

Charybdotoxin Selectively Blocks Small Ca-activated K Channels in *Aplysia* Neurons

A. HERMANN and C. ERXLEBEN

From the University of Salzburg, Institute of Zoology, A-5020 Salzburg, Austria, and the University of Konstanz, Faculty of Biology, D-7750 Konstanz, Federal Republic of Germany

ABSTRACT The action of charybdotoxin (ChTX), a peptide component isolated from the venom of the scorpion *Leiurus quinquestriatus*, was investigated on membrane currents of identified neurons from the marine mollusk, *Aplysia californica*. Macroscopic current recordings showed that the external application of ChTX blocks the Ca-activated K current in a dose- and voltage-dependent manner. The apparent dissociation constant is 30 nM at $V = -30$ mV and increases e-fold for a +50- to +70-mV change in membrane potential, which indicates that the toxin molecule is sensitive to ~35% of the transmembrane electric field. The toxin is bound to the receptor with a 1:1 stoichiometry and its effect is reversible after washout. The toxin also suppresses the membrane leakage conductance and a resting K conductance activated by internal Ca ions. The toxin has no significant effect on the inward Na or Ca currents, the transient K current, or the delayed rectifier K current. Records from Ca-activated K channels revealed a single channel conductance of 35 ± 5 pS at $V = 0$ mV in asymmetrical K solution. The channel open probability increased with the internal Ca concentration and with membrane voltage. The K channels were blocked by submillimolar concentrations of tetraethylammonium ions and by nanomolar concentrations of ChTX, but were not blocked by 4-aminopyridine if applied externally on outside-out patches. From the effects of ChTX on K current and on bursting pacemaker activity, it is concluded that the termination of bursts is in part controlled by a Ca-activated K conductance.

INTRODUCTION

A variety of toxins from animals and plants have proven useful as tools in the study of the electrophysiological and biochemical properties of ion channels in excitable membranes (Narahashi, 1974). A major advantage of some of the toxins is their specificity of action; i.e., they block or modify certain current components or ion channels without affecting others. Toxins such as tetrodotoxin or saxitoxin, which block Na channels, are outstanding in this respect and have been studied extensively (reviewed by Catterall, 1980).

Address reprint requests to Dr. A. Hermann, University of Salzburg, Institute of Zoology, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria.

Various scorpion venoms have been found to act on the Na as well as the K conductance in excitable tissue (Koppenhöfer and Schmidt, 1968; Narahashi et al., 1972; Romey et al., 1975; Gillespie and Meves, 1980). They generally slow Na inactivation and increase the maintained Na conductance, whereas their effect on K conductance is to reduce the steady state conductance and slow its activation. As might be expected from these diverse actions, the crude venoms consist of a variety of small polypeptides (usually three to five; cf. Tu, 1977), which probably have different modes of action. A peptide toxin isolated from the venom of the scorpion *Centruroides noxius*, for example, blocks the delayed, voltage-activated K current (Carbone et al., 1982). Another toxin from the scorpion *Leiurus quinquestriatus*, termed charybdotoxin (ChTX), has been reported to block Ca-activated K channels isolated from rat skeletal muscle and inserted into lipid bilayers (Miller et al., 1985).

In this article, we investigate the action of ChTX on the Ca-activated K current of *Aplysia* neurons. In particular, we were interested in the affinity of the toxin, its specificity of action, its voltage dependence, and its effect on action potentials and bursting pacemaker activity. A preliminary report has been presented elsewhere (Hermann, 1986).

METHODS

Aplysia californica were obtained from Marine Specimens Unlimited, Pacific Palisades, CA. For experiments, various identified cells in the abdominal ganglion (R2, R15, L3, L6, L7, and L11) and unidentified cells in the buccal and pleural ganglia were used. To facilitate the removal of connective tissue, the ganglia were bathed in artificial seawater (ASW) containing 10 mg/ml protease (type XIV, Sigma Chemical Co., St. Louis, MO) for 10–30 min, depending on the thickness of the connective tissue. The ASW contained 468 mM NaCl, 10 mM KCl, 10 mM CaCl₂, 45 mM MgCl₂, and 25 mM Tris at pH 7.7. After protease treatment, the ganglia were washed thoroughly for 15–30 min in ASW and then stored for 15 min to 2 h at 4°C in ASW containing 10 mM glucose. Similar experimental results were obtained with or without enzyme treatment.

