Mechanism of Charybdotoxin Block of the High-Conductance, Ca^{2+}-activated K^+ Channel

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ABSTRACT The mechanism of charybdotoxin (CTX) block of single Ca^{2+}-activated K^+ channels from rat muscle was studied in planar lipid bilayers. CTX blocks the channel from the external solution, and K^+ in the internal solution specifically relieves toxin block. The effect of K^+ is due solely to an enhancement of the CTX dissociation rate. As internal K^+ is raised, the CTX dissociation rate increases in a rectangular hyperbolic fashion from a minimum value at low K^+ of 0.01 s^{-1} to a maximum value of $\sim$0.2 s^{-1}. As the membrane is depolarized, internal K^+ more effectively accelerates CTX dissociation. As the membrane is hyperpolarized, the toxin dissociation rate approaches 0.01 s^{-1}, regardless of the K^+ concentration. When internal K^+ is replaced by Na^+, CTX dissociation is no longer voltage dependent. The permeant ion Rb also accelerates toxin dissociation from the internal solution, while the impermeant ions Li, Na, Cs, and arginine do not. These results argue that K ions can enter the CTX-blocked channel from the internal solution to reach a site located nearly all the way through the conduction pathway; when K^+ occupies this site, CTX is destabilized on its blocking site by $\sim$1.8 kcal/mol. The most natural way to accommodate these conclusions is to assume that CTX physically plugs the channel's externally facing mouth.

INTRODUCTION

Charybdotoxin (CTX), a protein from the venom of the scorpion *Leiurus quinquestriatus,* is a potent inhibitor of the high-conductance, Ca^{2+}-activated K^+ channel. The preceding study (Anderson et al., 1988) showed that CTX blocks this channel in a simple bimolecular process that is manifested at the single-channel level by the appearance of long-lived (10–100 s), nonconducting, or "blocked," intervals. Each of these blocked intervals represents the binding of a single CTX molecule to the single channel. We further found that CTX can bind to both the open and closed conformations of the channel, and that depolarization increases the rate of CTX dissociation from its blocking site.

In this study, we continue to pursue the interaction of CTX with the Ca^{2+}-activated K^+ channel. Specifically, we examine the effect of K ions added to the

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internal aqueous solution, on the side of the membrane opposite to the CTX-binding site. We find that internal K ions specifically enhance the dissociation of CTX to the external solution. The detailed characteristics of this "*trans*-enhanced dissociation" naturally rationalize the voltage dependence of CTX dissociation, and they argue strongly that CTX blocks the channel by physically occluding the K^+-conduction pore.

MATERIALS AND METHODS

All observations of CTX block of single Ca^{2+}-activated K^+ channels in planar lipid bilayers were made as described in the preceding article (Anderson et al., 1988). Single Ca^{2+}-activated K^+ channels from rat skeletal muscle plasma membranes were inserted into planar phospholipid bilayers, and purified CTX was added to the external solution to induce blocking events. The rate constants of association and dissociation of the toxin were determined, as in the preceding article, from the measured distributions of dwell times of the unblocked and blocked channels, respectively. In all experiments, the external aqueous composition was kept fixed (150 mM KCl, 10 mM MOPS, 0.1 mM EGTA, 6 mM KOH, pH 7.40). Many experiments required variation of K^+ in the internal medium. This was accomplished by inserting single channels into the bilayer with 150 mM KCl medium in the internal chamber. Immediately after the incorporation of a channel, the internal solution was perfused extensively with a low-K^+ medium (usually 50 mM KCl, 10 mM MOPS, 0.03 mM $CaCl_2$, 6 mM KOH, pH. 7.40). CTX block was then induced by adding the toxin to the external chamber, and records were collected on video tape for later analysis.

Most experiments here were concerned with the dissociation rate of CTX from its blocking site. In some experiments, the association rate was also studied, and in these cases it was always necessary to perform the measurement under "probability-clamp" conditions (Anderson et al., 1988), i.e., to maintain the open probability of the unblocked channel by addition or removal of internal Ca^{2+}. This precaution was necessary because the association rate (but not the dissociation rate) is sensitive to the gating state of the unblocked channel.

RESULTS

Trans-enhanced Dissociation: Internal K^+ Relieves CTX Block

K ions added to the internal solution relieve the block of externally applied CTX. Fig. 1 presents raw single-channel records from a membrane with 150 mM K^+ and 5 nM CTX in the external solution, while internal K^+ was varied. As internal K^+ is increased, CTX becomes a less effective blocker, as can be discerned from the shortened blocked intervals.

We examined this effect of K^+ quantitatively by measuring the probability distributions of blocked and unblocked dwell times (Fig. 2). Under all conditions, the dwell-time distributions are single-exponential. Increasing internal K^+ elevates the CTX dissociation rate (measured from the time constant of the blocked-time distribution; Fig. 2 *A*) and leaves the association rate unchanged (Fig. 2 *B*). This result shows that internal K^+ does not act competitively with CTX, since a competitive mechanism demands that K^+ must reduce the blocker's association rate. The K^+-induced increase in CTX dissociation rate shows that

