TRAPPING SINGLE IONS INSIDE SINGLE ION CHANNELS

CHRISTOPHER MILLER
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

ABSTRACT Single Ca^{++}-activated K^+ channels from rat muscle plasma membranes are inhibited by Ba^{++}. A single Ba^{++} entering the channel's conduction pore induces a long-lived blocked state. This study employs Ba^{++} as a probe of the channel's conduction pathway to show that the channel can be forced to close with a single Ba^{++} ion inside the pore. A Ba^{++} ion inside the closed channel is trapped and cannot escape until the channel opens. The results demonstrate that in the channel's closed state, the cytoplasmic side of the conduction pore is obstructed to the passage of ions.

INTRODUCTION

Many cells carry in their plasma membranes a high-conductance, K^+-specific channel which is activated by cytoplasmic Ca^{++} (1–4). Nothing is known about this Ca^{++}-activated K^+ channel's molecular structure other than the fact that it must be a membrane-spanning protein that contains a hydrophilic pore running from one side of the membrane to the other (5, 6). It is this pore that, in the channel's "open," K^+-conducting conformation, provides the pathway through which K^+ diffuses and that somehow becomes shut off when the channel closes. The purpose of this study is to develop a picture of the channel's conduction pathway, specifically of the way in which the conformational change leading to channel closure obstructs the pore to the passage of conducting ions.

These experiments use Ba^{++} ion, a high-affinity inhibitor of this channel, as a probe of the conduction pathway. While numerous K^+-selective channels are known to be inhibited by Ba^{++} (6–10), only in the case of this Ca^{++}-activated K^+ channel has Ba^{++} action been studied mechanistically at the single-channel level. Vergara and Latorre (10) showed that Ba^{++} blocks ion conduction competitively with K^+; Ba^{++} enters the pore, where it binds with an affinity at least 10^5-fold higher than that of K^+, and from which it dissociates at least 10^8-fold more slowly than does K^+.

By reconstituting single Ca^{++}-activated K^+ channels into planar lipid bilayers, it is possible to observe directly the interaction of single Ba^{++} ions with single channels. I use this capability here to show that the channel can be driven into its closed conformation with a Ba^{++} still on its blocking site within the conduction pore. Under these circumstances the Ba^{++} ion is occluded within the channel protein, unable to gain access to the aqueous solutions on either side of the channel. The results strongly suggest that in the closed conformation of the channel an obstruction to ion permeation exists on the cytoplasm-facing side of the conduction pore.

MATERIALS AND METHODS

Biochemical

Lipids used were 1-palmitoyl, 2-oleoyl phosphatidylethanolamine (POPE), and the analogous phosphatidylcholine (POPC), obtained from Avanti Polar Lipids Inc. (Birmingham, AL). Chemicals used were reagent grade. Transverse tubule vesicles were prepared from rat skeletal muscle as described (3) and were stored in 0.4 M sucrose at −70°C.

Planar Bilayers and Single Channel Analysis

Single Ca^{++}-activated K^+ channels were inserted into planar lipid bilayers exactly as described previously (11). Briefly, bilayers were formed by applying a phospholipid solution (21 mM POPE/9 mM POPC in *n*-decane) on a 300-μm diameter hole in a plastic partition separating the two aqueous solutions. The "internal" and "external" solutions were: 150 mM KCl, 30 μM $CaCl_2$, 10 mM Hepes-KOH, pH 7.4, and 0.1 mM EGTA, 10 mM Hepes-KOH, pH 7.4, respectively. Bilayers were voltage clamped, and ionic current was recorded with a laboratory computer system (Indec Systems). Transverse tubule vesicles were added to the internal solution (1–5 μg/ml) with stirring, and current was monitored until a single channel appeared in the membrane (usually <10 min). At this point, 170 mM NaCl was added to the external solution to suppress further channel insertion. With this procedure, channels always incorporate into the bilayer such that the "internal" solution corresponds to the cytoplasmic side of the channel. Voltage is reported according to the electrophysiological convention, with the external solution defined as zero voltage. Single-channel records were collected and analyzed as described (11).