Both dual-electrode voltage-clamp and patch-clamp techniques were used. The procedures for dual-microelectrode voltage-clamping were similar to those described previously (Hermann and Gorman, 1981a; Hermann and Hartung, 1982a). In brief, the voltage electrodes were filled with 3 M KCl, and the current electrodes were filled with 2.8 M K-acetate. The membrane potential was measured differentially between the intracellular voltage electrode and an extracellular microelectrode filled with 3 M KCl. Membrane currents were measured by a virtual ground circuit. For injection of Ca ions or EGTA into the cells, the electrodes were filled with 0.1 M CaCl₂ or 0.5 M K₂-EGTA solution, respectively. The injections were made ionophoretically using the voltage clamp as a current sink.

In Ca- or Na-free ASW, these ions were substituted by an equimolar amount of Co or Tris, respectively. In solutions containing 200 mM tetraethylammonium (TEA)-Cl or 5–10 mM 4-aminopyridine (4-AP), an equimolar amount of NaCl was replaced. Tetrodotoxin was dissolved in Ca-free ASW (1 mg/ml) and added directly to the experimental bath solution in a final concentration of ~50 μM.

Scorpion venoms are a composite of neurotoxic proteins, which have many physical and chemical properties in common (cf. Tu, 1977). The toxins isolated from the venom generally consist of a single peptide chain cross-linked by four disulfide bonds (Miranda

et al., 1970). The venom from *Leiurus quinquestriatus* contains five peptide components with 57–66 amino acid residues each. The toxins do not contain methionine and have a high amount of aromatic amino acids (7–10). ChTX, which is a component of the *Leiurus* venom, was obtained as a gift from Dr. C. Miller (Brandeis University, Waltham, MA). (The abbreviation “ChTX” is used here because the initials “CTX,” as suggested by the original investigators, have been used previously for *Condylactis toxin* from the Bermuda anemone [Narahashi et al., 1969].) ChTX is a basic polypeptide and has a molecular weight of ~7,000. The partially purified toxin (~30% pure) was obtained in a stock solution of 5 or 25 μM containing 200 mM NaCl (for purification techniques, see Smith et al., 1986). As the toxin is highly lipophilic and attaches easily to container walls, it was applied directly to the experimental bath. Since ChTX may also bind to surfaces in the experimental chamber and may bind nonspecifically to ganglion tissue, the concentrations given probably do not reflect the actual effective doses but should be considered as upper limits.

The bath temperature was kept at $17 \pm 1^\circ\text{C}$. Records were taken on a three-channel rectilinear pen recorder, or on Polaroid film, or were digitized, stored, and analyzed with a 15-bit digital oscilloscope (4094, Nicolet Instrument Corp., Madison, WI).

For single channel recordings, techniques based on those described by Hamill et al. (1981) were used. Patch electrodes (Garner Glass, Claremont, CA) were pulled in a dual-step procedure on a vertical puller (David Kopf Instruments, Tujunga, CA) and had resistances of 2–5 M Ω . The electrodes were coated by dipping the tips into Q-dope (Newark Electronics, Inglewood, CA). After 10–20 min of drying, the electrode tips were fire-polished. The tips of the electrodes were first filled with the appropriate filtered (0.2- μm membrane filter) solution by application of negative pressure and then back-filled. Within the patch pipette, a sintered Ag/AgCl wire was used and a larger Ag/AgCl pellet was used as bath ground.