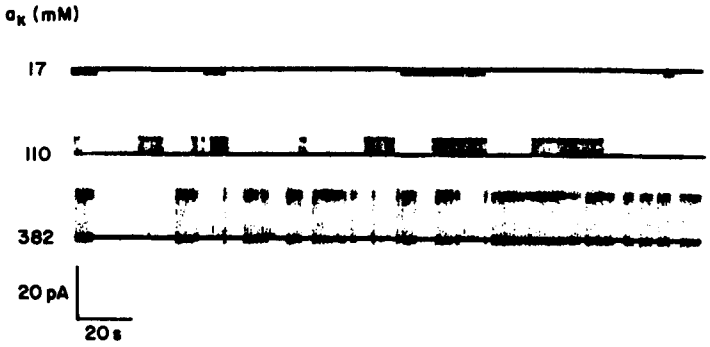

FIGURE 1. Relief of block by internal K^+. A single channel was observed at a holding voltage of 30 mV. The solution on the external side of the channel contained 100 mM KCl and 5 nM CTX, while KCl activity on the inside was increased, in a stepwise fashion, from 17 to 382 mM. Recordings are displayed at a low chart speed, so individual opening and closing events cannot be discerned.

internal K^+ is somehow able to destabilize CTX while the toxin resides on its externally facing blocking site. We term this effect "*trans*-enhanced dissociation." In the remainder of this study, we elucidate the nature of K^+-CTX interaction in this channel.

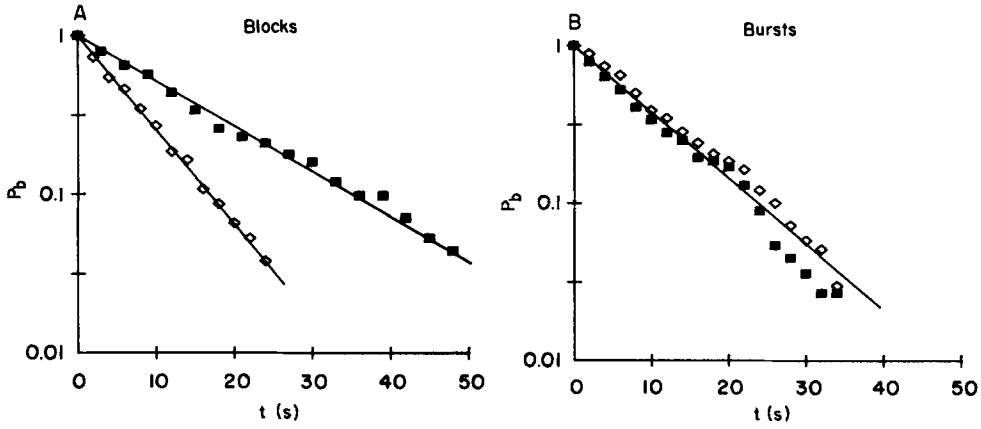

FIGURE 2. Effect of internal K^+ on dwell-time distributions. Unitary CTX blocking events in a single channel at 37 mV were recorded. The external solution contained 110 mM KCl, 23 nM CTX, and the internal solution contained 77 mM KCl (filled symbols) or 206 mM KCl (open symbols). Cumulative dwell-time distributions were constructed from more than 100 events for both blocked (*A*) and unblocked (*B*) intervals. A blocked interval was defined as a nonconducting interval of duration >500 ms, which is >50-fold longer than the mean closed time under these conditions. The time constants for the blocked distributions corresponding to 77 mM and 206 mM internal KCl were 15.7 and 7.5 s, respectively. The unblocked distributions fit a single exponential with a time constant of 10.6 s. The channel open probability, measured during the unblocked intervals, was maintained between 0.2 and 0.35 throughout the experiment.

Dependence of CTX Dissociation on Internal K^+ Concentration

As internal K^+ is raised, the CTX dissociation rate increases in a sublinear manner (Fig. 3). The inset of Fig. 3 presents a similar experiment in a lower K^+ concentration range and shows that the dissociation rate approaches a nonzero value of 0.01 s^{-1} as the K^+ concentration approaches zero. These results can be simply understood in terms of a model for CTX dissociation in which a channel with CTX bound (C:CTX) can be occupied by a K ion at some other site (K^+:C:CTX), as shown:

$$\begin{array}{ccc} \text{C:CTX} & \xrightarrow{\beta_{min}} & \text{C + CTX} \\ \text{fast}\ \uparrow\downarrow & & \\ \text{K}^+\text{:C:CTX} & \xrightarrow{\beta_{max}} & \text{K}^+\text{:C + CTX} \end{array} \qquad \text{(Scheme I)}$$

The basic idea is that CTX dissociation from the K^+-occupied channel is faster than that from the K^+-unoccupied channel (i.e., $\beta_{max} > \beta_{min}$). We assume that internal K^+ equilibrates with its site at much higher rates than CTX does with its site, and so the observed dissociation rate for CTX, k_{off}, is simply a weighted mixture of β_{min} and β_{max}:

$$k_{off} = \beta_{min} + \frac{a_K}{a_K + K_d}(\beta_{max} - \beta_{min}), \qquad (1)$$

where a_K is the internal K^+ activity and K_d is the apparent dissociation constant for K^+.

According to this scheme, as K^+ increases, the CTX dissociation rate should rise from a minimum value, β_{min}, eventually saturating at a maximum value, β_{max}. The data shown in Fig. 3 follow this expected behavior. The dissociation constant for K^+ is quite high, ~500 mM, and so we were not able to carry out experiments far above K_d, near saturation conditions. Nevertheless, all our data clearly show a sublinear increase in the rate constant with internal K^+, which is suggestive of the saturation of a K^+-binding site. The CTX dissociation rate at low K^+, β_{min}, is reproducible from membrane to membrane and has a value of ~0.01 s^{-1}, equivalent to a mean block time of 100 s, about sixfold longer than that seen with physiological internal K^+ (Miller et al., 1985; Anderson et al., 1988).

