Charybdotoxin Block of Single Ca^{2+}-activated K^+ Channels

Effects of Channel Gating, Voltage, and Ionic Strength

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ABSTRACT Charybdotoxin (CTX), a small, basic protein from scorpion venom, strongly inhibits the conduction of K ions through high-conductance, Ca^{2+}-activated K^+ channels. The interaction of CTX with Ca^{2+}-activated K^+ channels from rat skeletal muscle plasma membranes was studied by inserting single channels into uncharged planar phospholipid bilayers. CTX blocks K^+ conduction by binding to the external side of the channel, with an apparent dissociation constant of ~10 nM at physiological ionic strength. The dwell-time distributions of both blocked and unblocked states are single-exponential. The toxin association rate varies linearly with the CTX concentration, and the dissociation rate is independent of it. CTX is competent to block both open and closed channels; the association rate is sevenfold faster for the open channel, while the dissociation rate is the same for both channel conformations. Membrane depolarization enhances the CTX dissociation rate e-fold/28 mV; if the channel's open probability is maintained constant as voltage varies, then the toxin association rate is voltage independent. Increasing the external solution ionic strength from 20 to 300 mM (with K^+, Na^+, or arginine$^+$) reduces the association rate by two orders of magnitude, with little effect on the dissociation rate. We conclude that CTX binding to the Ca^{2+}-activated K^+ channel is a bimolecular process, and that the CTX interaction senses both voltage and the channel's conformational state. We further propose that a region of fixed negative charge exists near the channel's CTX-binding site.

INTRODUCTION

The high-conductance, Ca^{2+}-activated K^+ channel is present in the plasma membranes of many types of cells, both excitable and inexcitable (Latorre, 1986). Its

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identifying characteristics include high K^+ selectivity, high single-channel conductance, activation by both cytoplasmic Ca^{2+} and depolarizing voltages, and allosteric modulation of Ca^{2+} action by cytoplasmic Mg^{2+} (Marty, 1981; Barrett et al., 1982; Methfessel and Boheim, 1982; Moczydlowski and Latorre, 1983; Magleby and Pallotta, 1983; Petersen and Maruyama, 1984; Blatz and Magleby, 1984; Yellen, 1984; Eisenman et al., 1986; Golowasch et al., 1986; Oberhauser, A., O. Alvarez, and R. Latorre, manuscript submitted for publication). Recently, a high-affinity inhibitor directed against this channel was discovered in the venom of the scorpion *Leiurus quinquestriatus* (Miller et al., 1985), from which it was subsequently purified (Smith et al., 1986). This inhibitor, called charybdotoxin (CTX), is a highly basic protein of ~5 kD mol wt, which inhibits the Ca^{2+}-activated K^+ channel at nanomolar concentrations in the extracellular medium. Studies of CTX block in invertebrate neurons (Hermann and Erxleben, 1987) suggest that this toxin is selectively directed against Ca^{2+}-activated K^+ channels.

In this and the following study (MacKinnon and Miller, 1988), we seek to uncover the mechanism by which CTX inhibits the Ca^{2+}-activated K^+ channel. We know that the toxin induces in the single channel long-lived (~10 s), nonconducting intervals (Miller et al., 1985; Smith et al., 1986), each of which appears to represent the binding of a single CTX molecule to the channel. However, the mechanism by which toxin binding leads to channel inhibition is unknown. In an attempt to understand this process, we now ask several basic questions. Does the toxin bind to both open and closed states of the channel? How many toxin molecules are involved in inhibition? How does applied voltage affect toxin binding? Is toxin binding influenced by the ionic composition of the aqueous media on the two sides of the membrane?

In order to address these questions, we use the planar lipid bilayer, into which Ca^{2+}-activated K^+ channels from skeletal muscle plasma membranes can be reconstituted under well-defined conditions. We show that CTX binds to the channel in a simple bimolecular reaction, that both the open and closed states of the channel bind the toxin, that dissociation of CTX from the channel is enhanced by depolarization, and that the toxin association rate is strongly dependent on the ionic strength of the external medium. The following study (MacKinnon and Miller, 1988), by demonstrating that K^+ and other conducting ions specifically relieve toxin block from the opposite side of the membrane, quantitatively rationalizes all the results in terms of a model picturing CTX inhibition as a consequence of direct occlusion of the channel's conduction pore.

MATERIALS AND METHODS

Biochemical

The lipids were 1-palmitoyl,2-oleoyl phosphatidylcholine (POPC) and the corresponding phosphatidylethanolamine (POPE), both obtained from Avanti Polar Lipids, Inc., Birmingham, AL. Transverse-tubule membrane vesicles were prepared from rat skeletal muscle as described (Eisenman et al., 1986). Bovine serum albumin (BSA) was fraction V grade from Sigma Chemical Co., St. Louis, MO.