The pipette solution for inside-out patches (the cytoplasmic side of the membrane facing the bath solution) contained ASW. After a gigaseal was formed, the external ASW was exchanged for a solution containing 360 mM KCl, 292 mM sucrose, 25 mM HEPES, 2 mM MgCl_2 , 1 mM EGTA, and various concentrations of CaCl_2 . The free Ca concentration was adjusted to 10^{-9} , 10^{-7} , 3×10^{-7} , 10^{-6} , and 3×10^{-6} M at pH 7.4. The K equilibrium potential (E_K) for this solution is -90 mV and the Cl equilibrium potential (E_{Cl}) is -12 mV. For outside-out patches, the pipette solution contained 400 mM KCl, 50 mM HEPES, 1 mM EGTA, and CaCl_2 to give a free Ca concentration of 5×10^{-7} M and 10^{-6} M. In this case, $E_K = -93$ mV and $E_{Cl} = -10$ mV.

Patch recordings were taken from unidentified small cells (50–100 μm diam) in the abdominal, buccal, or pleural ganglia. Experiments were carried out at room temperature (20–24°C). Data were stored on magnetic FM tape (Store 4, Racal Recorders, Inc., Sarasota, FL) or on floppy disks. The data were low-pass filtered (0.3 or 1 kHz) and digitized (sample rate, 1 or 5 kHz, respectively) for further analysis with a computer (LSI 11/23, Indec Systems, Sunnyvale, CA). Measurements are given as means \pm standard error of the mean.

RESULTS

The effects of ChTX on macroscopic Ca-activated outward K currents were studied with two different experimental protocols: the current was activated either by brief depolarizing voltage pulses, which activates voltage-sensitive Ca channels in the plasma membrane, or by the intracellular injection of Ca ions (cf. Meech and Standen, 1975; Gorman and Hermann, 1979).

Effects of ChTX on Ca-activated K Currents Induced by Depolarization

In normal ASW, the outward K current of an R15 cell produced by brief, 150-ms depolarizing pulses to +50 mV consists of two distinct phases. After the application of 4-AP (10 mM), the fast rising phase of the current (which consists predominantly of the voltage-activated, delayed rectifier current) is reduced by