In the preceding study, we saw that the CTX dissociation rate is independent of the channel open probability (Anderson et al., 1988). In Fig. 4, we demonstrate that the ability of internal K^+ to relieve CTX block is also independent of the channel open probability. Here we measured the CTX dissociation rate constant as a function of internal K^+, at concentrations of internal Ca^{2+} adjusted to "clamp" the open probability to high (0.55–0.75) or low (0.05–0.2) values. The result of this experiment has both practical and mechanistic implications. In practical terms, it means that a rigorous "probability-clamp" protocol is not required for studying the effect of internal K^+ on the CTX dissociation rate. Mechanistically, the result means that *trans*-enhanced dissociation is not dependent on the rate of K ions entering the channel, but rather on the equilibrium occupancy of a K^+-binding site, as discussed below.

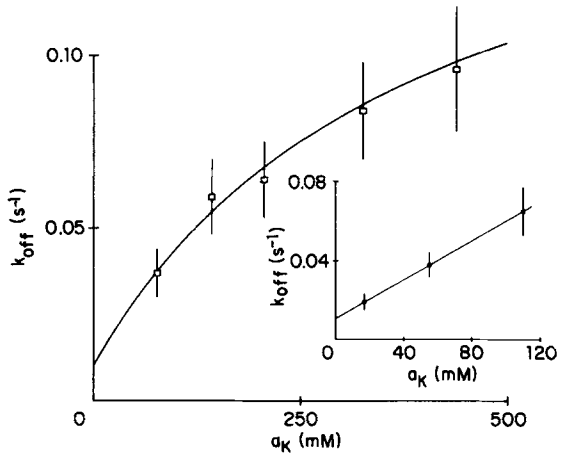

FIGURE 3. The effect of internal K^+ on the CTX dissociation rate. A single channel was observed in the presence of 35 nM CTX in the outside solution. Blocking events were recorded at various internal KCl activities. Each point represents the mean ± SEM of the CTX dissociation rate, k_{off}, determined from 23–50 blocking events. The solid curve is according to Eq. 1, with β_{min} = 0.01 s^{-1}, β_{max} = 0.177 s^{-1}, and K_d = 390 mM. The inset shows the relationship between k_{off} and internal KCl, measured in a different channel, over a low concentration range. The straight line through the points intercepts the ordinate at k_{off} = 0.01 s^{-1}. The external solution contained 110 mM KCl. The transmembrane voltage was 37 mV (graph) and 28 mV (inset).

Voltage Dependence of the Internal K^+ Effect

The preceding study (Anderson et al., 1988) showed that CTX dissociation is voltage dependent, and that depolarization speeds up the dissociation of the toxin. In this section, we propose an unusual mechanism for the voltage dependence of CTX: that it is not CTX itself that senses the transmembrane voltage,

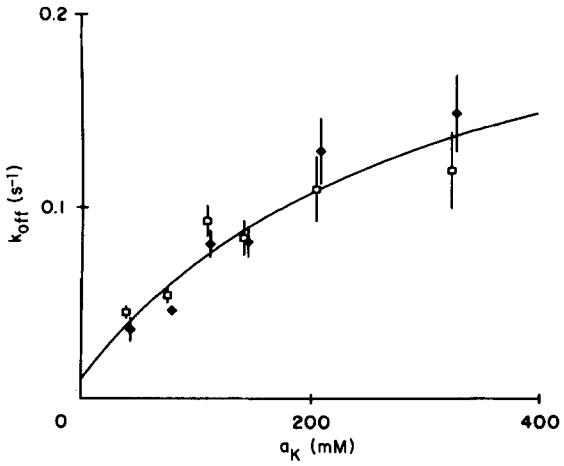

FIGURE 4. The effect of channel open probability on *trans*-enhanced dissociation. The CTX dissociation rate constant, k_{off}, was measured in single channels at low ($0.05 < p_o < 0.2$; diamonds) and high ($0.55 < p_o < 0.75$; squares) channel open probability. The open probability, monitored during unblocked intervals, was controlled by varying the internal Ca^{2+} concentration. Each pair of points represents the mean ± SEM of measurements made from three to six channels at the internal KCl concentration indicated on the abscissa. The external solution contained 110 mM KCl and 23 nM CTX. The transmembrane voltage was 37 mV. The curve is drawn according to Eq. 1, with β_{min} = 0.01 s^{-1}, β_{max} = 0.26 s^{-1}, and K_d = 310 mM.

but that depolarization renders K^+ more effective in accelerating CTX dissociation. Fig. 5 illustrates the effect of internal K^+ on CTX dissociation at two different voltages. From this double-reciprocal plot, it is evident that K^+ relief is stronger at the more depolarized potential. These results are consistent with the idea that only the K^+ dissociation constant is voltage dependent, and that the maximum off rate, β_{max} (Eq. 1), is independent of voltage. Unfortunately, the errors in the measurements of Fig. 5 are too large to make this claim rigorously; these experiments still allow the possibility that there is some voltage dependence to the maximum CTX off rate at saturating K^+; i.e., there is an inherent voltage dependence to the interaction of CTX with the channel. In the next

