

Potassium Channel Block by Internal Calcium and Strontium

CLAY M. ARMSTRONG and YORAM PALTI

From the Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6085; Department of Physiology and Biophysics, The Rappaport Family Institute & Faculty of Medicine, Technion, Haifa, Israel 31096; and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT We show that intracellular Ca blocks current flow through open K channels in squid giant fiber lobe neurons. The block has similarities to internal Sr block of K channels in squid axons, which we have reexamined. Both ions must cross a high energy barrier to enter the blocking site from the inside, and block occurs only with millimolar concentrations and with strong depolarization. With Sr (axon) or Ca (neuron) inside, I_K is normal in time course for voltages less than about +50 mV; but for large steps, above +90 mV, there is a rapid time-dependent block or "inactivation." From roughly +70 to +90 mV (depending on concentration) the current has a complex time course that may be related to K accumulation near the membrane's outer surface. Block can be deepened by either increasing the concentration or the voltage. Electrical distance measurements suggest that the blocking ion moves to a site deep in the channel, possibly near the outer end. Block by internal Ca can be prevented by putting 10 mM Rb in the external solution. Recovery from block after a strong depolarization occurs quickly at +30 mV, with a time course that is about the same as that of normal K channel activation at this voltage. 20 mM Mg in neurons had no discernible blocking effect. The experiments raise questions regarding the relation of block to normal channel gating. It is speculated that when the channel is normally closed, the "blocking" site is occupied by a Ca ion that comes from the external medium.

INTRODUCTION

The effects of extracellular Ca on the gating properties of ionic channels have been much studied, and some aspects are well known (see Hille, 1984 for a review). The action of intracellular Ca on voltage-gated channels, however, has received relatively little attention, partly because of technical difficulties: perfused axons require internal F, which is incompatible with a high Ca concentration. A prominent blocking effect with Sr was reported by Eaton and Brodwick (1980), with some similarity to block by internal Ba (Eaton and Brodwick, 1980; Armstrong and Taylor, 1980; Armstrong, Swenson, and Taylor, 1982).

Address reprint requests to Dr. Clay M. Armstrong, Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104-6085.

Because of a rekindled interest in the possibility that Ca ion has an important gating function (Brink, 1954; Frankenhaeuser and Hodgkin, 1957; Armstrong and Matteson, 1986; Armstrong and Lopez-Barneo, 1987) we have examined K channel block by Sr in axons, and by Ca and Sr in squid neurons. The axon experiments allowed precise control of the internal divalent concentration, but they are hard to interpret in some respects because of the known tendency of K to accumulate outside of the axon membrane. This is important because external K and other monovalent cations that permeate through K channels are known to interfere with block by internally applied agents, e.g., TEA derivatives (Armstrong, 1971) and Ba (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980). The same is true for Ca block, as shown below. Squid neurons, on the other hand, have the advantage that relatively little K accumulates outside, but one is never very sure of the concentration of an ion diffusing into the cell from the pipette.

This paper is a qualitative report of the surprising actions of internal Sr and Ca. We find that block can be seen only with the divalent cation in the millimolar range and at high voltage, in most cases above +50 mV. The results suggest that the blocking ion passes over a very high barrier to occupy a blocking site deep in the channel or near the outer surface.

METHODS

Giant axons and stellate ganglion neurons (the cell bodies whose axons fuse to form the giant axon) were obtained from the squid *Loligo pealei* at the Marine Biological Laboratory, Woods Hole, MA. Giant axons were dissected, cleaned, internally perfused, and voltage clamped using methods described elsewhere (e.g., Bezanilla and Armstrong, 1977). To facilitate internal perfusion, the axons were briefly exposed to internal pronase, which removed a part of the axoplasm. The standard internal medium contained 50 mM F, which for reasons that are still unknown, is necessary to prevent a large increase in the leak current. To apply Sr internally, it was necessary to remove F, so the exposures to Sr were kept as brief as possible to minimize rundown. That is, the internal medium was changed to F-free Sr containing medium for ~2 min and then returned to the standard F-containing medium. The internal volume of the axon was replaced every few seconds at the perfusion rates used, and complete exchange required ~1 min. A typical axon could endure several exposures to F-free medium.