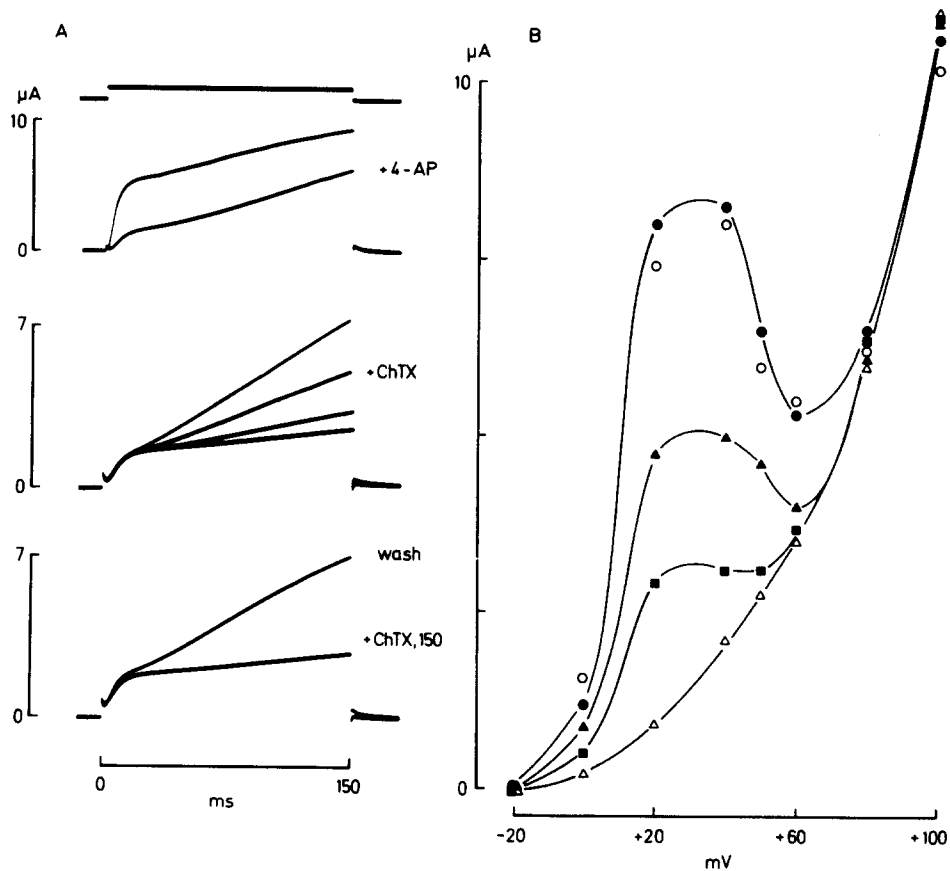


FIGURE 1. Effect of ChTX on outward K currents induced by depolarization. (A) Top traces: outward currents recorded in ASW and in a solution containing 10 mM 4-AP to suppress voltage-activated K currents. Middle traces: outward current under control conditions (ASW containing 10 mM 4-AP) and after the application of 50, 100, and 150 nM ChTX. Bottom traces: outward currents in ChTX- (150 nM) containing solution and after washout of the toxin. The membrane potential was clamped from a holding potential of -40 to $+50$ mV (see top trace). (B) Current-voltage relation of peak outward currents measured at the end of 150-ms pulses. Measurements were taken in ASW containing 10 mM 4-AP, before (filled circles) and after the application of 100 nM (filled triangles), 200 nM (filled squares), and 300 nM (open triangles) ChTX, and after washout of the toxin (open circles). Holding potential, -40 mV. R15 cell.

~80%, whereas the following slow rising phase (predominantly the Ca-activated K current) is slightly increased (Fig. 1A, top traces) (cf. Hermann and Gorman, 1981a). This experimental setup was chosen to suppress the voltage-dependent component of the outward current in order to maximize the visibility of the Ca-dependent component. ChTX suppressed the slow component of the outward current in a dose-dependent manner but had no effect on the residual fast outward current component. Fig. 1A (middle traces) shows records of the outward current before and after the addition of 50, 100, and 150 nM ChTX to the bath solution. The magnitude of the outward tail currents at the end of the voltage steps was also reduced by ChTX in a dose-dependent manner but the time course of decay was not altered after the application of the toxin (not shown). The blocking effect was completely reversible within 1–3 min after the toxin-containing ASW was replaced by normal ASW (Fig. 1A, bottom traces). The leakage current was somewhat increased after removal of the toxin, which may explain the apparent increase in the voltage-dependent current seen at the beginning of the trace.

Fig. 1B shows the current-voltage relationships of peak outward currents of another cell measured at the end of brief, 150-ms voltage pulses to various potentials. The current-voltage curve of R15 cells in ASW is typically N-shaped (Hermann and Gorman, 1981a). The outward currents were measured in a solution containing 10 mM 4-AP before and after the application of 100, 200, and 300 nM ChTX and after washout of the toxin. The “hump” of the current curve in the voltage range between +20 to +80 mV, where the Ca-activated K current is predominant (Meech and Standen, 1975), is suppressed by ChTX and is dependent on the dose of the toxin. The current appeared to be completely suppressed at 300 nM ChTX, since higher concentrations had no additional effect on the outward current. In another cell, 300 nM ChTX did not completely suppress the hump in the current-voltage relationship. The delayed outward K current at membrane voltages more positive than +80 mV was not appreciably altered by ChTX. Dose-response relationships of two cells showed that half-maximal inhibition of the Ca-activated K current occurred at 160 and 185 nM ChTX at $V = +20$ mV. The experimental values could be fitted by assuming a 1:1 toxin-receptor interaction (not shown). Correction of the currents for series resistance (typically 1.5 k Ω in these cells; cf. Hermann and Gorman, 1981a) did not alter the shape of dose-response curves but resulted in a shift of the curves, giving K_D values of 135 and 155 nM. In a later section, dose-response curves will be shown for K currents activated by Ca injection.