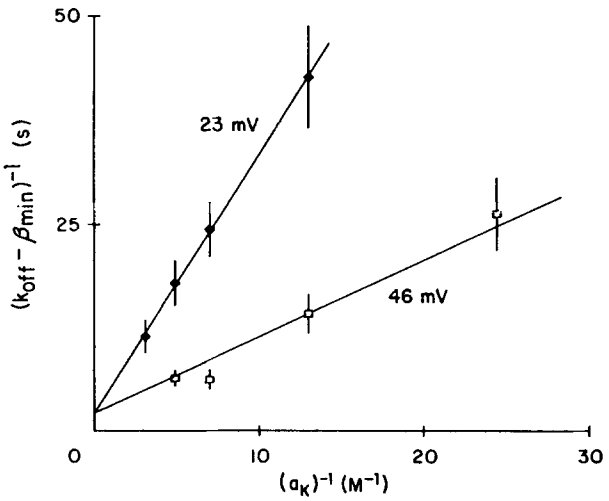

FIGURE 5. The voltage dependence of K^+-enhanced dissociation. The rate constant for CTX dissociation, k_{off}, was measured in a single channel as a function of internal KCl concentration at a holding voltage of 46 mV (open symbols) and 23 mV (filled symbols). The reciprocal of $k_{off} - \beta_{min}$ is plotted against the reciprocal of the internal K activity, according to a double-reciprocal transformation of Eq. 1. The straight lines were fitted by eye and correspond to $\beta_{min} = 0.01\ s^{-1}$, $\beta_{max} = 0.51\ s^{-1}$, $K_d(46\ mV) = 500$ mM, and $K_d(23\ mV) = 1{,}500$ mM. The external solution contained 110 mM KCl and 23 nM CTX.

section, however, we present strong evidence that CTX block is not inherently voltage dependent.

On the basis of this result, we extend Scheme I above to explain the voltage dependence of K^+-enhanced CTX dissociation. We propose, in harmony with Fig. 5, that the only voltage-dependent parameter in the interactions of CTX and K^+ with the channel is the dissociation constant for K^+. To explain this voltage dependence, we invoke a classic "Woodhull model" for the binding of internal K^+. The site at which K^+ exerts its effect on CTX dissociation is assumed to be located at an electrical distance δ across the transmembrane voltage drop, as measured from the inside. The K^+-site occupancy, and hence the

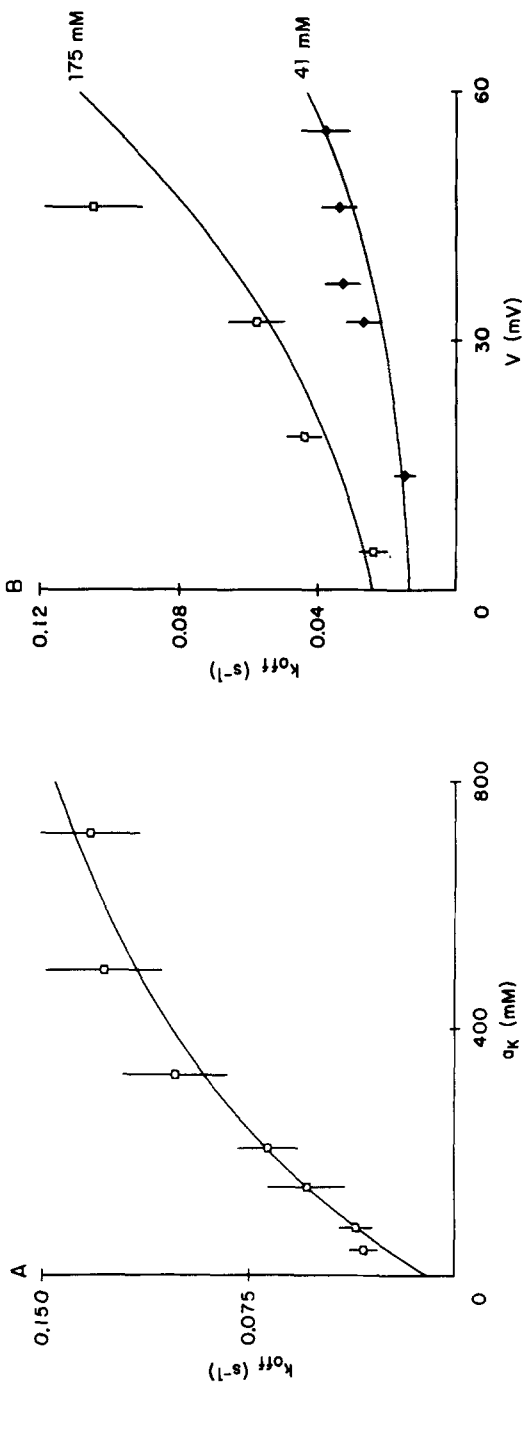

FIGURE 6. Internal K^+ and the voltage dependence of CTX block. (*A*) The CTX dissociation rate was measured as a function of internal KCl in a single channel at a holding voltage of 37 mV. The solid curve corresponds to Eq. 3 with $\beta_{min} = 0.01\ s^{-1}$, $\beta_{max} = 0.26\ s^{-1}$, $K_d(0) = 2{,}920$ mM, $\delta = 1.0$, and $V = 37$ mV. (*B*) In the same channel as in *A*, the CTX dissociation rate was measured as a function of transmembrane voltage at 41 mM (filled symbols) and 175 mM (open symbols) internal KCl. The solid curves correspond to Eq. 3 using β_{min}, β_{max}, and $K_d(0)$, which were independently determined in *A*. The external solution contained 110 mM KCl and 15 nM CTX.

