Relief of Na$^+$ Block of Ca^{2+}-activated K$^+$ Channels by External Cations

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ABSTRACT The flickery block of single Ca^{2+}-activated K$^+$ channels that is produced by internally applied Na$^+$ can be relieved by millimolar concentrations of external K$^+$. This effect of K$^+$ on the kinetics of Na$^+$ block was studied by the method of amplitude distribution analysis described in the companion paper (Yellen, G., 1984b, $J.$ $Gen.$ $Physiol.$, 84:157–186). It appears that K$^+$ relieves block by increasing the exit rate of the blocking ion from the channel, not by competitively slowing its entrance rate. This suggests that a K ion that enters the channel from the outside can expel the blocking Na ion, which entered the channel from the inside. Cs$^+$, which cannot carry current through the channel, and Rb$^+$, which carries a reduced current through the channel, are just as effective as K$^+$ in relieving the block by internal Na$^+$. The kinetics of block by internal nonyltriethylammonium (C$_9$) are unaffected by the presence of these ions in the external bathing solution.

INTRODUCTION

The preceding paper (Yellen, 1984b) treated the phenomenon of Na$^+$ blockade of Ca^{2+}-activated K$^+$ channels in chromaffin cell membranes, and explained a method of amplitude distribution analysis for measuring the kinetics of block under conditions where a blocker produces large fluctuations (flicker) in the open channel current. The flickery block produced by applying millimolar concentrations of Na$^+$ to the internal face of the channel can be relieved by applying millimolar concentrations of K$^+$ to the external face, as described in a preliminary report by Marty (1983). This paper describes experiments on the relief of Na$^+$ block by several species of external cations. By applying the amplitude distribution method for determining the rate constants for the block process, we can distinguish between two classes of possible mechanisms for relief: first, that K$^+$ relieves block by competing for the saturable Na$^+$ block site in the channel; and second, that K ions speed the exit of blocking Na ions by knocking them out or repelling them out of the channel.

MATERIALS AND METHODS

The materials and methods used in this paper are described in the companion paper (Yellen, 1984b).

RESULTS

Fig. 1 shows the effect of external K^+ concentration on the flickery block produced by 5 mM Na^+. Increasing the external K^+ concentration relieves the reduction in current and the flicker produced by a fixed concentration of internal Na^+.

External K^+ Speeds the Exit of Na^+ from the Channel

The competition and knock-out mechanisms make different specific predictions of how the rate constants of block will change as the external K^+ concentration
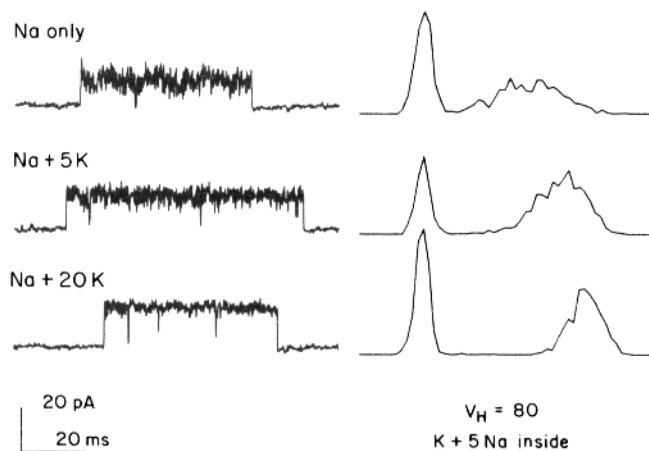

FIGURE 1. External K^+ relieves the block produced by internal Na^+. (*Left*) Single channel records with the specified outside solution (total concentration of $Na^+ + K^+$ = 160 mM) and with an internal solution containing 160 K^+ + 5 Na^+. The membrane voltage was +80 mV; filtered at 4 kHz. (*Right*) Amplitude histograms from the single records at left. The baseline peaks in the histograms have not been removed.

is raised. If K^+ and Na^+ are simply competing for a saturable site, we expect that increasing the K^+ concentration will reduce the association rate of Na^+ with the site, since Na ions cannot enter the site when it is occupied by K^+. We expect no change in the rate of exit of Na ions from the site. Alternatively, for knock-out, we expect that raising the K^+ concentration will increase the rate of blocker exit.