ChTX Effects on the K Current Induced by Ca Injection

The K current induced by ionophoretic, intracellular injection of Ca ions into voltage-clamped R15 cells has been identified by its reversal potential and by its pharmacological properties (for review, see Hermann and Hartung, 1983). Fig. 2A shows a plot of peak Ca-activated K currents vs. time during two applications of ChTX. The currents were produced by identical Ca injections (300 nA for 2 s) at 1-min intervals. Repeated Ca injection over a prolonged time (15–60 min)

caused a gradual, linear increase of the holding current and of the peak current amplitude. ChTX (100 nM) suppressed the peak outward current by 82 and 76% (Fig. 2A). The blocking effect was reversible within 2–3 min after washout of the toxin, recovery generally being slower than the onset of block. Fig. 2C shows recordings of Ca-activated outward K currents before and during the

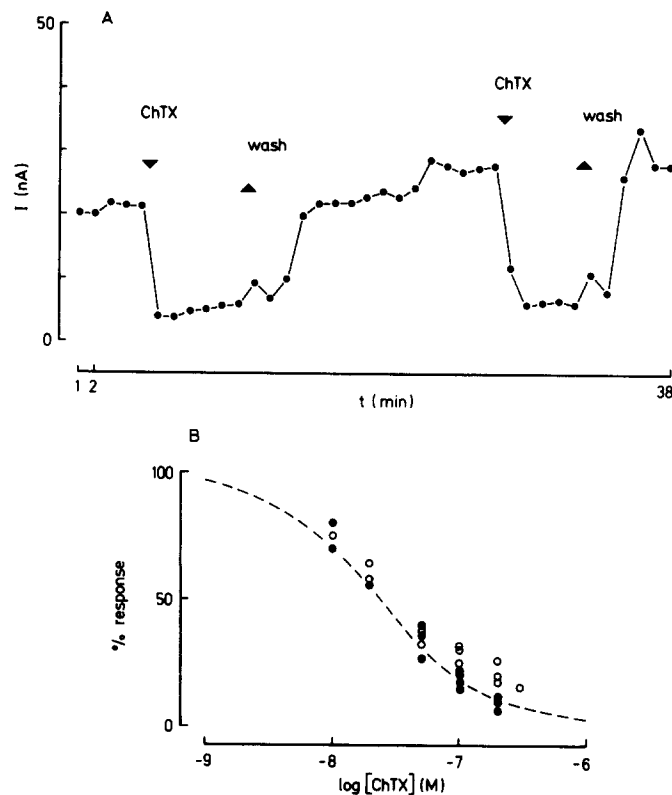


FIGURE 2. Action of ChTX on the Ca-activated outward K current induced by Ca injection. (A) Plot of peak outward currents activated by 300-nA, 2-s intracellular injections of Ca ions at a holding potential of -30 mV. The Ca injections were repeated every minute and ChTX was applied at a concentration of 100 nM at the times indicated. R15 cell. (B) Dose-response plot of the Ca-activated outward current at a holding potential of -30 mV, for five R15 cells (filled circles) and three L cells (one L3, two L6; open circles). The theoretical line drawn through the experimental points was calculated from the equation: $I_{K,ChTX}/I_K = K_D/(K_D + [ChTX])$, where $I_{K,ChTX}$ and I_K are the currents after the application of ChTX and in control solution, respectively, K_D is the apparent dissociation constant, and $[ChTX]$ is the external concentration of ChTX. (C) Records of outward currents activated by 400-nA, 2-s Ca injections at 1-min intervals before and after the application of 20, 50, and 100 nM ChTX. All records are from the same R15 cell. ChTX was washed out after each application.

application of ChTX. The currents reached a peak shortly after termination of the Ca injection and thereafter declined to their initial holding current level. ChTX applied at various concentrations (20, 50, and 100 nM) at the time indicated in the figure reduced the outward current in a dose-dependent manner. The time course of the decay of the outward current was not altered by the toxin. The dose-response relationship shown in Fig. 2B indicates that a 50%