CTX off rate, will therefore vary with voltage, V, since the dissociation constant for K^+ is given by (Woodhull, 1973; Coronado and Miller, 1979):

$$K_d(V) = K_d(0)\exp(-\delta FV/RT), \quad (2)$$

where $K_d(0)$ is the zero-voltage dissociation constant.

In principle, it is simple to determine δ by measuring K_d at several different voltages and applying Eq. 2. In practice, it is impossible to do this experiment for more than two voltages on a single channel, because of the time required to accumulate enough data at multiple K^+ concentrations and voltages. Therefore, by repeating the experiment of Fig. 5 on four separate channels, each at only two voltages, we obtained a rough estimate of δ of 1.1 ± 0.3. It appears, therefore, that the site accessible to internal K^+ is located all the way across the transmembrane voltage difference, as measured from the internal solution.

By inserting this explicit voltage dependence of K_d into Eq. 1, we arrive at the expected variation for k_{off} with both internal K^+ and voltage:

$$k_{off} = \beta_{min} + \frac{a_K(\beta_{max} - \beta_{min})}{a_K + K_d(0)\exp(-\delta FV/RT)}. \quad (3)$$

We now wish to subject this equation, and the ideas underlying it, to quantitative examination. Using a value for δ of 1, Eq. 3 allows us to predict k_{off} at any voltage and K^+ concentration, given a knowledge of the influence of K^+ on k_{off} at a particular voltage. Fig. 6 presents such a test of Eq. 3. First, a channel was held at 37 mV while internal K^+ was varied, to arrive at a value of K_d at that voltage (Fig. 6 *A*). Then the CTX off rate of the same channel was measured as a function of voltage at two different internal K^+ concentrations (Fig. 6 *B*). The results are well described by Eq. 3, using the previously estimated value of δ (1.0) and the values of K_d, β_{min}, and β_{max} determined at a single voltage in Fig. 6 *A*. As expected by the model, lowering K^+ lowers the value of k_{off} at all voltages and also lowers the apparent voltage dependence of the CTX dissociation rate.

Two Extreme Conditions: Zero Internal K^+ and Hyperpolarized Potentials

The model described by Eq. 3 makes several clear predictions under extreme conditions. Since the voltage dependence of the CTX off rate is postulated to be due to K^+ occupancy of a site accessible only from the internal solution, removing internal K^+ should render the CTX dissociation rate voltage independent:

$$k_{off}(a_K = 0) = \beta_{min} \quad \text{(independent of } V\text{)}. \quad (4)$$

The experiment of Fig. 7 shows that this prediction is verified. We compare the voltage dependence of the CTX off rate in 77 mM internal K^+ with that under K^+-free conditions (77 mM internal Na^+). With no K^+ present, the off rate is now invariant with applied voltage, with a value of 0.01 s^{-1}, which is identical to the value estimated in Fig. 1 by extrapolation to zero internal K^+. This is a remarkable result, but it must be interpreted cautiously. We find in the absence of internal K^+ that the CTX dissociation rate is enhanced by increasing the channel open probability. This is in contrast to the case when internal K^+ is present

(Anderson et al., 1988). We do not have an explanation for this difference, but we emphasize that it was necessary to apply probability-clamp conditions in order to carry out the experiment of Fig. 7.

A second "extreme" prediction of Eq. 3 is that as voltage is made very negative, the CTX dissociation rate should approach the same value approached at zero K^+, β_{min}, and hence should lose its voltage dependence.

$$k_{off}(V \rightarrow -\infty) = \beta_{min} \qquad \text{(independent of } V\text{)}. \tag{5}$$

The reason for this expected behavior is that the occupancy of the K^+ site becomes very low at negative voltages, and so the voltage-dependent K^+ effect

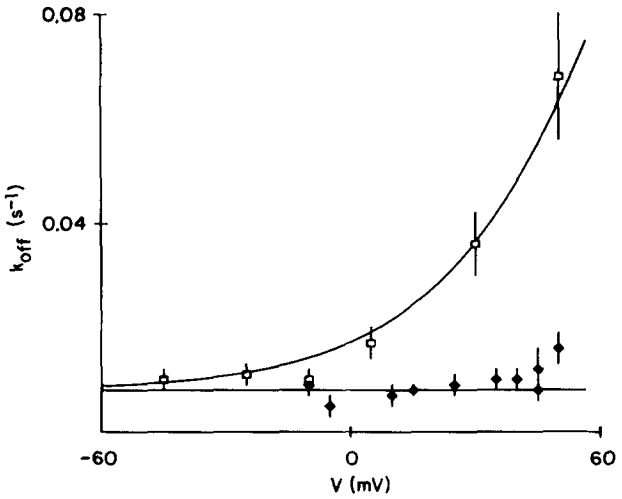

FIGURE 7. Voltage independence of CTX dissociation in the absence of internal K^+. The CTX dissociation rate in the absence of internal KCl (filled symbols) was measured in several channels at different voltages. The internal solution contained 77 mM NaCl. (In several experiments, which gave identical results, 5 mM KCl was also present.) The external solution contained 110 mM KCl and 12 nM CTX. The dissociation rate in the presence of internal 77 mM KCl or 110 mM KCl (−25 and −45 mV) was determined in two separate channels (open symbols). Internal Ca^{2+} was adjusted to maintain an open probability, measured during unblocked periods, of 0.10–0.30.

on CTX dissociation no longer operates. In practice, Eq. 3 predicts that for voltages more negative than −20 mV, k_{off} should approach β_{min} for all K^+ concentrations below 200 mM. In Fig. 7, it is clear that at negative voltages, k_{off} approaches the value of β_{min}, 0.01 s^{-1}.