Neurons were isolated from small squid, 2–3 in. in length, and maintained up to several weeks in primary culture using methods described previously (Llano and Bookman, 1986). The cells were subjected to whole-cell patch clamp using low resistance patch pipettes that were filled with the Sr- or Ca-containing solution to be tested. Frequency response of the clamp was enhanced by the use of “supercharging” (Armstrong and Chow, 1987). After breaking into the cell, there was a brief period when it was possible to obtain records that showed no sign of Ca or Sr block.

In some figures it was convenient to plot conductance rather than current. In these cases the conductance is the slope conductance of the I_K - V curve.

Solution compositions are given in Table I. In the figure legends solutions are given as external solution/internal solution. Internal Ca and Sr were made by mixing KCl_{ax} or KCl_n with isotonic $CaCl_2$ or $SrCl_2$.

TABLE I
Solutions

External							
	Na	Ca	Cl	Tris	HEPES		
ASW _{ax}	440	50	545	5			
ASW _n	470	50	575		10		
Internal							
	K	Cl	F	Glu	Tris	HEPES	Sucrose
275 K _{ax}	275		50	225	10		400
275 KCl _{ax}	275	275				10	400
550 KCl _n	550	50		500		10	

Concentrations in millimolar.

RESULTS

Blocking of axonal K channels by intracellular Sr is illustrated in Fig. 1. The four panels are at 50, 70, 90, and 130 mV, and each shows g_K for several Sr concentrations, as indicated. Block deepens as either voltage or the [Sr] is increased. At 50 mV, g_K activates with a near normal time course except for the 20-mM Sr trace, which is appreciably slowed. In all four traces there is a slight droop in the conductance 5–10 ms after the beginning of the step, presumably because of K accumulation in the Schwann cell clefts (Frankenhaeuser and Hodgkin, 1956).

The 20-mM Sr trace is distinctly smaller than the others at 70 mV, but block with 10 mM Sr is just detectable. The 10-mM Sr trace at 90 mV resembles fairly closely the 20-mM Sr trace at 70 mV, and 3 Sr at 90 mV is about equivalent in blocking potency to 10 Sr at 70 mV. Thus, block can be intensified by raising either voltage or concentration.

Most of the channels are blocked by the higher Sr concentrations at 130 mV. Several of the traces at 90 and 130 mV have a complex time course, and the current tends to creep upward after an initial period during which it decays. This phenomenon, which we will refer to as late relief of block, may be the result of K accumulation as first suggested to us by Dr. Gerry Oxford (University of North Carolina, Chapel Hill, NC) and discussed below.

The complex time course of g_K in the presence of Sr is further illustrated in Fig. 2, where the [Sr] was fixed at 10 mM. The conductance is almost normal in time course at 80 mV, but above this voltage there is evidence block. At 100 mV, for example, there is a hesitation in the rise of g_K ~ 2 ms after the step, which is accentuated at 110 mV. At 160 mV the current decreases and then slowly increases once again; i.e., there is apparently a late relief of the block.

K Channel Block and Sr in Neurons

The immediate rationale for repeating the experiments just described in neurons was to see if this strange time course was present in a preparation where K accumulation

is slight. We got a mixed but interesting answer: Sr block in neurons does not show "late relief," but block by internal Ca does.

Sr block of neuronal K channels is illustrated in Fig. 3. The pipette solution contained 20 mM Sr, but the left series was taken shortly after break-in, when little Sr had diffused into the cell. In this series no block is apparent until the voltage reaches 110 mV, where there is a slow droop that is probably related to Sr block. 112 s later, after more Sr has diffused into the cell, block is apparent at 70 mV, and very marked