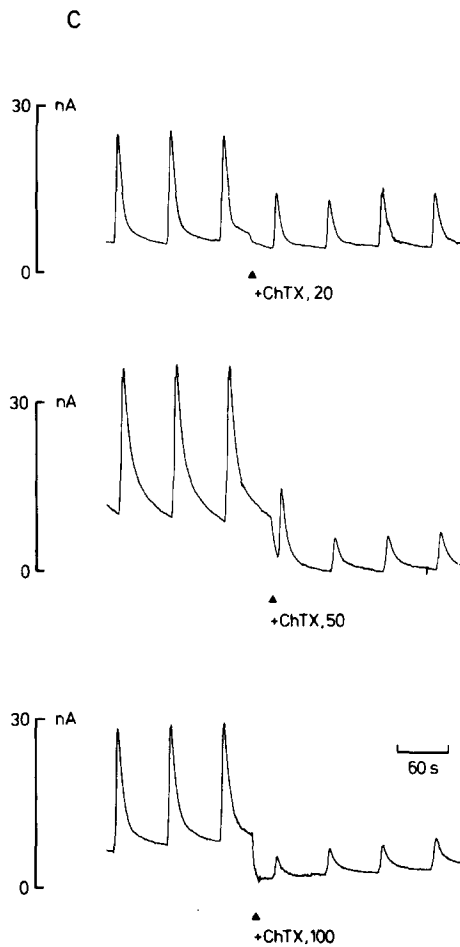


FIGURE 2C.

block of the current occurs at $\sim 30 \pm 28$ nM ChTX at $V = -30$ mV (eight cells). At $V = +20$ mV, the average half-maximal block was 170 ± 65 nM. The experimental data obtained from R15 cells could be fitted by assuming a 1:1 toxin-receptor interaction (dashed line in Fig. 2B). However, for L3 and L6 cells, the block was less than expected at toxin concentrations higher than 100 nM (three experiments). These cells have been reported to contain two components of Ca-activated K currents with different TEA sensitivities (Deitmer and

Eckert, 1985). This suggests that ChTX blocks the TEA-sensitive K current component (~98%) in R15 cells (Hermann and Gorman, 1981a), but spares a minor TEA-resistant K current component (~20%) in the L cells.

In Fig. 3A, peak outward currents produced by identical Ca injections are plotted against different membrane holding potentials. A clear reduction of the outward current by ChTX (100 nM) is observed at potentials more positive than -50 mV. The suppression of the current is less evident at more positive