To test this prediction further, we measured the kinetics of CTX block at negative potentials, where the channel is closed virtually all the time. We did this as in the previous study (Anderson et al., 1988), by the "test pulse" method, which measures the probability, $P_b(t)$, that a channel is blocked by CTX at time t, given that it was not blocked at time zero. Since we know that CTX binds to the closed channel, we now ask how transmembrane voltage, at negative voltages, influ-

ences the probability of block. This probability is given in terms of the fundamental rate constants of the CTX-channel interaction:

$$P_b(t) = \frac{k_{on}}{k_{on} + k_{off}} [1 - e^{-(k_{on}+k_{off})t}]. \quad (6)$$

Here k_{on} is the observed pseudo–first-order rate constant of CTX association and k_{off} is the rate constant for dissociation. Fig. 8 shows the time dependence of CTX binding to the fully closed channel, at two different voltages, −30 and −60 mV. The curves are identical, as are the rate constants of both association and dissociation.

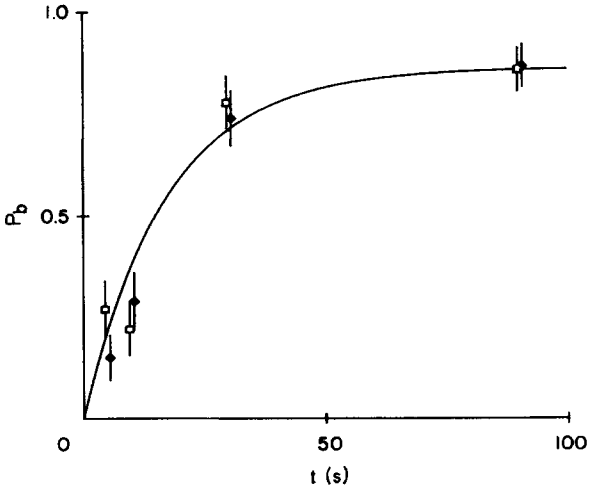

FIGURE 8. The voltage dependence of CTX block at negative voltages. The rate constants of the CTX association and dissociation reactions were measured at −30 and −60 mV in a single channel, using the pulse protocol described in the accompanying article (Anderson et al., 1988). At these voltages, the channel was closed >98% of the time. The channel was recorded at a "holding voltage" of 40 mV in the presence of 17 nM CTX. At a moment when the channel was clearly not blocked, the voltage was changed to a "test voltage" of −30 or −60 mV for a duration t, and then returned to the original holding voltage. The channel was observed to determine whether it was blocked or unblocked immediately after the return to the holding voltage. By repeating the sequence 24–43 times, for several durations at each test voltage, we obtain P_b, the probability that the channel is blocked at t, given that it was not blocked at $t = 0$. The variation of P_b with pulse duration is shown for test voltages of −30 mV (open symbols) and −60 mV (filled symbols). The solid curve is drawn according to Eq. 6, with $k_{on} = 0.050$ s^{-1} and $k_{off} = 0.008$ s^{-1}. The internal and external solutions contained 110 mM KCl and 110 mM NaCl, respectively.

and dissociation. Furthermore, the dissociation rate, 0.008 s^{-1}, is very close to the value of β_{min} determined at low K^+ and positive voltages above.

Aside from further verifying the proposal under examination, this result is important in that it rules out an alternative explanation for the voltage dependence of the CTX dissociation rate: that the dissociation constant of CTX itself varies with voltage according to Eq. 2, as originally proposed by Woodhull (1973) to explain the block of Na^+ channels by H^+. Such a model envisions the cationic CTX molecule as entering a significant fraction of the transmembrane voltage drop. But this cannot be the explanation for the voltage-dependent CTX

dissociation. Such a picture demands that the off rate decrease toward zero as voltage is made increasingly negative. In Figs. 7 and 8, we see that the CTX off rate clearly approaches a nonzero value at negative potentials. This finding is inconsistent with a Woodhull mechanism for voltage dependence.

Ion Selectivity of CTX Dissociation

We now ask whether ions other than K^+ in the internal solution are competent to relieve CTX block. In Fig. 9, we show that of all the cations tested, only K^+ and Rb^+ accelerate CTX dissociation. This is an important result, since these are the only ions in the series tested that permeate this channel (Blatz and Magleby, 1984; Yellen, 1984*a*; Eisenman et al., 1986). For technical reasons, we are