CTX was purified from the lyophilized venom of *L. quinquestriatus* (Latoxan, Rosans,

France) by an improvement of the procedure described previously (Smith et al., 1986). Crude venom (250 mg) was dissolved in 40 ml of 250 mM NaCl. 50 ml of acetone, prechilled at −20°C, was then added slowly with stirring. The mixture was incubated at −20°C for 30 min, and was centrifuged in glass bottles at 2,000 *g* for 5 min. 110 ml of chilled acetone was added to the supernatant slowly with stirring, and the mixture was incubated for 30 min at −20°C. This was centrifuged as above, and the pellet was dissolved in 50 ml of buffer A (40 mM NaCl, 10 mM Na-borate, 10 mM Na_2CO_3, pH 9.0). After the small amount of particulate material in this solution was centrifuged out, the supernatant was applied to a 5-ml column of SP-Sephadex C-25 (medium), and the column was washed with 100 ml of buffer A, flowing at 2 ml/min. A linear gradient (150 ml total volume) of buffer A to buffer B (20 mM NaCl, 10 mM Na_2CO_3, 10 mM Na_3PO_4, pH 12.0) was then applied, with flow restricted to 0.4 ml/min. CTX activity eluted in a well-defined peak at pH 10.8–11.0. Fractions containing CTX activity were neutralized to pH 7.0 with acetic acid, and the pooled material was fractionated by reverse-phase high-pressure liquid chromatography (HPLC), using a Dynamax C-8 column (300-Å pore, Rainin Instruments, Woburn, MA). Samples (200 μg per run) were applied to the column, which was then washed with 5 ml of buffer C (0.1% trifluoroacetic acid). A linear gradient of buffer C to 50% acetonitrile over 40 min was run at 1 ml/min, and the absorbance at 280 nm was monitored. CTX eluted in a sharp peak at ~30 min. The CTX-containing fractions were immediately neutralized with Na_3PO_4, NaCl was added to a final concentration of 100 mM, and acetonitrile was removed by evaporation in a stream of N_2. The final preparations were >95% pure and were stored frozen at CTX concentrations >5 μM.

Concentrations of CTX stock solutions were determined by absorbance at 280 nm, using an extinction coefficient of 10,500 $M^{-1}cm^{-1}$, ~1.7-fold lower than that determined previously (Smith et al., 1986). The extinction coefficient was determined by quantitative amino acid analysis on highly purified samples, using HCl hydrolysis and *o*-phthaldehyde or phenylthiohydantoin detection on C18 reverse-phase columns (Jarrett et al., 1986; Bergman et al., 1986). Our previous preparations contained a small amount of a contaminating peptide, which led to an erroneous estimation of molecular weight based on amino acid composition (Smith et al., 1986). Recent sequencing work (Garcia et al., 1988) shows that CTX contains 37 amino acids and has a molecular weight of 4.3 kD.

Planar Bilayers and Single-Channel Data Collection

Single Ca^{2+}-activated K^+ channels were inserted into planar lipid bilayers formed from neutral phospholipids (POPE/POPC, 7:3) using rat skeletal muscle plasma membranes as the source of channels (Eisenman et al., 1986). Channels were inserted under conditions promoting fusion of the muscle membrane vesicles with the planar bilayer; vesicles (1–5 μg/ml) were added to the internal solution, which was composed of 150 mM KCl, 10 mM MOPS, 0.05 mM $CaCl_2$, 5 mM KOH, pH 7.3; the external solution contained 10 mM MOPS, 0.1 mM EGTA, 5 mM KOH, pH 7.3. With these solution conditions, the channels always incorporated with unambiguous physiological orientation, i.e., with the Ca^{2+} activation and CTX blocking sites facing the internal and external solutions, respectively. After the incorporation of a single channel, further insertion was suppressed by reducing the transmembrane osmotic gradient and removing the vesicles from the chamber; this was done by adding the desired concentration of salt (KCl, NaCl, or arginine-Cl) to the external solution and perfusing the internal solution with vesicle-free medium of the desired ionic composition. Using this method, it was routinely possible to maintain single-channel bilayers for several hours, as required in most experiments.

In order to obtain quantitatively reproducible values for the CTX blocking kinetics, we found it necessary to stir the CTX-containing chamber continuously, and to include

50 μg/ml bovine serum albumin in this chamber to suppress nonspecific binding of the toxin to the polystyrene walls of the chamber. After each experiment, we washed the bilayer chambers extensively with 0.5 M NaCl to remove the last traces of CTX.

Data Analysis

The association and dissociation rates of CTX were determined from the statistical distributions of the long-lived blocked and unblocked dwell times measured directly from the single-channel record. Blocking events could be recognized unambiguously as nonconducting intervals longer than 300 ms, since channel closing events were short-lived, with average dwell times in the range of 1–5 ms (Miller et al., 1985). Data were collected on video tape and analyzed by a pattern-recognition program using a laboratory computer (Indec, Sunnyvale, CA). The details of the computer analysis were identical to those used to analyze block of this channel by Ba^{2+} (Miller, 1987; Miller et al., 1987). Apparent rate constants of CTX association and dissociation were measured from the mean blocked and unblocked intervals in a given channel record, τ_b and τ_u, respectively:

$$k_{on} = 1/\tau_u; \tag{1}$$

$$k_{off} = 1/\tau_b. \tag{2}$$

In calculating these mean dwell times, the usual corrections for missed events were employed (Miller et al., 1987; Anderson, 1987).

Because the kinetics of CTX blocking are bimolecular, the apparent dissociation constant for CTX was calculated from the measured probability of channel block, P_b, at a given CTX concentration (Smith et al., 1986):

$$K_d = [\text{CTX}](1 - P_b)/P_b. \tag{3}$$

RESULTS

The inhibition of Ca^{2+}-activated K^+ channels by CTX is illustrated in Fig. 1. Here, a single Ca^{2+}-activated K^+ channel from rat muscle plasma membrane was incorporated into a planar bilayer formed from neutral lipids, and the ensuing unitary fluctuations were examined in the presence of internal Ca^{2+} at a depolarized holding potential of 40 mV. The opening and closing kinetics of this channel operate on the millisecond time scale and are therefore too rapid to discern in the records shown. Addition of CTX to the internal solution at a high concentration has no effect on the channel, while the toxin added to the external solution elicits a characteristic response. The toxin causes the appearance of nonconducting, or "blocked," intervals that are much longer-lived (~10 s) than the channel's closed states. This toxin blocking effect is reversed by perfusion of the external chamber with CTX-free medium (Miller et al., 1985).