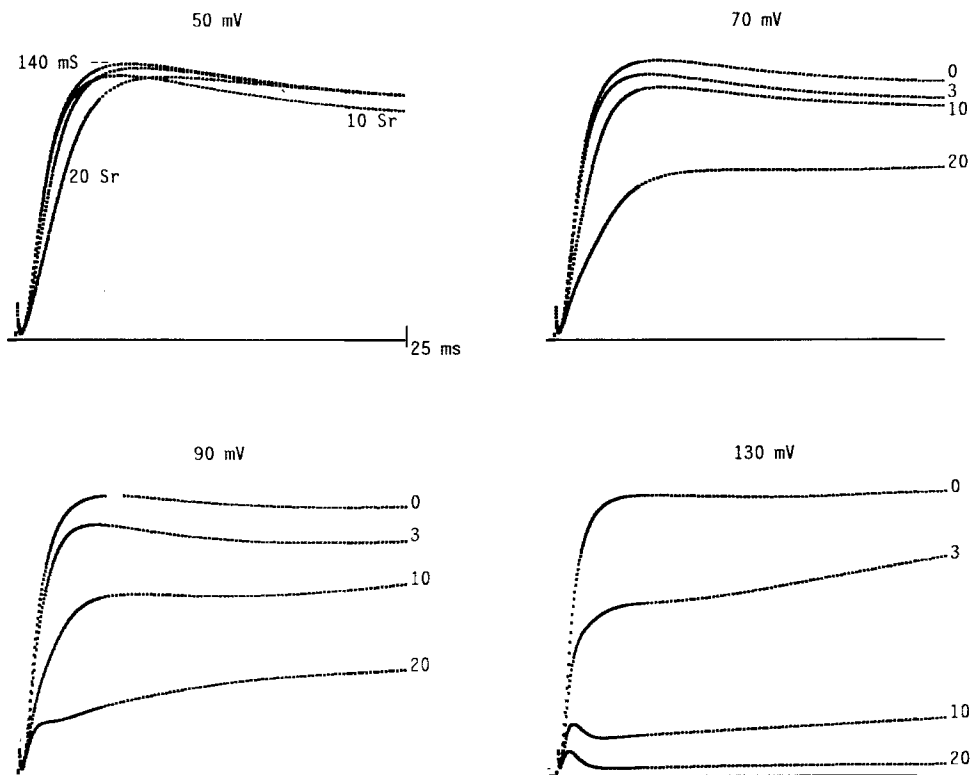


FIGURE 1. K channel block by internal Sr. Ionic currents were recorded from a giant axon perfused internally with the indicated medium. The conductance was determined from the slope conductance of the control I_K - V curve. Block is more intense as either voltage or Sr concentration is increased. ASW_{ax}/KCl_{ax} ; HP -70 mV; 8°C .

at 110 mV. None of the traces show a late increase of the current, and this held true in all seven of the cells where block by internal Sr was tested. The tentative conclusion is that K accumulation does play a part in generating the complex time course seen with Sr block in axons.

Block of Neuronal K Channels by Ca

Ca block of K channels in neurons has the same temporal characteristics as Sr block in axons. (No intracellular Ca experiments were performed on axons). Fig. 4 shows

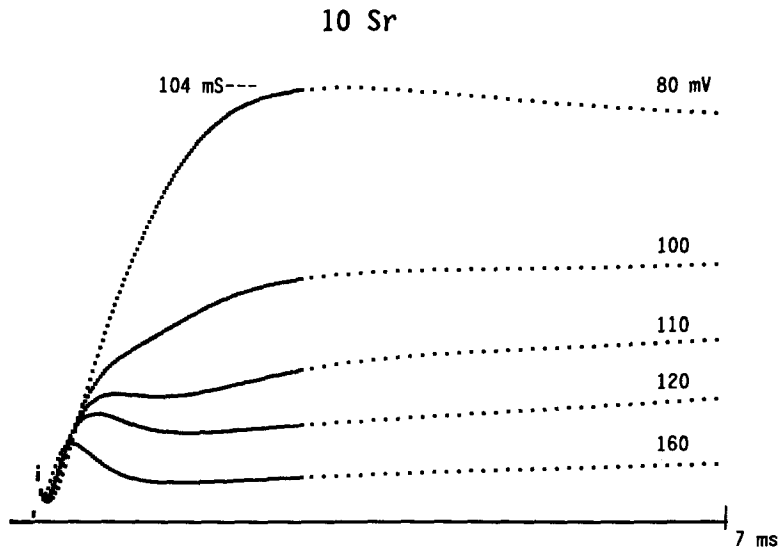


FIGURE 2. K channel block deepens with voltage at a constant internal Sr concentration (10 mM). The traces were taken from a giant axon and scaled according to the slope of the control I_K - V curve. ASW_{ax}/KCl_{ax} ; HP -70 mV; 8°C .

the result of putting 20 mM Ca in the pipette. Three traces are shown for each voltage, the first (*i*) shortly after breaking into the cell, then 42 (*ii*) and 102 (*iii*) s later. The traces form a concentration series, but with the concentration unknown.