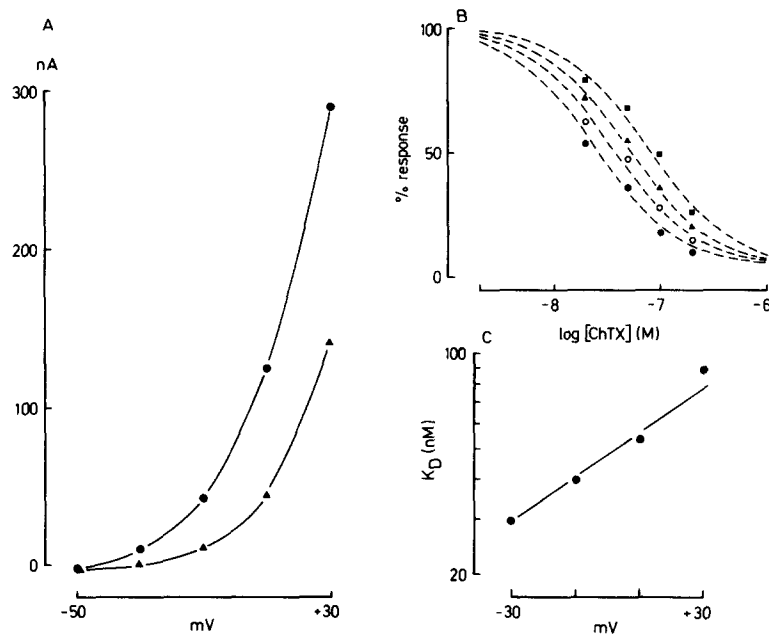


FIGURE 3. Effect of ChTX and membrane potential on the Ca-activated K current. (A) Current-voltage relationship of peak outward K currents activated by intracellular injection of Ca ions (200 nA, 2 s) before (circles) and after (triangles) the application of 100 nM ChTX. (B) Dose-response curves of the Ca-activated outward K current at different test pulse potentials: $V = -30$ (●), -10 (○), $+10$ (▲), and $+30$ (■) mV. The dashed lines fitted to the experimental points were calculated after the equation outlined in Fig. 2B. (C) Semilogarithmic plot of the apparent dissociation constant, K_D , vs. membrane potential. All currents were measured in Ca-free ASW containing 10 mM Co and 50 μ M tetrodotoxin. The line drawn to the experimental points is from a linear least-squares best fit with an e-fold change of K_D per 63-mV change in membrane voltage; $r = 0.97$. R15 cell.

membrane potentials, which indicates that the block is voltage dependent. Fig. 3B shows dose-response plots at different membrane potentials. The experimental points were fitted by a series of curves whose half-maximal values are shifted to the right along the concentration axis at more positive membrane voltages. The semilogarithmic plot in Fig. 3C shows the relationship between K_D and the membrane potential. K_D changes e-fold for a 63-mV change in potential. In a different cell, K_D increased e-fold per 69-mV change in membrane potential.

The effective valence (which is the product of the true valence and the fraction of the membrane electric field experienced at the blocking site) for the voltage dependence of the block is 0.3–0.35.

The time course of the ChTX block was studied in experiments where Ca ions were injected continuously at a low injection intensity until the outward current reached a stationary level. Fig. 4A shows that ChTX (50 nM) applied at the time indicated in the figure suppressed the outward current by 80% in <10 s. In Fig. 4B, a similar experiment is shown on a slower time base, where the Ca injection was continued for some time after the application of ChTX. The toxin initially blocked the current completely within 13 s, but the block appeared to be overcome by the prolonged Ca injection.

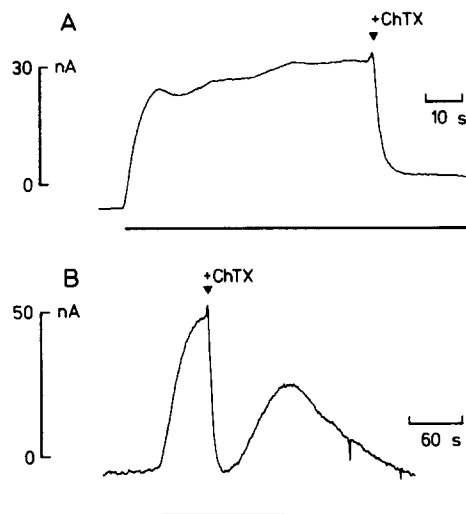


FIGURE 4. Time course of the ChTX block on the Ca-activated K current. (A) The outward current was activated by a 50-nA Ca injection for the time indicated by the line beneath the current trace. The time at which ChTX (50 nM) was added to the bath solution is indicated above the response. Holding potential, -30 mV. R15 cell. (B) Similar experiment on a slower time base. ChTX (100 nM) application (arrowhead) blocks the outward current, but the block is partially overcome by the prolonged Ca injection (bar beneath the current injection).

Specificity of ChTX Action

To determine the specificity of ChTX action, inward Na and Ca currents, as well as delayed and the transient outward K currents, were investigated. The currents were studied under conditions that allowed for their optimal separation. Under the appropriate conditions, ChTX had no significant effect on the inward Na current (two cells) or the inward Ca current (two cells) (Fig. 5, A and B). ChTX also had no effect on the delayed rectifier current (three cells) or the transient outward K current (two cells) (Fig. 6, A and B). The transient K current was elicited after removal of its inactivation by a brief hyperpolarizing prepulse. The current recording in Fig. 6B shows a record of the apparent inward leakage

current (I_L) in response to a hyperpolarizing voltage pulse, which is followed by the transient outward K current (I_A) on the repolarizing phase of the pulse. The outward current activated by the injection of Ca ions is shown on a slower time base. After application of ChTX (50 nM), the Ca-activated K current and the leakage current were reduced, but the transient outward current was not altered.