We can distinguish between changes in exit rate and changes in entry rate by a qualitative inspection of the amplitude histograms of the flicker. The amplitude distribution of a very fast filtered process is a narrow peak; for slower processes, the peak broadens. If we reduce the entry rate of Na^+, the overall flicker process will be slower and the amplitude distribution will be broader. We can experimentally reduce the entry rate by lowering the Na^+ concentration; at lower Na^+ concentrations, the amplitude distribution is broader (Fig. 2A), as expected.

Alternatively, if we increase the exit rate of Na^+, the overall process will be faster and the amplitude distribution will become narrower. This is the effect observed when external K^+ concentration is raised to relieve the Na^+ block. As the K^+ concentration is increased (Fig. 2B), the amplitude distribution shifts to higher amplitudes (reflecting the reduced average block) and becomes narrower.

The quantitative analysis of the amplitude distributions for K^+ relief of block bears out this qualitative impression. Fig. 3 shows the blocking and unblocking rate constants for different external K^+ concentrations at a fixed voltage and

REDUCED BLOCKER CONCENTRATION

A

20 Na   5 Na   2 Na

ELEVATED EXTERNAL K CONCENTRATION
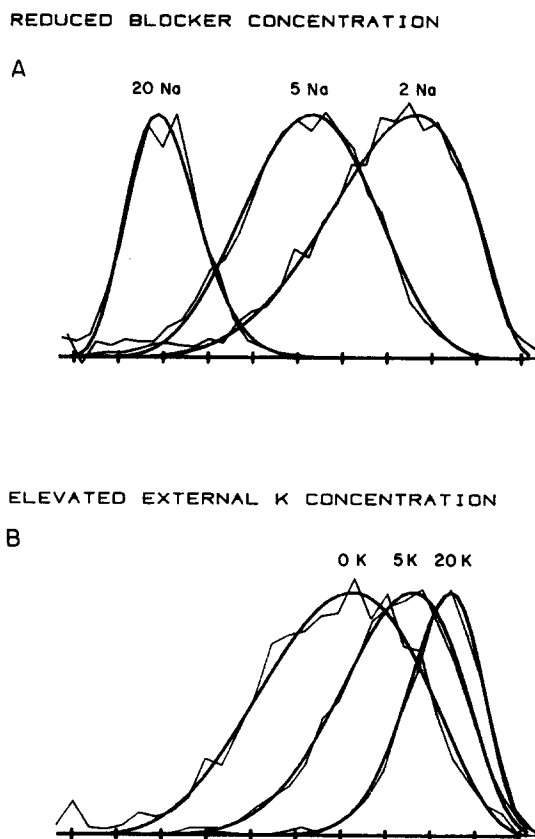
B

0 K   5 K   20 K

FIGURE 2.   Effects on entry and exit rates can be qualitatively distinguished from the shape of the amplitude distributions. (A) Amplitude histograms (light lines) and fits (heavy lines) for three different blocker concentrations (indicated), with an external solution containing 160 Na^+. The histograms are from records at +80 mV filtered at 4 kHz. Reducing the blocker concentration decreases the entry rate and broadens the histogram. (B) Amplitude histograms (light lines) and fits (heavy lines) for three different concentrations of external K^+ (indicated) to relieve the block by 5 mM internal Na^+. The histograms are from records at +80 mV filtered at 4 kHz. Increasing the concentration of external K^+ increases the exit rate and narrows the histogram.

internal Na^+ concentration. The blocking rate (corresponding to the entry of Na^+) is constant over the range of external K^+ concentrations studied, which indicates that there is no significant competition between external K^+ and internal Na^+ under these conditions. The unblocking rate (the rate of exit of Na^+) has a value of $9.8 \pm 0.02 \times 10^4$ s^{-1} (38 measurements on 4 inside-out patches, corresponding to $\tau = 10.1$ μs) with no external K^+ and increases linearly with external K^+ concentration. At 10 mM external K^+, the exit rate of Na^+ is increased 3.3-fold (at +60 mV) over its value with no external K^+.