Stoichiometry of CTX Block

The CTX-induced blocking events were originally proposed to represent the binding of single CTX molecules with the channel in a simple bimolecular reaction (Miller et al., 1985), according to:

$$\text{Channel} + \text{CTX} \rightleftharpoons \text{blocked channel.} \qquad \text{(Scheme I)}$$

This scheme demands that both blocked and unblocked times be exponentially distributed, that the pseudo–first-order rate constant of blocking (association)

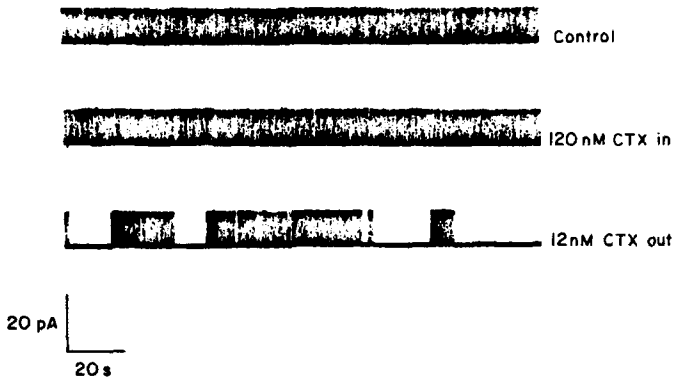


FIGURE 1. Block of Ca^{2+}-activated K^+ channels by purified CTX. A single channel was inserted into a planar bilayer and observed in symmetrical 150 mM KCl medium, at a holding voltage of 40 mV. Recordings are displayed at a slow chart speed, so that individual opening and closing events are not visible. Upper trace: control record, no CTX. Middle trace: 120 nM CTX added to internal solution. Lower trace: 12 nM CTX added to external solution.

be linearly dependent on CTX concentration, and that the dissociation rate be independent of CTX. Figs. 2 and 3 confirm these predictions. The dwell-time distributions are exponential for both blocked and unblocked channels (Fig. 2). The pseudo–first-order rate constant of blocking increases linearly with CTX concentration, while that of unblocking remains constant over the entire CTX concentration range (Fig. 3). Under the conditions of Fig. 3, the CTX concentration for half-inhibition (K_d, defined by Eq. 3) is ~10 nM. In separate exper-

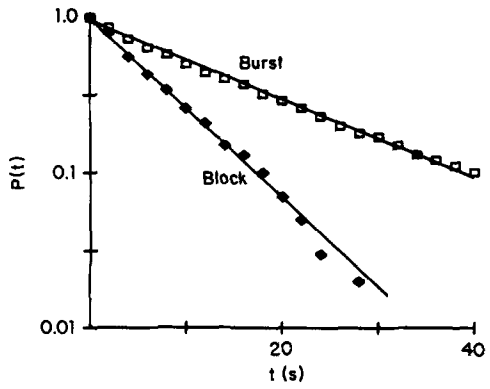


FIGURE 2. Exponential distribution of CTX blocked and unblocked times. A single channel was observed at 30 mV in symmetrical 150 mM KCl medium containing 33 nM external CTX, and dwell-time distributions were constructed for both blocked and unblocked intervals, using the cumulative distribution function, $P(t)$, defined as the probability that a given dwell time is greater than or equal to time t. The open probability of the unblocked channel was ~0.5. A blocked interval was defined as a nonconducting interval of duration >300 ms, which is >50-fold longer than the mean closed time under these conditions. Open symbols: unblocked intervals; filled symbols: blocked intervals. The time constants for the unblocked and blocked distributions, τ_u and τ_b, were 7.8 and 16.6 s, respectively. Distributions were constructed from a total of 830 dwell times.

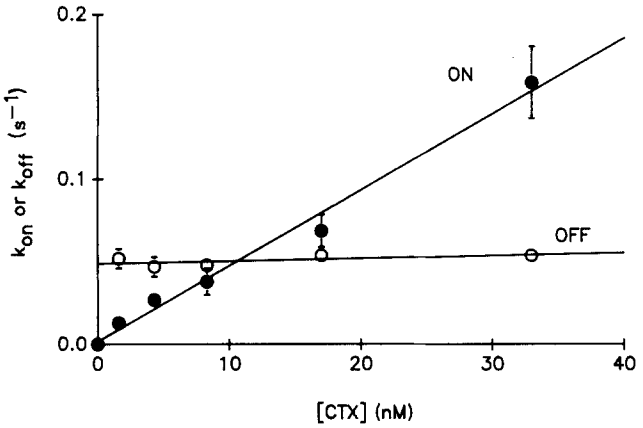


FIGURE 3. Bimolecular kinetics of CTX interaction. Rate constants of blocking (k_{on}, open symbols) and dissociation (k_{off}, filled symbols) were measured from single-channel blocked and unblocked intervals as a function of CTX concentration, under the same conditions as in Fig. 2. Each point represents the mean ± SE of three to five separate determinations, each on a separate channel in a separate bilayer. The internal phase contained 150 mM KCl medium, and the external phase was 150 mM NaCl medium. The holding voltage was 30 mV.

iments (not shown), we have confirmed the linearity of blocking rates at concentrations spanning the range of 0.1–20 K_d. The single-exponential time distributions and strict linearity of the on rate with CTX concentration argue strongly in favor of Scheme I. We conclude that CTX binds to the channel in a simple bimolecular fashion, and that each blocking event observed in the single-channel record represents a segment of time in which a single toxin molecule is bound to the channel.