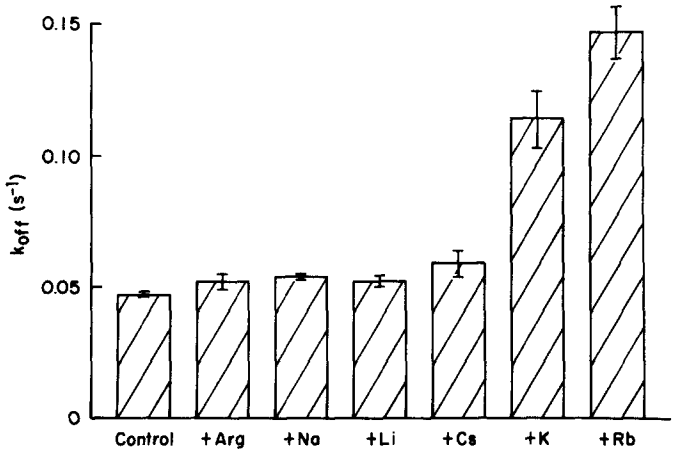

FIGURE 9. Ionic selectivity of *trans*-enhanced dissociation. CTX blocking events in a single channel were recorded in the presence of 100 mM internal KCl at 37 mV. The measured k_{off} was defined as control. Then 200 mM of the indicated chloride salt was added to the internal solution, in addition to the 100 mM KCl already present. The CTX k_{off} was again measured. Each value represents the mean ± SEM (or range of the mean) of two to five determinations, each in a separate channel. In all cases, the external solution contained 150 mM KCl and 23 nM CTX. Salt concentrations are not reported as activities in this figure.

unable to test the other two permeant ions, NH_4^+ and Tl^+. In addition to demonstrating that the K^+ effect on CTX dissociation is highly specific, this result suggests that only by permeating the channel's conduction pathway can an ion reach the site at which CTX dissociation is affected.

DISCUSSION

In this study, we attempt to explain the mechanism by which CTX blocks the high-conductance, Ca^{2+}-activated K^+ channel. The key observation of this study is the finding that K^+ on the inside of the channel increases the dissociation of CTX, which blocks the outside of the channel. Through a detailed investigation of this effect of internal K^+ on CTX block, we have developed a simple model

that leads to a physical picture of the way in which CTX blocks the channel, and offers a rather surprising mechanism by which CTX gains its voltage dependence.

A Physical Picture of CTX Inhibition

A cartoon of the model we consider here is shown in Fig. 10. We propose that CTX physically plugs the pore of the Ca^{2+}-activated K^+ channel. This proposal does not follow rigorously from our results, but it is a conclusion that fits so naturally with the observations made here, and explains them so economically, that we are willing to offer it as a strong suggestion. We will now summarize our experiments as they relate to the physical-occlusion model for CTX action.

We have demonstrated that internal K^+ specifically relieves channel inhibition by external CTX, and that this effect is due wholly to an enhancement of the

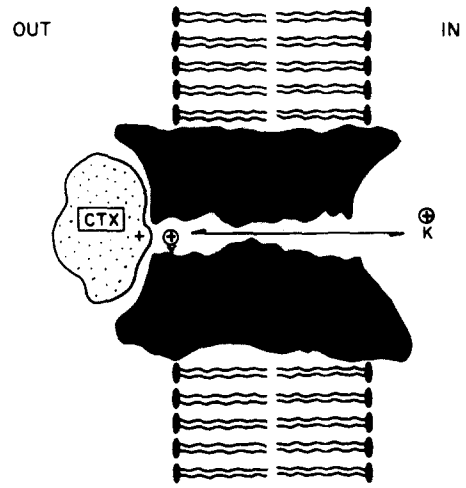

FIGURE 10. CTX block of the high-conductance, Ca^{2+}-activated K^+ channel. The cartoon depicts a membrane-spanning ion channel with a central ion-conduction pore. CTX blocks ion conduction by binding to the outside of the channel and plugging the pore. A K^+-binding site in the channel is located close to the external mouth of the pore. When CTX is bound to the channel, K^+ from the inside can bind to the site and repel the blocker.

CTX dissociation rate by K^+. Crudely stated, internal K^+ "pushes" CTX off its blocking site. Because K ions coming from the internal solution interact with CTX binding from the external solution, we propose that CTX physically occludes the conduction pathway, as expressed in Fig. 10.

As internal K^+ is raised, the CTX off rate increases in a rectangular hyperbolic fashion from a minimum value of 0.01 s^{-1} to a maximum value of ~0.2 s^{-1}. Although the precise value of the maximum off rate is uncertain owing to the low apparent affinity for K^+, it is clear that there is an ~20-fold enhancement of the CTX off rate by internal K^+. It is the saturating behavior that leads us to propose that K^+ binds to a site in the channel at which it exerts its effect on the CTX off rate. When the site is unoccupied, the rate constant for CTX dissociation is low, 0.01 s^{-1}, and when K^+ occupies the site, CTX dissociation is ~20-fold faster. Because CTX blocks the channel on a time scale of seconds, while

K^+ presumably equilibrates with its site on a time scale of nanoseconds, the observed CTX off rate reflects the time-averaged occupancy of the K^+ site, as expressed in Eq. 3.

We have shown that the relief of CTX block by K^+ is independent of the channel open probability (Fig. 4). If the channel is able to undergo its normal gating while CTX is bound to its externally facing site, then a blocking CTX is "exposed" to the internal solution in proportion to the open probability of the channel. Why then is internal K^+ not more effective when a channel is open most of the time than when it is mostly closed? Our result appears to run counter to intuition, but in fact it is a natural consequence of the proposed model. We assume that K^+ equilibrates with its site in the channel in a few nanoseconds (the conduction time scale), and CTX resides on the channel for many seconds. Since channel gating occurs in the millisecond time domain, even a "mostly closed" channel will open many times during an average CTX block event, and the K^+ will have ample opportunity to reach equilibrium occupancy with its site. Therefore, K^+-site occupancy, and hence K^+-enhanced CTX dissociation, is expected to be independent of the channel's open probability.