Thus, rather than simply competing for a saturable site in the channel, the relieving ions appear to speed the exit of the blocking ion. This behavior corresponds to the "knock-out" behavior seen in other systems, notably in the delayed rectifier K^+ channel (Armstrong, 1971, 1975a; Bezanilla and Armstrong,
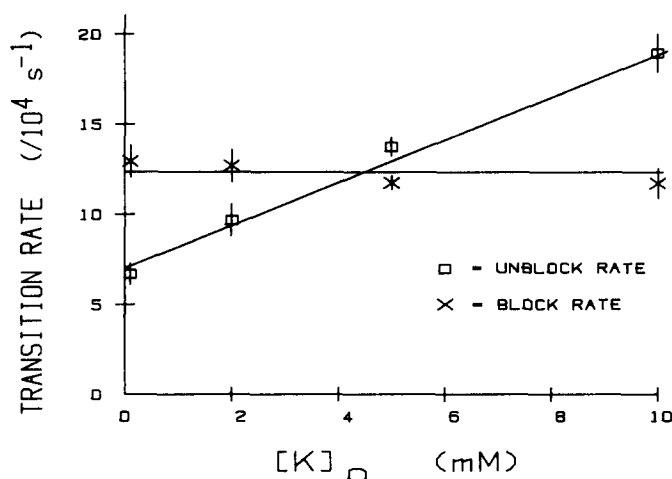

FIGURE 3.   External K^+ speeds the exit of Na^+. Block and unblock rates determined by amplitude histogram analysis. Each point is the mean $\pm$ SD of two to four determinations on the same patch. The membrane voltage was +60 mV and the internal solution contained 160 K^+ + 20 Na^+.

1972; Armstrong and Hille, 1972), for the effect of external cations on internal blockers.

Relief of block appears to show a slight voltage dependence, with more effective relief at less positive voltages. For technical reasons, it is difficult to compare rate constants for relief of block at different voltages. These experiments are performed on outside-out patches (with changes of the external solution only), so rather than directly measuring the open channel current in the absence of blocker, one must extrapolate the true open channel current from its value under conditions where most, but not all, of the block is relieved by K^+. The value of the open channel current is needed for the amplitude histogram analysis. Uncertainty in this value does not compromise the results obtained at a single voltage, since the same value is used for all of the experimental conditions. It does, however, make it more difficult to compare values at different voltages

with complete confidence (it also accounts for the discrepancy between the unblock rates measured in outside-out patches and the more accurate determinations in inside-out patches). When careful estimates are used for the open channel currents, the degree of relief by K^+ appears to be greater at less positive voltages (10 mM external K^+ speeds exit by 2.1 ± 0.1-fold at +80 mV, $n = 2$, and by 3.3 ± 0.15-fold at +60 mV, $n = 5$). This suggests that the relieving ions traverse part of the transmembrane electric field in the process of expelling the blocker. The voltage dependence of relief of block should be unaffected by the diffusion limitation phenomena discussed in Yellen (1984b), since there is no current flow through the channel when it is occupied by a blocking ion.

Cs^+ and Rb^+ Are as Effective as K^+ in Relieving Block

Since K^+, Cs^+, and Rb^+ show differences in their ability to carry current through the channel, it is interesting to ask how these differences affect their ability to relieve Na^+ block. Cs^+ cannot carry current through the channel, although external Cs^+ does appear to bind to the channel and block outward K^+ current in a very voltage-dependent fashion. This voltage-dependent binding and block suggest that Cs^+ can interact with the permeation pathway of the channel, so Cs^+ may work effectively to relieve Na^+ block. Rb^+ carries current through the channel and appears to bind more tightly to the channel than K^+, as reflected by the smaller current carried by Rb^+. If Rb^+ binds more tightly to the channel than K^+, and if relief is the result of binding of the external cation and repulsion between the bound ion and the blocking Na ion, Rb^+ might be expected to be more effective at relieving block than K^+, since its occupancy of the binding site would be higher.