Dependence of CTX Block on the Channel's Gating State

Next, we examine the state dependence of CTX block. Does CTX bind exclusively to the open or to the closed channel, or can it bind to both of these con-

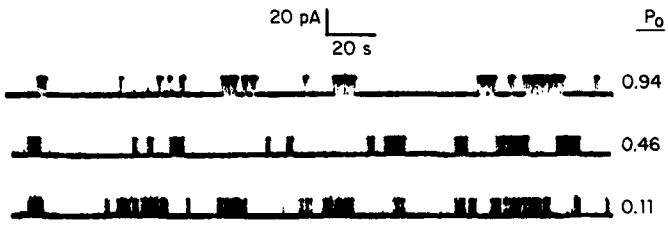


FIGURE 4. Effect of channel open probability on CTX block. A channel was observed under conditions of Fig. 2, in the presence of 17 nM CTX. In these experiments, the internal Ca^{2+} concentration was varied (in the range 5–20 μM) to change the open probability of the unblocked channel, P_o, indicated on the figure. Open probability was measured only during unblocked intervals.

formations? We answer this question by systematically varying the channel's open probability and studying the CTX inhibition kinetics. These experiments are straightforwardly interpreted, since the opening and closing processes are much faster than the CTX kinetics. Fig. 4 shows records of a single channel in the presence of a fixed concentration of CTX, with the Ca^{2+} concentration adjusted to vary the open probability. Qualitatively, we see that CTX inhibits the channel effectively over a wide range of open probability, and this result argues that the toxin can bind to both the open and the closed conformations of the channel.

Fig. 5 examines this question quantitatively, by displaying the association and dissociation rates of CTX as a function of the channel's open probability. We see that the association rate increases with open probability; moreover, as the open probability approaches zero, the CTX association rate approaches a non-

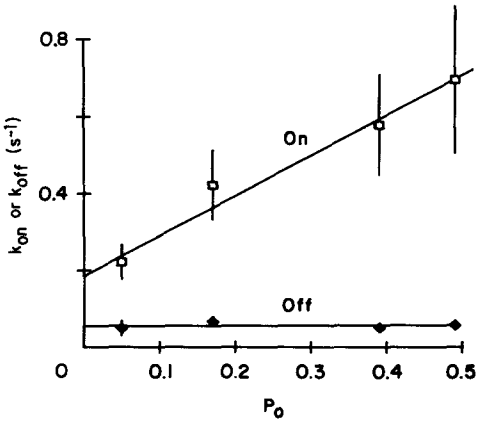


FIGURE 5. Effects of open probability on CTX blocking kinetics. A single channel was observed under the conditions of Fig. 4, with 46 nM CTX present and 20 μM internal Ca^{2+}. The open probability of the unblocked channel was varied by lowering internal Ca^{2+} by adding known concentrations of EDTA. The association and dissociation rates were measured at each value of open probability, from 70–100 blocking and unblocking events (standard errors of each measurement shown). Solid line for k_{on} is drawn according to Eq. 6, with $\alpha = 3 \times 10^7\ M^{-1}s^{-1}$ and $\alpha' = 4 \times 10^6\ M^{-1}s^{-1}$.

zero value. This result demonstrates that both the open and closed channel conformations are competent to bind toxin, and that the association rate is about sevenfold greater for the open than for the closed channel. CTX dissociation rates do not depend on the channel open probability (Fig. 5 *B*). In most experiments, we used the Ca^{2+} concentration to control the channel's open probability. In other experiments (not shown), we held Ca^{2+} fixed and activated the channel with Mg^{2+} or Mn^{2+}, which are known to act allosterically at a site distinct from the Ca^{2+} activation site (Golowasch et al., 1986); using these allosteric activators, we obtained results similar to those in Fig. 5 (Anderson, 1987).

Kinetics of CTX Block of the Fully Closed Channel

To test further whether CTX can bind to the closed channel, we exploited the voltage dependence of this channel's gating. In these experiments, we used the

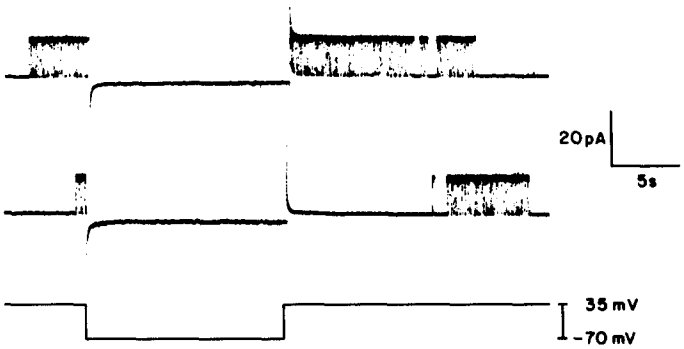


FIGURE 6. Channel-triggered pulse protocol for CTX block of a closed channel. A single channel was observed under the conditions of Fig. 2, at a holding potential of 35 mV, with 17 nM CTX present. The potential was changed to a test voltage of −70 mV during an unblocked interval, to shut the channel. After 15 s, the voltage was returned to the holding potential to assess whether the channel was blocked or unblocked at this time. The figure illustrates the two possible outcomes of this experiment: blocked (top trace) and unblocked (bottom trace).