Block becomes more pronounced with the passage of time, as Ca diffuses into the cell, and as voltage is increased. It is detectable at 70 mV only in trace *iii*, where [Ca] is highest, but at 130 mV block is detectable in trace *i*. As in the axon experiments, raising the concentration is roughly equivalent to raising the voltage. For example, trace *iii* at 90 mV has a time course very similar to trace *ii* at 110 mV.

The pattern of initial Ca block followed by late relief of the block is similar to Sr block in axons, as can be seen by comparing Figs. 2 and 4.

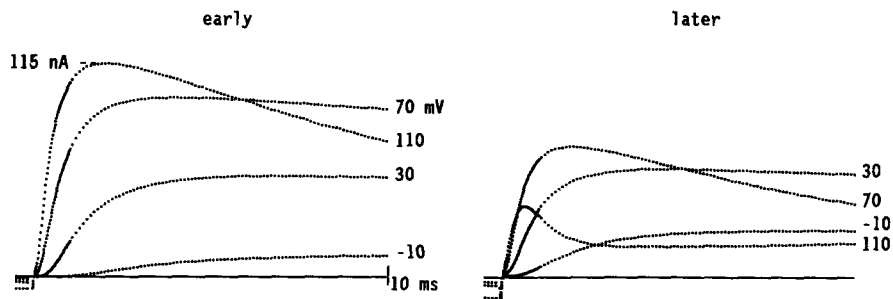


FIGURE 3. Internal Sr blocking K channels in a neuron. The traces on the left were taken shortly after breaking into the cell with a pipette containing 20 mM Sr. The right traces were taken ~ 1 min later, when more Sr had diffused into the cell. At the higher Sr concentration there is a clear "inactivation" of the current. ASW_n/KCl_n ; HP -80 mV; 15°C .

The Voltage Dependence of Sr Block

The voltage dependence of block is apparent in the I_K - V curves of Fig. 5 *A*, recorded from an axon. The 0 Sr curve (open squares) was measured in KCl, and the other curves have been scaled for linear rundown. With 3 mM Sr, block is first detectable at 70–90 mV. The I_K - V curve peaks at 100–110 mV, and above 110 mV the current declines as V_m is made more positive. The 10 and 20 Sr curves are generally similar, but their peaks are shifted to the left; i.e., block is evident at much lower V_m .

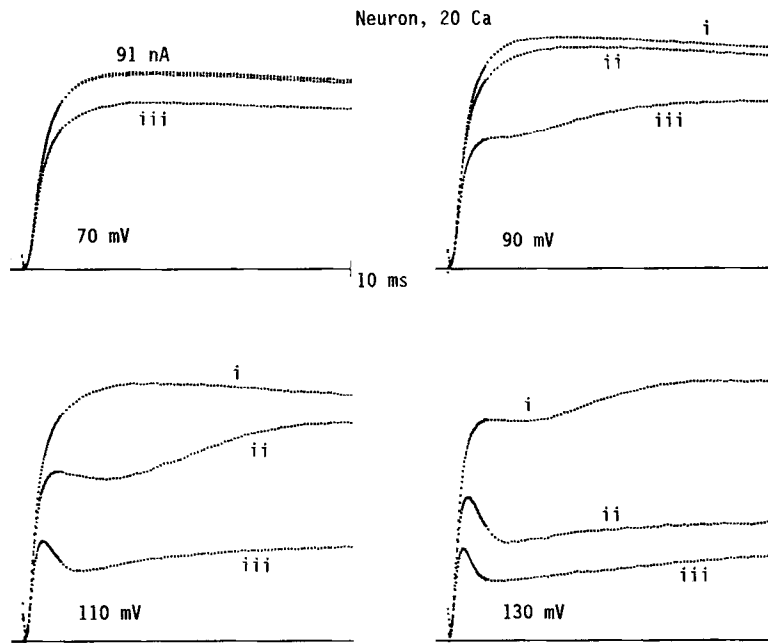


FIGURE 4. Block of neuronal K channels by internal Ca. The three traces at each voltage were recorded at ~ 1 -min intervals as Ca diffused into the cell from the patch pipette. At 70 mV there is block only for the latest trace (highest concentration). ASW_n//KCl_n; HP -70 mV; 15°C .