Fig. 4A shows that the impermeant ion Cs and the permeant blocking ion Rb are both effective in relieving block by Na ions when applied to the outside. In fact, a direct comparison of the relief produced by external Rb^+ or Cs^+ with that produced by external K^+ shows that the three ions are quantitatively indistinguishable in their ability to relieve Na^+ block (Fig. 4B; the data for Rb^+ are not shown, but compare the relief in Fig. 4A). This suggests that whatever their differences in interacting with the rest of the channel, K, Cs, and Rb ions have very similar properties in the outermost part of the channel with which they interact in the process of displacing Na ions from the channel, a process we may call "knock-out."

Two Alternative Schemes for Knock-Out

The data presented here on the Ca^{2+}-activated K^+ channel in chromaffin cells and the work by Armstrong (1971, 1975a, b) showing the effects of external K^+ on nonyltriethylammonium (C9) block of the squid delayed rectifier K^+ channel demonstrate convincingly that external cations can speed the exit of a blocker from these K^+ channels. The physical mechanism that underlies this phenomenon remains obscure, however. There are two general schemes that can account for the results.

The first scheme, which I will term kinetic knock-out, was formulated by Armstrong (1975a) to explain his and his collaborators' results on the delayed

rectifier channels of squid (Armstrong and Binstock, 1965; Armstrong, 1966, 1969, 1971, 1975a, b; Bezanilla and Armstrong, 1972) and frog node of Ranvier (Armstrong and Hille, 1972). The idea is that an occasional external ion has enough kinetic (thermal) energy to enter far enough into the channel, which is
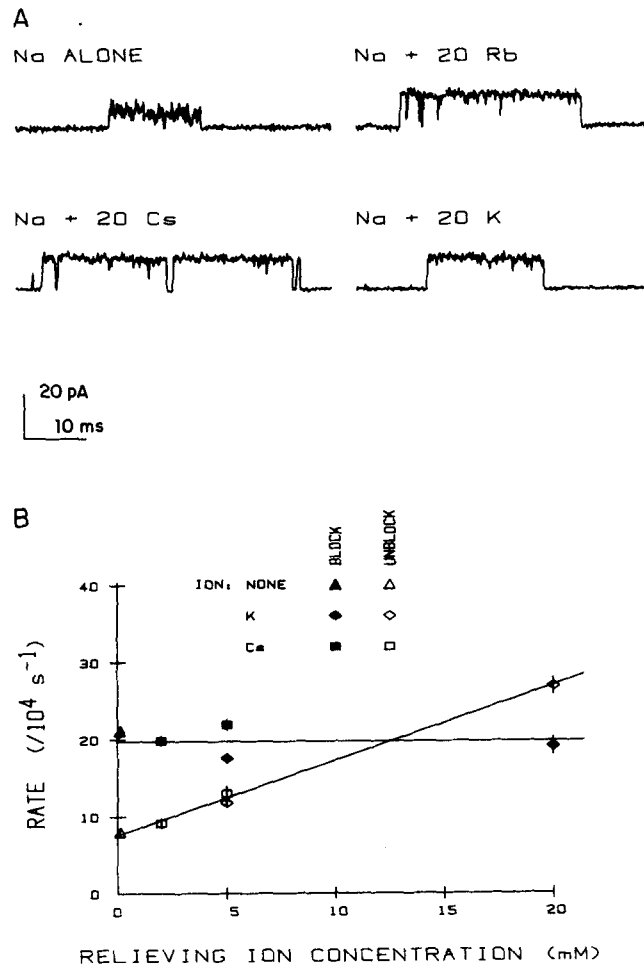
A

FIGURE 4.   External Cs^+ and Rb^+ also speed the exit of Na^+. (A) Single channel records with the indicated external solution, and an internal solution containing 160 K^+ + 20 Na^+. Membrane voltage was +60; data were filtered at 4 kHz. (B) Block and unblock rates with different concentrations of external K^+ or Cs^+. The lines (drawn by eye) fit the rate constants for both K^+ and Cs^+.

occupied by the blocking ion, to repel the blocker over its intrinsic energy barrier for exit. The probability that an external ion will have a high enough energy to knock out the blocker increases linearly with the external concentration of relieving ion.