method previously developed for studying Ba^{2+} block of closed channels (Miller et al., 1987), shown for CTX in Fig. 6. Here, a single channel was observed at a depolarized holding potential (35 mV) in the presence of CTX. At a moment when the channel was clearly not blocked, the voltage was changed to a hyperpolarized test potential of −70 mV, at which the channel closed within 1 ms. Voltage was held hyperpolarized for several seconds and was then returned to the holding potential. We examined the channel record immediately after the return to the holding voltage to ask whether the channel had become blocked

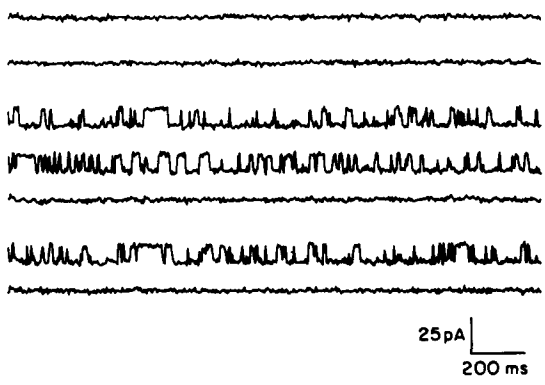


FIGURE 7. CTX blocking of the closed channel. CTX blocking of the fully closed channel was examined by the channel-triggered pulse method, as in Fig. 6. A channel was observed at a holding potential of 40 mV, in the presence of 17 nM CTX, and a test potential of −60 mV was applied during an unblocked interval and maintained for 10 s. The voltage was then returned to the holding potential. The figure shows seven consecutive trials, each consisting of the 2 s immediately following the return to the holding potential. Of these seven trials, four were blocked. In the experiment from which these trials are taken, the probability of being blocked (measured from 27 trials) was 0.41.

by CTX during the hyperpolarized test interval. Fig. 6 shows two such trials to illustrate the two possible outcomes, in which the channel is either blocked or not blocked at the end of the test interval.

By performing this kind of experiment repetitively, we obtained the relative frequencies of the two outcomes. Fig. 7 shows the results of seven consecutive trials in an experiment of this kind, displaying the first 2 s after returning to the holding voltage. Four of the seven trials shown were clearly blocked and must have become blocked during the hyperpolarized interval. Qualitatively, this result demonstrates that the closed channel is competent to bind CTX, since at the hyperpolarized test voltage, the channel is closed virtually all the time.

This method can be used to measure the kinetics of CTX interaction with the closed channel. A repetitive series of trials as in Fig. 7 gives us a direct measure of the probability, P_b, that the channel is found blocked after a hyperpolarized

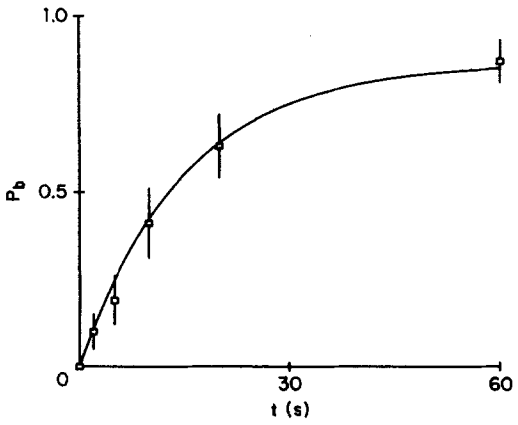


FIGURE 8. Kinetics of CTX block of the closed channel. The pulse protocol of Fig. 7 was applied to a single channel in the presence of 17 nM CTX, at varying test pulse durations, t. At each test pulse duration, a set of 27–31 trials was collected, and the probability of being blocked at the end of the test pulse, P_b, was measured. The solid curve is drawn according to Eq. 5, with k_{on} = 0.058 s^{-1} and k_{off} = 0.009 s^{-1}. The holding voltage was +30 mV; the test voltage was −60 mV.

test interval of duration t, given that it was not blocked at the beginning of this test interval:

$$P_b(t) = \text{Prob(blocked at } t\text{/unblocked at } t = 0). \tag{4}$$

By assuming that binding and dissociation are both simple Poisson processes, it is easy to show that:

$$P_b(t) = \frac{k_{on}}{k_{on} + k_{off}} [1 - e^{-(k_{on}+k_{off})t}]. \tag{5}$$

This is merely the microscopic analogy of a simple first-order macroscopic relaxation; the expression is valid regardless of how many times the channel becomes blocked and unblocked during the test interval. The importance of Eq. 5 is that it allows us to determine both the association and dissociation rates of CTX with the fully closed channel, even though we cannot directly observe the individual toxin blocking events.