The curves in Fig. 5 *A* suggest that Sr is driven into the membrane when the voltage is positive, to occupy a site that is somewhere within the membrane field. To get an idea of the location of the blocking site in the channel, we fitted the following formula to the curve representing the fraction of blocked channels as a function of voltage, using a least-squares method:

$$\text{fraction blocked} = 1 / \{1 + \exp [(V_m - V_{1/2}) \delta z F / RT]\}$$

$V_{1/2}$ is the voltage where half the channels are blocked, z is the valence of the blocking ion ($2e$), and δ is the fraction of the field through which the ion must move to reach its blocking site. The fraction blocked was measured ~ 1.5 ms after the application of the voltage step, when block was most intense. A representative fit is given in Fig. 5 *B*. Determined at 3, 10, 10, and 20 Sr, δ was, respectively, 0.75, 0.9, 1.25, and 0.75, for

an average value of 0.88. The precise meaning of this electrical distance depends on the model of block used (see Discussion), but in general it suggests that the blocking site is deep in the channel and possibly close to the outer edge of the membrane.

Block in Neurons Studied with a Two-Pulse Protocol

In Figs. 1 and 2 the time course of g_K reflects both the opening of the channels and their block by Sr. To monitor blocking time course at least partially separated from opening, we used a two-pulse protocol to give the results illustrated in Fig. 6. Two traces are shown in each panel, one recorded from a neuron with no Ca inside (i), and the second from a neuron of similar size, with 20 mM Ca internally. A first pulse, to 30 mV, opened most of the channels without causing significant block; i.e., the

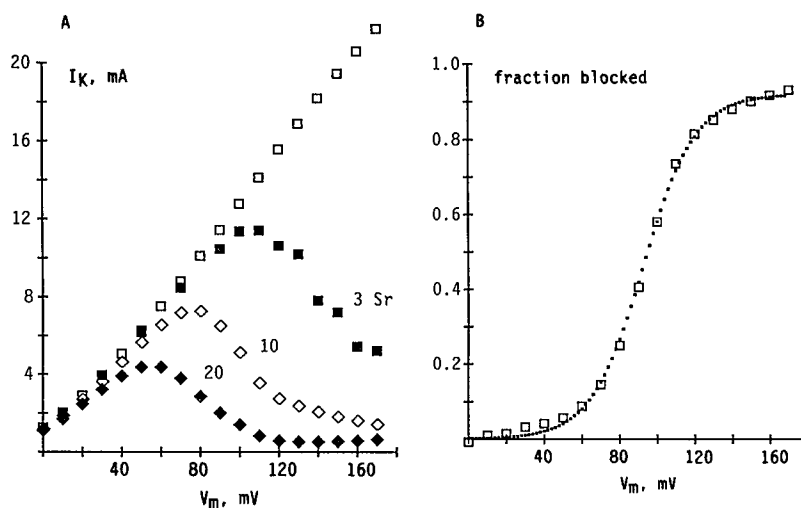


FIGURE 5. The voltage dependence of axonal K channel block by Sr. (A) I_K was measured at the end of a 10-ms pulse at the Sr concentration indicated. The plots have been scaled for linear rundown. (B) The fraction of blocked channels (*open squares*) is plotted as a function of V_m for 10 mM Sr. The dots are the fit of a Boltzmann curve, as described in the text. For this fitting, δ was 0.9, and half of the channels were blocked at $93 \text{ mV} \cdot \text{ASW}_{\text{ax}} / K_{\text{ax}}$ or KCl_{ax} ; HP -70 mV ; 8°C .

traces with and without internal Ca are nearly identical. A second step to the indicated voltage caused an immediate jump due to increased driving force, followed by additional opening of channels in the absence of Ca (traces labeled i). With Ca internally (ii) block is evident at all voltages, slight at 90 mV, but rapid and intense at 150 mV.