The second scheme, which I will term equilibrium knock-out, is an implicit

property of permeation models with multiple ion occupancy and repulsion between ions in the channel (Hodgkin and Keynes, 1955; Hladky, 1972; Urban and Hladky, 1979; for review see Hille and Schwarz, 1978). The possibility that such models could explain many of the complexities of K^+ channels was explored by Hille and Schwarz (1978), who pointed out that the repulsive interactions between ions in the channel provided a possible mechanism for relief of block by permeant ions. One particular scheme, which may apply to the knock-out effects discussed here, involves external (relieving) ions binding and unbinding from the outer binding site in the channel; this binding is in rapid equilibrium compared with the long residence of a blocking ion in the inner site. Whenever the outer site is occupied, the net energy required for blocker exit is reduced by
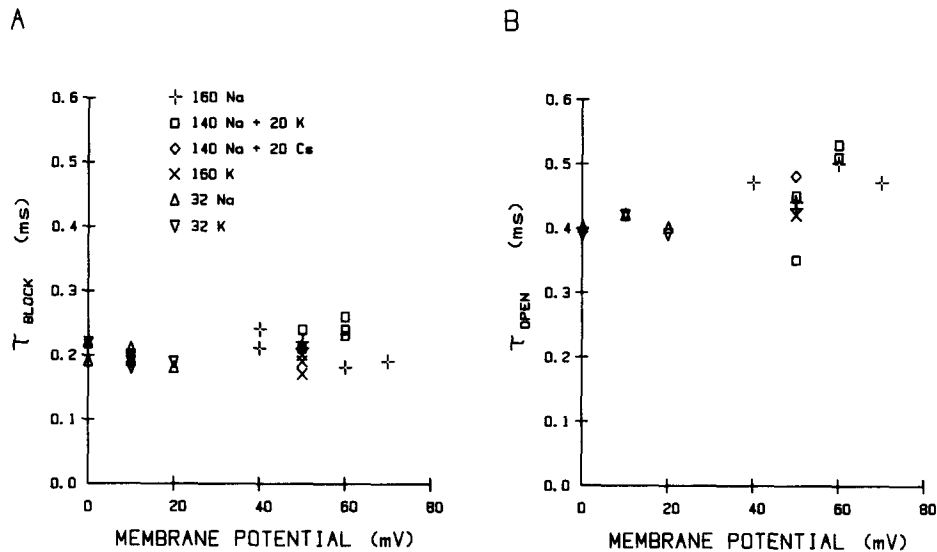

FIGURE 5.   Block by internal C_9 is not relieved by external K^+. Blocked and open lifetimes were determined by fitting an exponential function to the closed and open duration distributions. The internal solution for all experiments was 160 K^+ + 10 μM C_9; the external solution was as indicated. Each point is from the fit to a single histogram; data from three different patches are shown.

the positive energy of repulsion between the blocker and the relieving ion. The average rate of blocker exit is the average of the exit rates with and without the outer site occupied, weighted by the probability that the outer site will be occupied. This occupancy probability depends on the external concentration of relieving ion.

In principle, these two schemes can be distinguished experimentally. The kinetic scheme predicts that the rate of blocker exit will increase indefinitely as the relieving ion concentration is increased; the equilibrium scheme predicts that at high relieving ion concentrations, a maximum exit rate will be reached when the outer site (to which the relieving ions bind) is saturated. Of course, an equilibrium model may saturate only at extremely high, experimentally inacces-

sible concentrations, and even a channel obeying the kinetic model will show some saturation phenomena at high enough concentrations. Nonetheless, because of the low ion concentrations required for relief, it seems conceivable that saturation would occur at moderate concentrations. Indeed, the permeation model used in Yellen (1984b) predicts saturation for relief with a $K_{1/2}$ of ~150 mM.