Fig. 8 illustrates the results of such an experiment. As the duration of the test interval increases, the probability of block rises from zero to a steady value, with a time course reflecting the CTX kinetics. CTX block occurs on the time scale of tens of seconds, and the blocking kinetics here are somewhat slower than

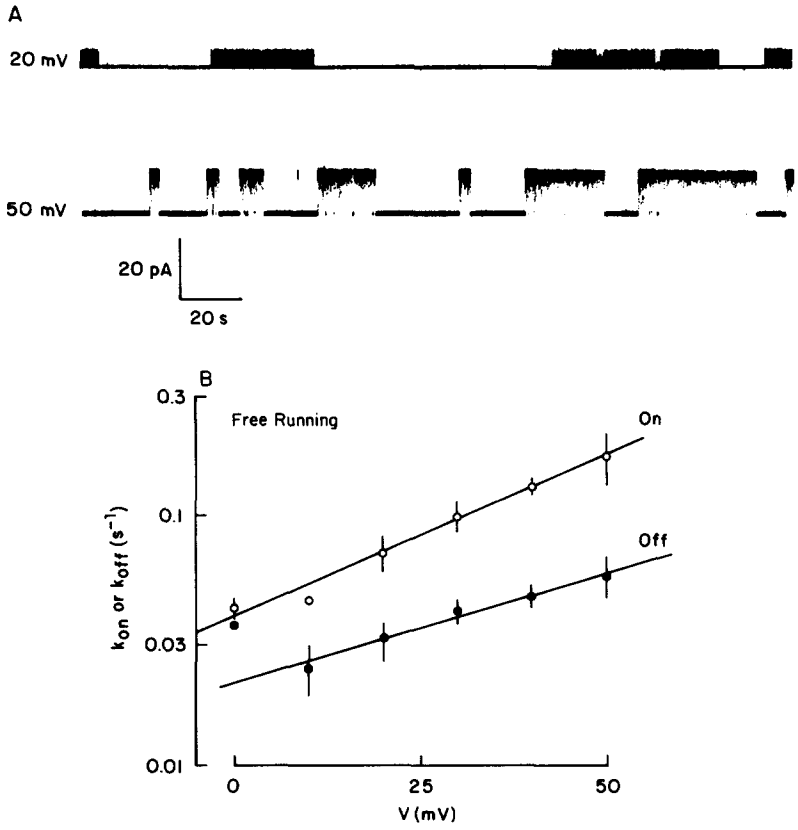


FIGURE 9. Voltage dependence of CTX block: "free-running" conditions. (*A*) Raw single-channel records from a channel under the conditions of Fig. 2 in the presence of CTX (17 nM) at the voltages indicated. (*B*) A channel was observed with 150 mM KCl medium internal and 100 mM NaCl external, at the indicated applied voltages, at 10 μM internal Ca^{2+} and 21 nM CTX. The rate constants for CTX association (open symbols) and dissociation (filled symbols) are displayed.

those observed for the open channel. This is the expected result, since we know from Fig. 4 that the CTX blocking rate slows as the open probability decreases. Again we conclude that the closed channel is competent to bind CTX.

Voltage Dependence of CTX Block

The kinetics of CTX block depend on applied voltage, but in an unusual way. Fig. 9 *A* displays raw records of CTX block at two different voltages. It is appar-

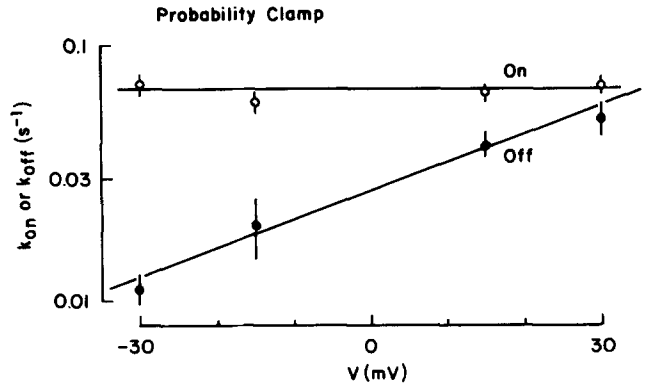


FIGURE 10. Voltage dependence of CTX block: "probability-clamp" conditions. A channel was observed in symmetrical KCl media as in Fig. 2, at the indicated voltages, and k_{on} (open symbols) and k_{off} (filled symbols) were measured. As the voltage was made more negative, Ca^{2+} was added to the internal solution to keep the unblocked open probability constant (between 0.14 and 0.20). The CTX concentration was 33 μM.

ent that both the blocked and unblocked times become shorter as the membrane is depolarized. Fig. 9 *B* summarizes this effect quantitatively and shows that the association and dissociation rates both speed up with depolarization; the affinity of the toxin, measured from the ratio of these rates, is weakened only slightly by depolarization. Since CTX is a polycationic molecule, the increase in the dis-

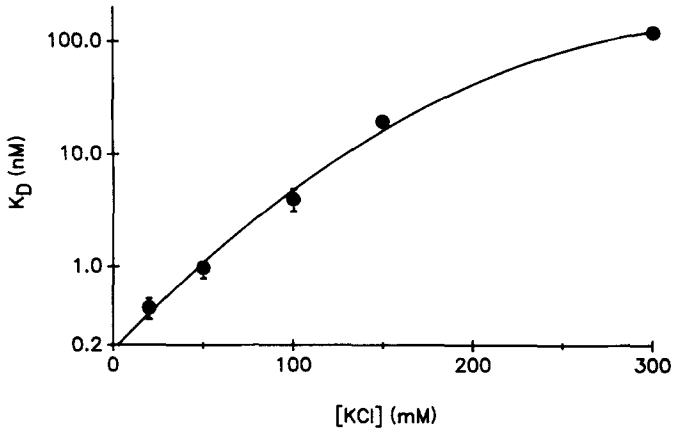


FIGURE 11. Effect of K^+ on the CTX dissociation constant. The dissociation constant for CTX was measured as a function of KCl concentration, which was varied symmetrically on the two sides of the membrane. Each point represents the mean ± SE of three to five separate determinations in different bilayers. Dissociation constants were estimated from the measured probability of block at a given CTX concentration, as described in Materials and Methods. The CTX concentrations used in these experiments varied from 0.7 to 135 nM. The solid curve was drawn by eye and carries no theoretical connotations.

sociation rate with depolarization might be rationalized in terms of an electrostatic mechanism (Woodhull, 1973). However, the parallel variation in the association rate is puzzling; why should the binding of an externally applied cationic toxin become more, rather than less, favorable as the membrane is depolarized?