Because of the additional opening of channels during the second step, the separation between opening and blocking is not complete in this experiment. Nonetheless, it is clear that block occurs only at high V_m , and that it is much faster at high voltage.

Recovery from Sr Block Is Rapid

A similar two-pulse protocol was used to investigate Sr block and recovery in an axon. The left panel in Fig. 7 compares current at 30 mV (*a*), where there is no block, with that at 130 mV, where block is rapid and intense. After 10 ms at 30 mV (*a*), the potential was jumped to 130 mV and trace *b* was recorded. An immediate jump in current due to the increased driving force is followed by a rapid decay of the current as Sr ions enter and block the channels. The final current level is not quite as low as

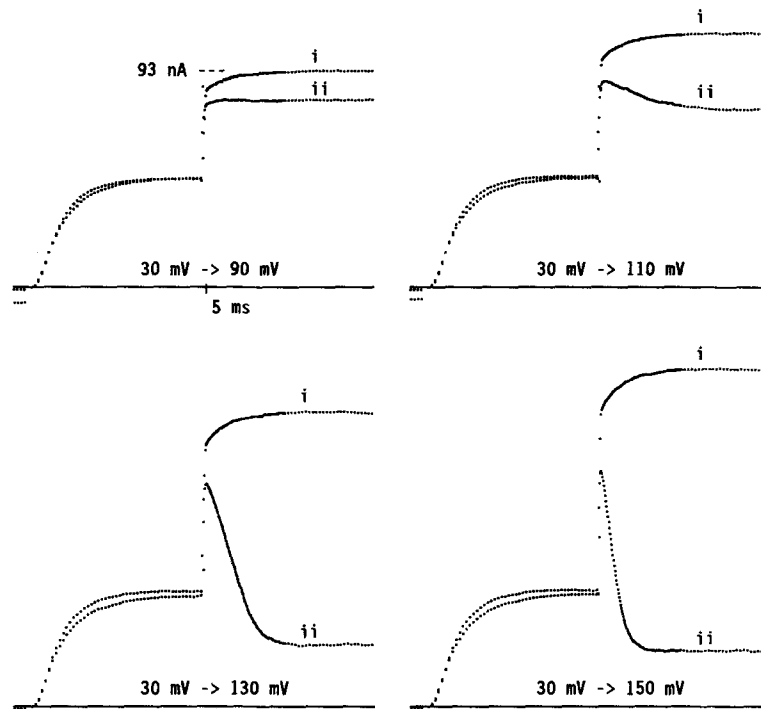


FIGURE 6. Ca block of K channels in a neuron. In each panel, traces from a cell with (nominally) 20 Ca inside (*ii*) are compared with traces from a control cell of similar size with no internal Ca. (*i*). In all panels a 5-ms step to 30 mV is followed by a second step to the voltage indicated. The time course of the current at 30 mV is unaffected by internal Ca. $ASW_n//KCl_n$; HP -70 mV; 15°C .

for a single step to 130 mV, perhaps because of external K accumulation during the 10-ms step to 30 mV.

The left panel shows the converse experiment. I_K for a pulse to 30 mV is given by trace *d*. Traces *e* and *f* show I_K for a pulse to 130 mV followed by a pulse to 30 mV. At 130 mV the channels open and are quickly blocked by Sr. On stepping to 30 mV, current in theory decreases instantaneously because of reduced driving force, although this was not captured in the record. Current then increases as the channels recover from block.