Unfortunately, I have insufficient time resolution in my experiments to measure the extremely rapid exit rate of Na$^+$ at high external K$^+$ concentrations. This experiment would be practical for a blocker whose intrinsic exit rate was much slower; unfortunately, C$_9$, which does have a much slower exit rate for this K$^+$ channel, is completely unaffected by external K$^+$ concentration (Fig. 5). Ba ion block, which has been studied in a similar Ca^{2+}-activated K$^+$ channel from T-tubule membrane, is relieved by external K$^+$, but this appears to be a true competition instead of an effect on Ba^{2+} exit (Vergara and Latorre, 1983). Perhaps this critical experiment can be performed if a slow blocker of the Ca^{2+}-activated K$^+$ channel is found that is susceptible to knock-out, or if C$_9$ block of squid delayed rectifier K$^+$ channels (which is susceptible to knock-out; Armstrong, 1971, 1975a, b) is studied to determine the concentration dependence of K$^+$ relief of block at high K$^+$ concentrations.

It is difficult to decide between the two schemes on the basis of the present evidence. The equilibrium scheme requires more moderate quantitative assumptions than the kinetic knock-out scheme (see Yellen, 1984a); a two-site model with repulsion is therefore used in modeling permeation in the channel (Yellen, 1984b).

DISCUSSION

Block and Relief in This Channel Are Similar to That in K$^+$ Channels of Other Preparations

The ionic block reported here for the Ca^{2+}-activated K$^+$ channels of chromaffin cells is very similar to that observed in the well-studied delayed rectifier K$^+$ channels of squid giant axon (Chandler and Meves, 1965; Adelman and Senft, 1966; Bezanilla and Armstrong, 1972) and node of Ranvier (Bergman, 1970). The K$^+$ channels in both of these preparations are blocked by Na$^+$ and Cs$^+$, and this block is enhanced by depolarization.

Relief of block by external cations is also well known in other preparations. Bezanilla and Armstrong (1972) showed that the block of K$^+$ channels by internal Na$^+$ or Cs$^+$ could be relieved by raising external K$^+$ concentrations; relief could even be noticed when external K$^+$ was raised by the accumulation of K$^+$ in the region of restricted diffusion around the axon (Frankenhaeuser and Hodgkin, 1956) produced by prolonged outward K$^+$ current. Dubois and Bergman (1977) showed that external Cs$^+$ increased the outward K$^+$ current of the node of Ranvier. This observation suggested that even impermeant ions can relieve block; it is consistent with the present direct observation that external Cs$^+$ can relieve the block of the Ca^{2+}-activated K$^+$ channel produced by internal Na$^+$.

The flickery block produced by internal Na$^+$ and the relief of block by external K$^+$ or Cs$^+$ also occur in channel currents of the Ca^{2+}-activated K$^+$ channels in

another secretory cell, the continuous pituitary cell line GH_3 (American Type Culture Collection No. 82.1; G. Yellen, unpublished results).

The most telling experiments on the mechanism of relief of block are the studies by Armstrong (1966, 1969, 1971, 1975a, b) on the blocking action of tetraethylammonium ion (TEA^+) and its derivatives. C_9, a long-chain derivative of TEA^+, effectively blocks K^+ channels from the inside with high affinity. Block by C_9 is voltage dependent, and it can be relieved by raising the external K^+ concentrations (Armstrong, 1971; also shown for nodal K^+ channels by Armstrong and Hille, 1972). C_9 is driven out of the channels by a brief hyperpolarizing voltage pulse between two depolarizing pulses (which open the channels); the rate of C_9 exit from the channels can be evaluated by changing the duration of the hyperpolarizing pulse. C_9 exit, as measured in this way, can be speeded by raising the external K^+ concentration. The interpretation of this result is slightly complicated because C_9 exit is affected by the gating of the K^+ channel, which is itself affected by external K^+ (see Swenson and Armstrong, 1981), but the straightforward interpretation of this result is that external K ions can expel a C_9 ion, perhaps by knocking it out.