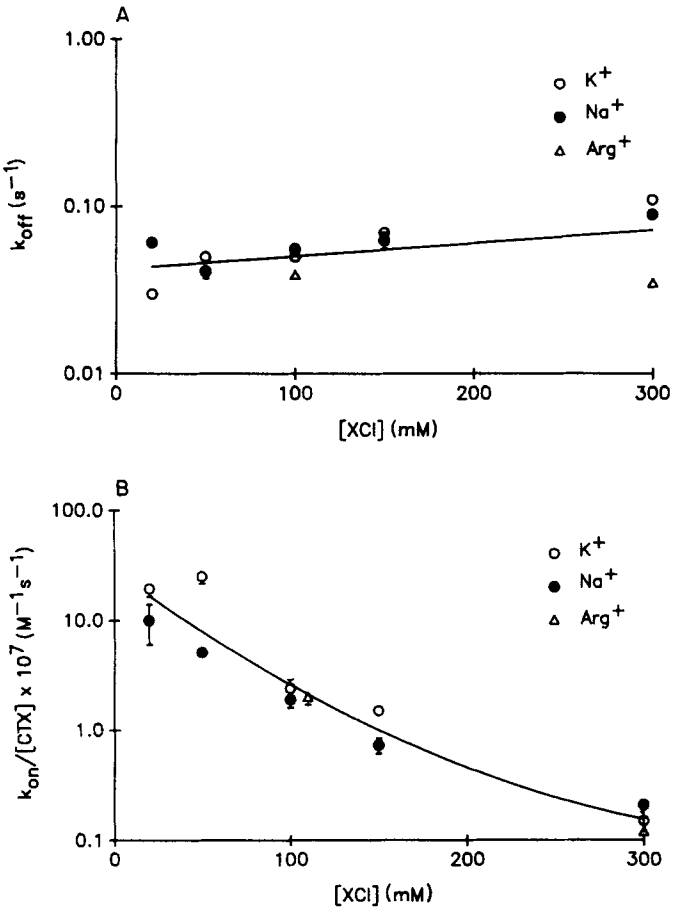


FIGURE 12. Sensitivity of CTX blocking kinetics to external ionic strength. Association and dissociation rates were measured as a function of ionic composition in the external solution, at 30 mV. The internal solution was 150 mM KCl medium in all cases. The unblocked channel open probability was maintained near 0.5 by adjusting the internal Ca^{2+} concentration. The external ionic strength was raised by addition of either KCl, NaCl, or arginine-Cl. The solid curves carry no theoretical significance. (*A*) Dissociation rate. (*B*) Association rates reported as second-order rate constants, $k_{on}/[CTX]$.

One possibility suggested by our earlier results (Fig. 4) is that the variation in the association rate with voltage may be influenced by the variation in opening probability that occurs with voltage.

To test this idea, we performed the "probability-clamp" experiment shown in

Fig. 10. We measured CTX blocking kinetics as a function of voltage while simultaneously holding the channel's open probability constant by adding or removing Ca^{2+} as the voltage changed. By continuously monitoring the unblocked channel's open probability, we could control this parameter to within $\pm 20\%$. Under these conditions, we found that only the dissociation rate depends on voltage; we observed no change in the association rate over a 60-mV range, across which the dissociation rate varies about eightfold (equivalent to an e-fold increase per 28 mV). The unusual co-variation in the binding and unbinding rates observed initially (Fig. 9) was due to the fact that we had allowed both voltage and open probability to change together in those experiments.

Effects of Ionic Composition on CTX Block

With an isoelectric point of about 10.8 (Smith et al., 1986), CTX carries a net positive charge at neutral pH. If the external surface of the channel protein carries negative charge, we might expect there to be a substantial electrostatic component to the interaction between the toxin and the channel. To examine this possibility, we studied the variation of CTX block with ionic composition. First, we measured CTX affinity as a function of KCl concentration varied symmetrically on both sides of the bilayer (Fig. 11). The affinity of the toxin is weakened enormously as KCl increases, with K_d increasing from the 100-pM range at 10 mM KCl and approaching 1 μM above 500 mM KCl.

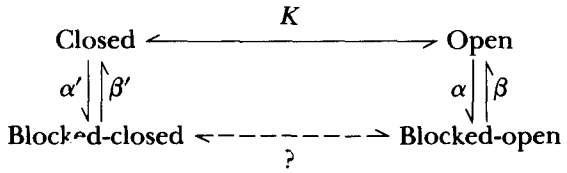
To investigate this large salt effect further, we varied the ionic composition only on the external side of the membrane, the side from which CTX binds, and we examined the kinetics of block. These results, shown in Fig. 12, lead to two conclusions. First, only the association rate varies strongly with salt concentration; the variation in the dissociation rate is small in comparison, if present at all. Second, the effect of salt on the association rate is nonspecific: the binding rate is reduced equally by K^+, a conducting ion, by Na^+, a chemically similar nonconducting ion, and by arginine, a large nonconducting organic cation. These results point to the involvement of nonspecific electrostatic contributions to the interaction of CTX with the Ca^{2+}-activated K^+ channel, i.e., a local negative surface potential near the CTX binding site.