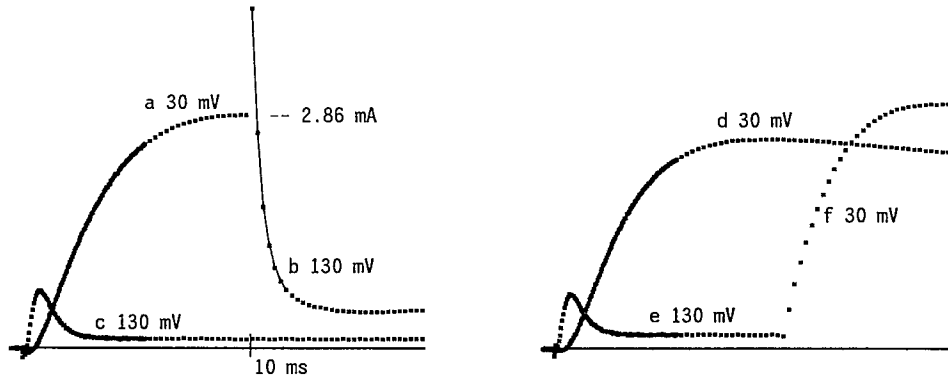


FIGURE 7. Sr block and recovery in an axon. The axon contained 10 mM Sr. (*Left*) *a* and *b* show current for a step to 30 mV, followed by a second step to 130 mV. At 30 mV the current has a normal time course. At 130 mV the open channels are rapidly blocked, with an approximately exponential time course. Current declines almost to the level expected for a single pulse to 130 mV (*c*). Right. Trace *f* shows the recovery at 30 mV of channels that were blocked by a step to 130 mV. The time course of recovery from block at 30 mV (*f*) and for simple opening of the channels at 30 mV (*d*) are similar. ASW_{ax}//KCl_{ax}; HP -70 mV; 8°C.

Similar results were obtained, but not illustrated, with Ca block of neuronal K channels.

A point of considerable interest is that recovery (*f*) has almost the same time course as does opening during the single pulse to 30 mV (*d*). This suggests that block and channel gating may be related, as briefly considered in the Discussion.

Mg_m Does Not Cause Readily Detectable Block

20 mM Mg was introduced into four cells without noticeable effect on the time course of the current.

External Rb Prevents Ca Block

There are several cases in the literature in which block by an agent applied on one side of the membrane is modified by an ion applied to the other side (e.g., Armstrong, 1971; Armstrong and Taylor, 1980; Neyton and Miller, 1988*a, b*). Fig. 8

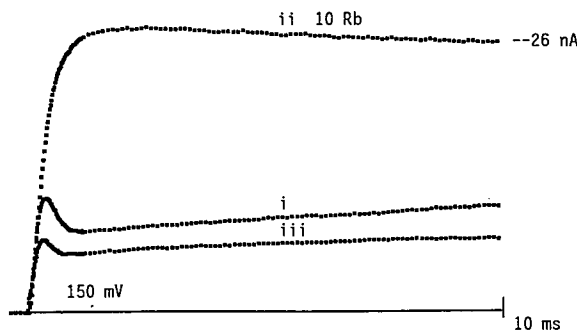


FIGURE 8. External Rb prevents block by internal Ca. The traces (taken in the order *i, ii, iii*) show block by 20 mM internal Ca (*i* and *iii*) and its reversible abolition by the addition of 10 mM Rb to the external medium. ASW_n//KCl_n; HP -70 mV; 15°C.

shows such an effect of externally applied Rb on block by intracellular Ca. The traces, recorded in the order *i*, *ii*, *iii*, show a profound block in the control external solution, and little or no block when 10 mM Rb was present outside. Apparently occupancy of some site in the channel by Rb almost completely prevents Ca from reaching its blocking site.

DISCUSSION

There are two strong arguments that Ca and Sr decrease current by actually entering the channel itself. The first is the voltage dependence, which suggests that the blocking ion moves a considerable distance into the membrane. Obviously a convenient avenue for this movement would be the channel itself. A second and stronger argument is that block can be abolished by including a K channel permeant cation (Rb) on the side of the membrane opposite the blocking ion. The simplest explanation is that Rb enters the channel from the outside, and its presence there prevents Ca entry from the inside.

Our results suggest that Ca and Sr ions entering the channel from the inside face a very high barrier, which can be surmounted only when the membrane potential is very positive (50 mV or more). The entering ion then occupies a site that is deep in the channel or near its outer end. The ability of external Rb, at relatively low concentration and at high voltage, to interfere with block by internal Ca suggests that the blocking site is near the outer end of the channel, as does the simple interpretation of the electrical distance. On the other hand, there must be a barrier that prevents access by divalent cations present in the external medium, because neither extracellular Ca nor Sr has a large blocking effect on I_K (Armstrong and Matteson, 1986).