The Channel Has at Least Two Selectivity Sites

The behavior of Na^+ in this channel argues for at least two distinct selectivity sites, one near the outside solution and one near the inside. First, there must be a selective barrier slowing Na^+ entry from the outside, since block by external Na ions, if it occurs, is much weaker than block by internal Na^+. Second, there must be a barrier that makes Na^+ entry from the inside solution more difficult than K^+ entry. The net flux of K ions through the channel is a lower limit for the rate of K^+ entry; a current of 20 pA corresponds to an entry rate of ~7.2 × 10^8 M^{-1} s^{-1}. The rate for Na^+ block is much smaller: ~5 × 10^6 M^{-1} s^{-1} under the same conditions. This corresponds to an additional barrier for Na^+ entry of at least ~4 kT (2.4 kcal/mol).

The outer part of the channel allows K^+, Rb^+, and Cs^+ to enter, but not Na^+ (see Table I for a summary of permeation behavior). If the equilibrium scheme for knock-out is correct, then the outer part of the channel binds these three ions equally well (since they relieve block equally well), but the rate of binding and unbinding may be different for the different ions. Either the rate of Na^+ binding or the affinity of Na^+ for the outer site must be much lower, since external Na^+ is much less effective (if at all) in relieving internal Na^+ block.

The inner part of the channel probably binds Rb^+ more tightly than K^+. The reduced conductance when Rb^+ carries current through the channel (Yellen, 1984b, Fig. 2) indicates that Rb^+ blocks; since the outer site should have the same affinity for K^+ and Rb^+, as judged by their efficacy in relieving block, the Rb^+ block probably occurs at the inner site.

The complexity of the behavior of different cations in this channel indicates that the channel selectivity is not located at a single identifiable "selectivity filter" site. Either there are multiple specific sites in the channel that select between ions, or selectivity is a more physically diffuse property of the channel, as one might expect from a semi-rigid pore that selects between ions on the basis of

their close fit to the coordination groups within the channel (Mullins, 1959; Bezanilla and Armstrong, 1972; Hille, 1975).

Evidence for a High-Affinity Channel

The low concentrations of K^+, Rb^+, or Cs^+ required to relieve the block by internal Na^+ suggest that the channel has a very high affinity for these ions, even when it is occupied by a second (blocking) ion. K ions will leave this high-affinity site slowly, unless their leaving rate is increased by the binding of a second ion to the channel. Thus, the proposed model for this channel (Yellen, 1984b, Fig. 11) has two high-affinity sites for K^+. When both sites are filled, both bound ions leave the channel rapidly because of the repulsion between them; when only a single ion is in the channel, it leaves slowly because of the tight binding interaction. In the model, then, K ions flow through the channel by repeated knock-out: a singly occupied channel becomes filled by a second ion, and then the doubly occupied channel expels one of the two ions. The net current flow is determined by which side—the inside or the outside—provides most of the ions that enter the channel, and to which side most of the ions from doubly occupied

TABLE I

Summary of Ion Permeation Properties in the Chromaffin Cell Ca^{2+}-activated K^+ Channel

Ion	Carries current?	Blocks?		Relieves block?
		Inside	Outside	
K	Yes	(Not applicable)		Yes
Na	No	Flickery	Weak if any	No*
Cs	No	Weak	Weak; very V-dependent	Yes
Rb	Yes (less than K)	?	Permeant block	Yes
TEA	No	$K_d \approx 30$ mM	Flickery; $K_d \approx 0.2$ mM	

* Na^+ serves as the reference ion for relief of block.

channels are expelled. A similar mechanism has been proposed by Hess and Tsien (1984) to explain permeation and selectivity in a voltage-activated Ca^{2+} channel of cardiac muscle.