RESULTS AND DISCUSSION

Fig. 1 illustrates the effect of Ba^{++} on the channel. In the absence of Ba^{++}, the channel opens and closes stochastically on a rapid time scale (1–10 ms); low concentrations (0.1–1 μM) of Ba^{++} added to the internal solution induce long-lived nonconducting intervals separated by "bursts"

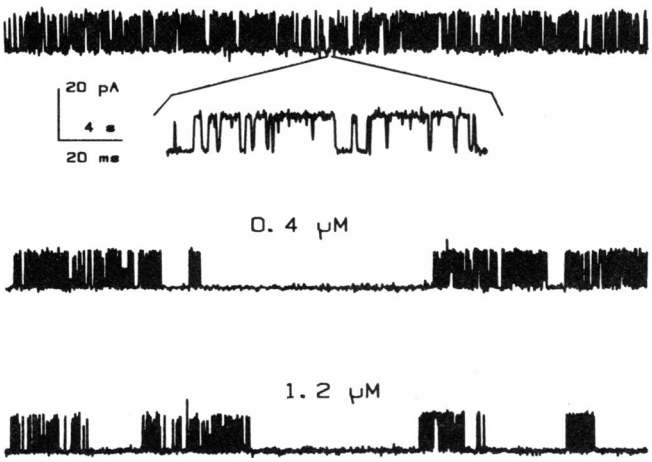

FIGURE 1 Ba^{++} block in the Ca^{++}-activated K^+ channel. A single Ca^{++}-activated K^+ channel from rat muscle plasma membranes was reconstituted into a planar lipid bilayer and was recorded at 45 mV holding potential, in the absence of Ba^{++} (upper trace), or with 0.4 or 1.2 μM Ba^{++} added to the internal solution. Expanded time scale trace is also displayed to show the rapid opening and closing events.

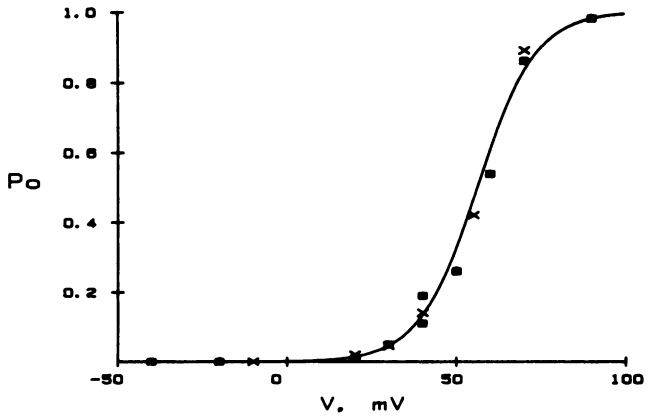

FIGURE 2 Voltage-dependent gating in the presence and absence of Ba^{++}. A channel was incorporated into a planar bilayer as in Fig. 1, and records were collected at the indicated holding voltages (30–60 s at each voltage). Probability of being in the open state, p_o, was calculated as the fraction of time spent open during each record. Ba^{++} (1.9 μM) was then added to the internal solution, and open-state probability was again measured, but now only during intervals of time in which the channel was not in a blocked state. Solid squares and crosses indicate the absence and presence of Ba^{++}, respectively.

of channel activity. Vergara and Latorre (10) showed that each of these long-lived "blocking" events represents the binding of a single Ba^{++} ion to the channel, and they presented strong evidence that the site of Ba^{++} binding is located within the K^+ conduction pore. They proposed a simple model in which the closed channel must enter an open state before it can be blocked by Ba^{++}.

$$\text{CLOSED} \rightleftharpoons \text{OPEN} \rightleftharpoons \text{BLOCKED}$$

Scheme I

This model envisions a single blocked state in which the channel protein remains in its "open" conformation with Ba^{++} on its blocking site. It is also possible, however, that the channel can close even while Ba^{++} remains within the conduction pathway.

