particularly at long times toward the end of a voltage step. We attribute this quantitative discrepancy to the assumption that the rate constants for the reaction from CLOSED BLOCKED to OPEN BLOCKED differ from those for the reaction from CLOSED to OPEN only a multiplicative factor. This is almost surely not true because the ratio of opening to closing rates for a channel when a Ba$^{2+}$ or Sr$^{2+}$ ion is occupying the channel will probably be quite different than the same ratio when the channel is empty or occupied by a univalent ion like $K^+$. 
A reaction scheme that allows closed blocked channels is also attractive in explaining some of the Ba\(^{2+}\) data as well. We often noticed that the maximum available potassium conductance after treatment with Ba\(^{2+}\) was significantly less than that which one could predict on the basis of the rate of the reaction from OPEN to OPEN BLOCKED. This fact suggested to us that there might be a very slow process of Ba\(^{2+}\) removal from some channels. But our experiments to measure the Ba\(^{2+}\) "off" process (Fig. 5 A and B) gave no indication of such a slow reaction. The multiple reaction sequence with CLOSED BLOCKED channels offers a reasonable alternative, since under normal conditions the CLOSED BLOCKED channels will not contribute to the total conductance and the rate of transition from CLOSED BLOCKED to OPEN may be very slow. Credence is lent to this idea since on reexamination of data particularly at temperatures between 12 and 18\(^\circ\)C there were suggestions of multiple time constants in the current-time records after Ba\(^{2+}\) treatment that were reminiscent of the effects of Sr\(^{2+}\). Taylor and Armstrong (1978), during their investigation of the effects of internal Ba\(^{2+}\), have also concluded from the time-course of Ba\(^{2+}\) unblocking that K\(^+\) channels may close with the divalent in place.

In summary, Ba\(^{2+}\) represents the most potent K\(^+\) channel blocker known. This blocking ability makes Ba\(^{2+}\) a useful ion with which to explore the properties of the potassium conductance mechanism. We hope that in the future a more detailed comparison of the effects of Ba\(^{2+}\) and other divalent cations will provide specific information about the conformation of the potassium channel protein.

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