The repulsive interaction between ions bound to the two sites in the channel will appear also as a negative cooperativity in ion binding to the channel. This negative cooperativity may help explain the biphasic concentration dependence of the conductance observed for the Ca^{2+}-activated K^+ channel from T-tubules. Vergara et al. (1984) have shown that the conductance of this channel increases very sharply when the concentration is raised from 0 to 1 mM, and then slowly saturates as the concentration is raised further. This behavior might be expected from a two-ion model with repulsion: the first ion would bind to the channel with high affinity, but the second ion would bind with lower affinity because of repulsive interaction with the ion already bound.

What Physiological Relevance May Block and Relief Have?

The concentration of internal Na^+ required for significant block of the Ca^{2+}-activated K^+ channel seems comparable to the amount of Na^+ inside cells. Indeed,

if a cell-attached patch on a chromaffin cell is depolarized by ~100 mV over the cell's resting potential, channel currents through the Ca^{2+}-activated K^+ channels appear very flickery (G. Yellen, unpublished observation), which is consistent with the presence of intracellular Na^+ concentrations on the order of 5–20 mM. Similarly, the millimolar concentrations of K^+ required to relieve block are in the same range as extracellular K^+ levels. The block process is very voltage dependent, so block and its relief will only be important at depolarized voltages (about +40 mV and above), such as those reached during action potentials.

Ca^{2+}-activated K^+ channels probably make a significant contribution to the repolarization of the action potential in secretory cells. In both chromaffin cells and cultured pituitary cells (GH$_3$), there are many (5–10) Ca^{2+}-activated K^+ channels in an average patch; each open channel has a shunt conductance of 260 pS, as compared with a total resting conductance of about 500–1,000 pS (1–2 GΩ) for the entire cell. Even at resting Ca^{2+} levels, Ca^{2+}-activated K^+ channels can be 3% activated at +80 mV (with 0.3 μM internal Ca^{2+}). Thus, even small changes in the Ca^{2+}-activated K^+ conductance produced by intracellular accumulation of Na^+ or extracellular accumulation of K^+ may significantly change the repolarizing current and therefore the duration of the action potentials in secretory cells.

Extracellular K^+ levels can change significantly during activity; these changes may be sufficient to relieve tonic block of K^+ channels by intracellular Na^+, and thus affect the duration of the action potential in some excitable cells. Bezanilla and Armstrong (1972) showed that K^+ accumulation around the squid axon (Frankenhaeuser and Hodgkin, 1956) during a depolarizing voltage clamp pulse was sufficient to relieve significantly the block of the delayed rectifier by internal Na^+. Raising external K^+ is known to shorten the action potentials in cardiac muscle (Ringer, 1883); this effect is partly explained by the "crossover effect" of external K^+, an increase in the outward K^+ currents (McAllister and Noble, 1966; see Noble, 1979).

Significant changes in extracellular K^+ levels with activity are also known in other systems (Baylor and Nicholls, 1969; Hounsgaard and Nicholson, 1983). Hounsgaard and Nicholson have shown that extracellular K^+ concentrations rise during spontaneous firing of Purkinje cells in cerebellar slices; the concentration they measure with extracellular ion-sensitive microelectrodes changes from 6 to 7 mM, but this change is a lower limit for the changes that occur in the confined extracellular space between closely approximated cells. Yarom and Spira (1982) have shown that a single action potential in one giant interneuron of the cockroach can significantly depolarize the adjacent giant interneuron; this depolarization appears to be produced by local extracellular accumulation of the K^+ that flows out of the neuron during the action potential. This depolarizing "postsynaptic" potential has an extrapolated reversal potential of ~0 mV, which indicates a significantly elevated external K^+ concentration produced by just a single action potential. It seems possible that one way in which elevated external K^+ might modify the electrical activity of excitable cells is by its effect on block of Ca^{2+}-activated K^+ channels.

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