This study is designed to investigate the possibility of trapping Ba^{++} inside the closed channel. To do this, I exploit the fact that this channel is voltage-dependent, and that its open conformation may be favored by changing the transmembrane voltage in the positive, or "depolarizing" direction (1–4). Fig. 2 illustrates this voltage dependence; the probability of channel opening increases with depolarization in the absence of Ba^{++} and an identical voltage dependence is observed for the unblocked channel in Ba^{++}-containing medium. Based upon the rapidity (1–10 ms) with which the channel responds to instantaneous voltage changes (4), a "Ba^{++} trapping" experiment is constructed as follows. A single channel is recorded in the presence of Ba^{++} at a "holding voltage" of 40–50 mV, at which the unblocked channel is often open. When a Ba^{++} blocking event occurs, a strongly hyperpolarizing "test voltage" of -70 mV is applied in an attempt to drive the channel, now blocked by a Ba^{++} ion, into its closed conformation. The test voltage is maintained for a time long compared with the average time Ba^{++} normally resides in the open channel (~3 s). Now the voltage is returned to the original holding voltage, and we ask whether the channel is blocked or unblocked at the moment after this voltage change.

Fig. 3 displays a Ba^{++} trapping experiment. After a Ba^{++} blocked state is recognized at a holding voltage of 45 mV, a test voltage of -70 mV is applied for 15 s (top

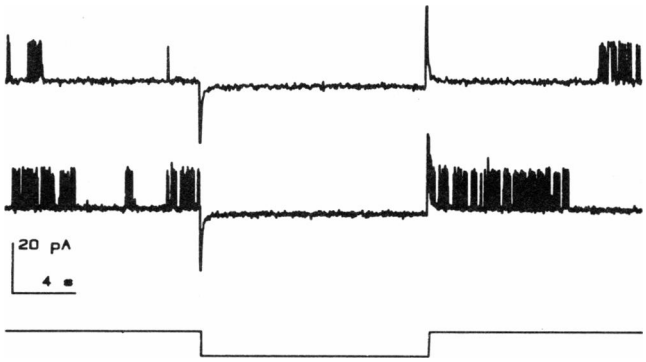

FIGURE 3 Ba^{++} trapping experiment. A channel was exposed to 1.9 μM internal Ba^{++} at a holding potential of 45 mV, and the voltage pulse protocol was applied as described in the text. Upper trace: a 15-s hyperpolarizing test pulse to -70 mV was applied during a blocked interval. The computer recognized a blocking event as a nonconducting state lasting for longer than 800 ms. Under these conditions, the average block time is 3 s, while the average closed time is 2 ms; the identification of the blocked state is therefore unequivocal. Lower trace: a similar pulse applied during a burst, when the channel is not blocked. To identify whether the channel is blocked or unblocked after the test pulse, the channel record is examined immediately after returning to 45 mV; if the channel fails to open during the first 200 ms after returning to 45 mV, the channel is scored as being blocked. In this example, the first latency to opening was 11 s in the upper trace, and <10 ms in the lower trace.

trace). Immediately after returning to the holding voltage, the channel is still blocked. As long as the test voltage is made strongly negative, these results are obtained every time the experiment is repeated: the channel is always blocked after a test pulse applied during a blocked interval. It is essential to realize that this is not a trivial consequence of an inherently voltage-dependent rate of Ba^{++} dissociation from the open channel; the open-channel dissociation rate is, in fact, independent of voltage (10), and the polarity of the test voltage is in a direction tending to promote, rather than retard, the exit of Ba^{++}. If the same test pulse is given at a time when the channel is unblocked (Fig. 3, second trace), then the channel is still unblocked upon return to the holding voltage. This latter experiment shows that Ba^{++} does not block the channel at -70 mV, and thus rules out the possibility that the channel becomes unblocked and reblocked during the hyperpolarized test interval. We therefore conclude that when a hyperpolarizing test pulse is given during a blocking event, Ba^{++} remains on its blocking site throughout the entire test interval.

The effect of test pulse duration is examined in Fig. 4. Test pulses of varying lengths were applied during either a block or a burst. For each pulse duration, a set of 10–30 repetitive pulses was given, and the probability of remaining blocked during the pulse was calculated from each set. We see that a channel initially blocked remains blocked, even up to pulse durations of a minute, which is 20-fold longer than the average block time of the open channel. Likewise, a channel initially unblocked remains unblocked. It is as though time stands still for Ba^{++} as long as the channel is held at a high negative potential.