DISCUSSION

In this study, we wished to lay out some of the basic characteristics of the interaction of CTX with the high-conductance, Ca^{2+}-activated K^+ channel. We chose to study this process in a planar bilayer rather than the physiological membrane because of the simplicity and flexibility of the model membrane system. The results allow us to reach four firm conclusions.

First, the binding of CTX is a simple, bimolecular process, in which a single CTX molecule causes inhibition by binding to a site on the channel. This one-to-one stoichiometry is strongly supported by the linearity of the blocking rate and the independence of the dissociation rate with CTX concentration, and by the single-exponential distributions of both the blocked and unblocked dwell times.

Second, the toxin binds to both the open and closed conformations of the channel. The experiments employing variations of the open probability (Figs. 4 and 5) are consistent with the following scheme:



(Scheme II)

Here, the two blocked states represent the toxin-blocked channel protein in either the closed or open conformation. The observed pseudo–first-order association rate constant, k_{on}, is given in terms of the true second-order rate constants of association with open and closed channels, α and α', respectively:

$$k_{on} = \{\alpha' + P_o(\alpha - \alpha')\}[\text{CTX}], \quad (6)$$

where P_o is the open probability of the unblocked channel. The results of Fig. 5 are consistent with this scheme and require that CTX binding to the open channel be seven- to eightfold faster than that to the closed channel. The association rate constant under "standard conditions" (150 mM ionic strength, 30 mV) is on the order of 10^7 $M^{-1}s^{-1}$, quite high for a protein-protein interaction, but still well below the diffusion limitation.

We cannot make a definite prediction for the behavior of the dissociation rate according to Scheme II, since we do not know whether opening and closing sitions occur between the two blocked states. In either case, however, the fact that the off rate is independent of the channel open probability (Fig. 4) forces us to conclude that the toxin dissociation rates are identical for both open and closed channel conformations. We speculate that the two conformations of the CTX-blocked channel can in fact interconvert, since previous work has clearly shown such interconversion in the Ba^{2+}-blocked channel (Miller et al., 1987).

Third, block by CTX depends upon applied voltage in an apparently complex way. As voltage is varied under "free-running" conditions, with voltage and channel open probability changing together, both the association and dissociation rates vary roughly in parallel, both increasing with depolarization. If the channel's open probability is kept fixed as voltage varies, however, then only the dissociation rate increases with depolarization. The association rate is inherently voltage independent.

Viewed together, the above state-dependent and voltage-dependent kinetics provide a particularly clean set of results. The association rate is sensitive to the channel's gating conformation, but the dissociation rate is not. Exactly in contrast, the dissociation rate depends on voltage, while the association rate does not. Recently, Hermann and Erxleben (1987) showed that CTX block of a small Ca^{2+}-activated K^+ channel in *Aplysia* is weakly voltage dependent, being relieved by depolarization, as seen here for the large, Ca^{2+}-activated K^+ channel.

Fourth, the ionic composition of the external medium exerts a huge influence on the interaction of the toxin with the channel, in a way expected for a nonspecific electrostatic effect. The apparent affinity of CTX is vastly lowered by

increasing the ionic strength, whether this maneuver is accomplished with K^+, Na^+, or arginine as the cation. This absence of ion specificity in effect argues that a specific ion-binding site on the channel (or the toxin) is not involved.

Rather, we favor the simplest idea: that the channel carries a region of fixed negative charge density near the toxin binding site. This sets up a local electrostatic potential near the site, which raises the local concentration of CTX. This effect should be strongly dependent on ionic strength, as was observed, since increasing the salt concentration screens the local surface charge and reduces the apparent affinity of toxin block. The phenomenon should be independent of the specific salt used to raise ionic strength, again as observed. Finally, the salt effect should be exerted only upon the association rate of the toxin, as was the case here. The lack of a salt effect on the dissociation rate also argues against a proposal that the toxin itself changes conformation as ionic strength is raised.

If a local electrostatic mechanism is responsible, as we propose, then the surface charge involved must be located on the channel protein, since the lipids used here carry no net charge. Local electrostatic effects have now been documented in several different channels reconstituted into planar bilayers (Bell and Miller, 1984; Moczydlowski et al., 1985; Affolter and Coronado, 1986). Recently, Green and co-workers (1987) reported similar experiments on Na^+ channels and demonstrated the existence of a region of high negative charge near the binding site for tetrodotoxin. In the present case, we are unable to deduce the magnitude of the negative charge density on the protein, since neither the valence of nor the charge distribution on the CTX molecule is known.

Several obvious questions about CTX blocking remain unanswered. Why is the CTX dissociation rate voltage dependent? How does the ionic composition of the internal solution affect CTX block from the external solution? By what physical means does CTX prevent ion conduction through the channel? In the companion study (MacKinnon and Miller, 1988), we investigate the ability of internal K^+ to relieve the block by CTX. The results immediately suggest both an explanation for the voltage dependence of CTX dissociation and a physical picture of the blocked state itself.

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