The effect of internal K^+ is voltage dependent. As membrane potential is made increasingly positive, internal K^+ becomes increasingly effective in promoting CTX dissociation. The effect of voltage appears to be on the affinity of the K^+-binding site, but this is the weakest point in our argument. Because of the very low affinity of the K^+ site at all voltages, we cannot rigorously claim that there is no effect of voltage on the maximum CTX off rate; our results, however, are fully consistent with the sole effect of voltage being on the K^+-binding affinity.

The proposal that depolarization increases the affinity of K^+ for its site enables us to explain the voltage-dependent dissociation of CTX observed initially (Anderson et al., 1988). The interaction of CTX with the channel is actually independent of voltage; it is the antagonistic effect of K^+ that is voltage dependent. As the membrane is depolarized, the K^+-site occupancy increases, and CTX dissociation is speeded up. This interpretation is particularly fortified by the observation (Fig. 7) that merely replacing internal K^+ by Na^+ eliminates the voltage dependence of CTX dissociation.

The voltage dependence of the CTX off rate (Fig. 5) allows us to calculate the "electrical distance," δ, of the K^+-binding site. We find that this site is located all the way across the channel ($\delta \approx 1$), so that internal K^+ must traverse the entire transmembrane voltage drop before it reaches the binding site. It is this observation that leads us to propose that the K^+-binding site is physically located on the external side of the K^+-conduction pore, and that therefore the CTX-binding site is located in the externally facing mouth of the pore, close enough to the K^+ site to allow direct interaction of K^+ with the toxin.

This conclusion is further strengthened by the observation (Fig. 9) that of all the ions tested, only the two conducting ions, K^+ and Rb^+, increase the off rate of CTX. Particularly striking in this regard is the failure of internal Na^+ and Cs^+ to enhance CTX dissociation. Both of these ions are known to enter the con-

duction pore deeply and block the channel (Marty, 1983; Yellen, 1984*b*); the voltage dependence of internal Na^+ block locates this ion's blocking site 80% of the way through the voltage drop along the pore (Yellen, 1984*b*), and yet it cannot exert an effect at the K^+ site located only slightly further on.

So far we have not addressed the mechanism by which K^+ accelerates CTX dissociation. Why does K^+ occupancy of the channel speed up CTX dissociation 20-fold? We have no way to answer this question, but again the physical-occlusion model provides a plausible rationale. If the K^+-binding site is located near to a positively charged group on the CTX bound in the channel mouth, as pictured in Fig. 10, then simple electrostatic repulsion between this group and bound K^+ could easily account for the rate acceleration. Even in a high-dielectric-constant environment, two groups of like charge 0.5 nm apart can mutually destabilize each other's binding by up to 4 kcal/mol (Kirkwood and Westheimer, 1938). A 20-fold rate enhancement is equivalent to a destabilization of CTX binding of only 1.8 kcal/mol. Therefore, we suggest that the K^+-binding site is located close to a positively charged group on the surface of CTX adjacent to the channel mouth, and that the electrostatic repulsion between K^+ and CTX leads to an increased rate constant for CTX dissociation.

Internal K^+ relieves CTX block by enhancing the dissociation rate. We argue that K^+ in the channel somehow repels CTX. Why is the association rate of CTX not affected by internal K^+? By assuming a physical plugging mechanism for CTX block, the answer to this question is straightforward. In the blocked state, with CTX occluding the channel's externally facing mouth, the K^+ site is in a true equilibrium with the internal aqueous solution, since K^+ can leave the pore only by the same path that it entered. In the unblocked channel, however, K^+ permeates. If the K^+ site is situated very close to the external mouth of the channel, as our data suggest, then the occupancy of the site will be dominated by K^+ in the external solution when the channel is unblocked. Since internal K^+ will not influence the occupancy of the K^+ site in the unblocked channel, the CTX association rate should not vary with internal K^+.

Trans-enhanced Dissociation in Other Channels

There are several other examples of *trans*-enhanced dissociation in ion channels, in which an ion entering the channel from one side speeds the exit of another ion to the opposite side of the pore. This phenomenon was first observed in the squid axon K^+ channel (Bezanilla and Armstrong, 1972; Adelman and French, 1978), in which channel block by externally applied Cs^+ was relieved by internal K^+. Similarly, Yellen (1984*b*) found that block by Na^+ in the high-conductance, Ca^{2+}-activated K^+ channel is relieved by external K^+ or Cs^+. A related observation is the ability of Ca^{2+} entering one end of the pore of the L-type Ca^{2+} channel to accelerate the exit of a second Ca ion near the other end of the pore (Hess and Tsien, 1984; Almers and McClesky, 1984). In each of these cases, the observation of *trans*-enhanced dissociation was taken as *prima facie* evidence that the two interacting ions simultaneously occupy the conduction pathway.

In contrast, for the case here, we have not been willing to take the mere observation of K^+-enhanced CTX dissociation as direct proof that CTX occupies

the pore. Whereas it is easy to envision K^+ analogues such as Cs^+ and Na^+ binding within a pore designed for K^+, this analogy is much more tenuous for a protein like CTX. Therefore, we have attempted to carry out a full range of quantitative tests to address this question. While our results do not provide airtight proof of the physical occlusion of the channel by CTX, the idea is compelling to the extent that it provides a simple and natural explanation for a diverse set of characteristics of the interaction of CTX with this channel.

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