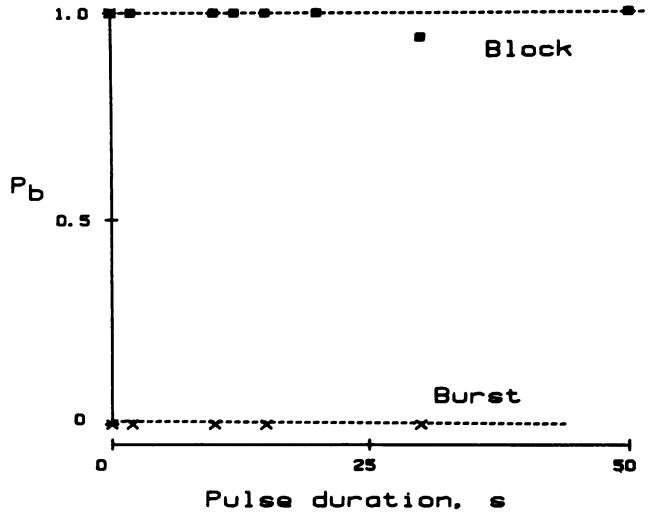

FIGURE 4 Time dependence of Ba^{++} trapping. Experiments as in Fig. 3 were performed, except that the test pulse duration was varied. For each duration a set of 12–30 repeated test pulses was applied, and the state of the channel (blocked or unblocked) immediately after the pulse was determined. The probability of being blocked after the pulse, P_b, was calculated for each set of pulses. Test pulses were initiated during either a blocked (■) or a bursting (×) interval.

A straightforward explanation for this result is that in response to the application of negative potential, the channel closes rapidly (in less than a millisecond) and traps the Ba^{++} ion on its binding site within the pore. This picture envisions the Ba^{++} ions as occluded within the channel protein, unable to communicate with the internal aqueous solution from which it had originally entered the channel. If this picture is correct, it should be possible to keep the Ba^{++} ion trapped while removing all Ba^{++} from the aqueous solution. Fig. 5 shows that this expectation is borne out. A Ba^{++} ion is trapped as before, and then, with the negative test voltage maintained, all the Ba^{++} is removed by perfusion with EDTA-containing medium. (This maneuver also removes the Ca^{++} needed for channel activation and further ensures that the channel will stay closed during the test interval.) After 5 Ba^{++}-free minutes, Ca^{++} is added back, and the voltage is returned to 55 mV. The figure shows that the channel is still blocked; several seconds later this channel became unblocked, never to be blocked again, since the internal aqueous solution now contains only the single Ba^{++} ion released by the channel. This perfusion experiment has been repeated on seven different channels, with the same unequivocal result.

The interpretation of these experiments is clear: Ba^{++} ions can be trapped in the Ca^{++}-activated K^+ channel for long periods of time by applying a strong hyperpolarizing voltage. The natural explanation for Ba^{++} trapping is that hyperpolarization rapidly closes the blocked channel in much the same way as it does the normal, unblocked channel. Ba^{++} thus becomes occluded within the conduction pathway and cannot escape until the channel is allowed to open. Ba^{++} shows no tendency to "leak" out of the occluded state even after 5 min, which is two orders of

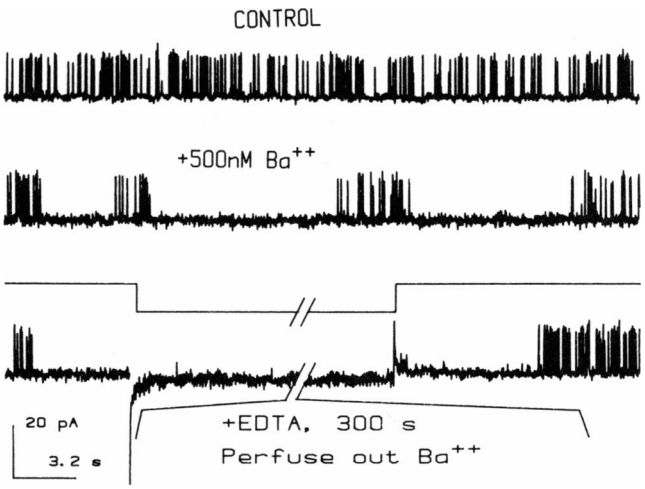

FIGURE 5 Occlusion of Ba^{++} within the channel. A channel was recorded at a holding potential of 55 mV and was shifted to a test voltage of -75 mV as indicated. The internal chamber was then perfused with 20 vol of normal medium containing 0.5 mM EDTA. After 4.5 min, $CaCl_2$ was added to a final concentration of 0.55 mM, and voltage was returned to 55 mV. In this example, the first latency of channel opening was 7.6 s.