Experimentally, the precise location of the binding site depends on two questions that will require further work.

(a) Interpretation of the electrical distance depends on how many ions (in addition to Ca/Sr) move through the channel during the blocking event. K channels are thought to be occupied by several ions at once (e.g., Hodgkin and Keynes, 1955; Hille and Schwartz, 1978; Begenisich and DeWeer, 1980). A precise number is not available for voltage-dependent K channels at present, although Neyton and Miller (1988*a*, *b*) have made interesting progress in examining the Ca-activated K channel.

(b) Does the divalent ion exit to the cytoplasm or to the external medium? On leaving the blocking site the ion could in theory either reenter the cytoplasm or exit to the outside medium. If dissociation is to the outside, Ca/Sr is not at equilibrium in the channel, and the measured electrical distance is influenced or determined by the position of the barrier peak as well as, or rather than, the position of the binding site. At present it is not clear which way the divalent ion moves in exiting the channel.

Block by internal Ca is clearly very sensitive to external Rb, and this can be used to give a qualitative explanation for the curious time course of Sr block in axons. It is known that the K concentration can approach 100 mM during a large depolarization (Adelman, Palti, and Senft, 1973), and it seems plausible that such a high concentration of K could interfere with Sr block, just as external Rb interferes with Ca block. This agrees well with the absence of both accumulation and the complex time course of Sr block in neurons. How, then, can the time course of Ca block in neurons be

explained? It may be that for unknown reasons Ca more than Sr block is sensitive to interference from external cations, and accumulation of K even at low concentrations is sufficient to influence Ca block.

It is of interest that relatively large changes in divalent cation concentration do not result in detectable surface charge effects (alterations of gating kinetics and shifts of the conductance voltage curve along the voltage axis), at least in axons. In neurons there was a tendency for channels to open more rapidly after lengthy internal dialysis, but this seemed to occur independently of the divalent ion content of the internal medium. The literature contains evidence for a high density of negative surface charge on the axon membrane's inner surface, giving rise to a surface potential that should be altered by changes of the internal divalent concentration (Chandler, Hodgkin, and Meves, 1965). It is somewhat perplexing that we see no evidence for changes of surface potential in axons perfused with Sr.

A question of clear interest is whether the blocking site for intracellular cations is related to the site that is occupied by external Ca ions as the channel is closing (Armstrong and Matteson, 1986; Armstrong and Lopez-Barneo, 1987). The similarity in time course between normal opening and recovery from block (Fig. 7) suggests that this may be the case. Specifically, one can speculate that a late step in the opening of a K channel involves the release of a divalent cation from the blocking site in the channel lumen into the external medium. This step can be reversed by forcing internal Sr or Ca into the channel at high voltage. When V_m is then returned to a low value (30 mV) where these ions do not enter, the Sr or Ca ion blocking the channel clears to the outside at the same rate as during normal opening, leaving the channel in a conducting state.

Comparison with Existing Literature

The blocking effect that we describe here has been briefly reported by Eaton and Brodwick (1980) using Sr ion. We cannot, however, confirm their finding that 1 mM Sr causes measurable block. In numerous experiments, our lowest effective concentration was at least 1,000 times higher. Grissmer and Cahalan (1989) have shown that the inactivation rate of K channel in T lymphocytes speeds up when either intracellular or extracellular Ca is raised. Although the effect cannot be attributed to a simple block, we postulate a Ca-binding site in the outer end of the pore. Our conclusions regarding the location of a Ca-binding site are almost identical.

Block with Ca or Sr has some similarities to the effects of internal Ba, as noted by Eaton and Brodwick (1980). In both cases there is voltage-dependent block and an "inactivating" time course, but the peculiar kinetic patterns seen with Ca and Sr (e.g., Figs. 1 and 2) have not been seen with Ba. The electrical distance to the blocking site for Ba and for Ca/Sr is similar: 1.0 for Ba (Armstrong et al., 1982) and, as reported here, 0.88 on the average for Sr. The precise location of the blocking site within the membrane field is model dependent. Ba apparently enters the channel more readily than Ca or Sr, and block can be seen at relatively low V_m . A major difference is in the time course of recovery from block, which is milliseconds for Ca or Sr, but minutes for Ba block.

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