magnitude longer than its average residence time in the open channel. We must therefore modify the model in Scheme I to include both open and closed conformations for the blocked channel:

$$\begin{array}{ccc} \text{CLOSED} & \overset{\text{fast}}{=\!=} & \text{OPEN*} \\ & & \Vert\ \text{slow} \\ \text{CLOSED/blocked} & \overset{\text{fast}}{=\!=} & \text{OPEN/blocked} \end{array}$$

Scheme II

Of these four states, only the OPEN* state allows ion conduction. Transitions between the CLOSED state and CLOSED/blocked state containing occluded Ba^{++} do not occur; Ba^{++} dissociates only from the channel's open conformation.

The phenomenon of Ba^{++} occlusion reinforces the evidence, already compelling (10), that the Ba^{++} blocking site lies within the channel's normal K^+ conduction pathway, where the ion binds with high affinity. The possibility has been discussed (6) that Ba^{++}, because of the similarity in its size to K^+, acts as a transition-state analogue for K^+ permeation through this channel. Since Ba^{++} equilibrates with its blocking site only from the internal aqueous solution (10), these results identify the internally facing side of the channel protein as carrying a "gating structure," where the ion conduction pore becomes obstructed when the channel assumes its closed conformation. It is intriguing that the same sidedness of a gating region of a different K^+ channel, the delayed rectifier of squid axon, was deduced many years ago by Armstrong (12), on the basis of occlusion of tetraethylammonium ions.

I thank Drs. Paul Adams and Alison Gurney, whose skepticism provoked these experiments.

This research was supported by National Institutes of Health grants GM-31768 and AM-19826.

Received for publication 16 February 1987.

REFERENCES

1. Marty, A. 1981. Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature* (*Lond.*). 291:497–500.
2. Barrett, J. N., K. L. Magleby, and B. S. Pallota. 1982. Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol.* (*Lond.*). 331:211–230.
3. Latorre, R., C. Vergara, and C. Hidalgo. 1982. Reconstitution in planar lipid bilayers of Ca^{2+}-dependent K^+ channel from transverse tubule membranes isolated from rabbit skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 79:805–809.
4. Latorre, R. 1986. The big beautiful one. *In* Ion Channel Reconstitution. C. Miller, editor. Plenum Publishing Corp., New York. 431–467.
5. Armstrong, C. M. 1974. Ionic pores, gates, and gating currents. *Q. Rev. Biophys.* 7:179–210.
6. Latorre, R., and C. Miller. 1983. Conduction and selectivity in potassium channels. *J. Membr. Biol.* 71:11–30.
7. Armstrong, C. M., and S. R. Taylor. 1980. Interaction of barium ions with potassium channels in squid giant axons. *Biophys. J.* 30:473–488.
8. Eaton, D. C., and M. S. Brodwick. 1980. Effect of barium on the potassium conductance of squid axons. *J. Gen. Physiol.* 75:727–750.
9. Miller, C. 1978. Voltage-gated cation conductance channel from fragmented sarcoplasmic reticulum. *J. Membr. Biol.* 40:1–23.
10. Vergara, C., and R. Latorre. 1983. Kinetics of Ca^{++}-activated K^+ channels from rabbit muscle incorporated into planar bilayers: evidence for a Ca^{++} and Ba^{++} blockade. *J. Gen. Physiol.* 82:543–568.
11. Eisenman, G., R. Latorre, and C. Miller. 1986. Multi-ion conduction and selectivity in the high-conductance Ca^{++}-activated K^+ channel from skeletal muscle. *Biophys. J.* 50:1025–1034.
12. Armstrong, C. M. 1971. Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J. Gen. Physiol.* 58:413–437.