ChTX-induced Inward Current

The reduction of the apparent leakage current by ChTX suggested a selective effect on a channel that is active near the resting potential of the cell. ChTX induced an inward shift of the steady state current recorded at -30 mV, whose

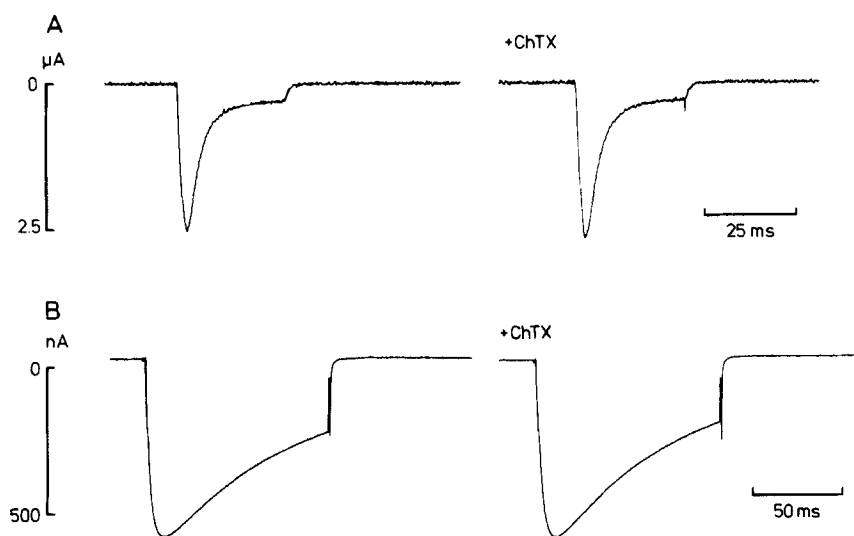


FIGURE 5. Specificity of ChTX action. (A) ChTX (50 nM) does not affect the inward Na current. The currents were measured in Ca-free solution containing 10 mM Co, 200 mM TEA, and 5 mM 4-AP. Holding potential, -40 mV; depolarizing voltage jump to $+30$ mV. R2 cell. (B) ChTX (100 nM) also has no effect on the inward Ca current. The currents were recorded in ASW containing 200 mM TEA, 5 mM 4-AP, and $50 \mu\text{M}$ tetrodotoxin. Holding potential, -50 mV; depolarizing voltage jump to $+20$ mV. R15 cell. The currents in A and B were corrected for capacitive and leakage current.

magnitude increased with the concentration of the toxin (Fig. 7A and cf. Fig. 2B, seven cells). The apparent inward current was accompanied by a decrease of the membrane resting conductance (cf. Fig. 6B). The current was also dependent on membrane voltage and reversed at -70 to -80 mV (Fig. 7B, two cells). The current was increased 10–20% after the removal of external Na (Na replaced by Tris, two cells), but it was reduced by $\sim 80\%$ in Ca-free, 10 mM Co solution (three cells). The inward current persisted in a solution containing 1 mM serotonin (two cells) and in a solution containing 4-AP (10 mM, two cells), but it was reduced by 80–90% in solutions containing TEA (100–200 mM, three cells). In two experiments, a fraction of the inward current (20–35%) persisted

after the injection of EGTA (500 nA for 5 min) into the cells. The experiments suggest that ChTX blocks a resting K conductance that is activated by internal Ca ions.

Ca-activated Single K Channel Recordings

It is known that ChTX blocks a large-conductance, Ca-activated K channel from vertebrate cells (Miller et al., 1985), but there is to date no information about

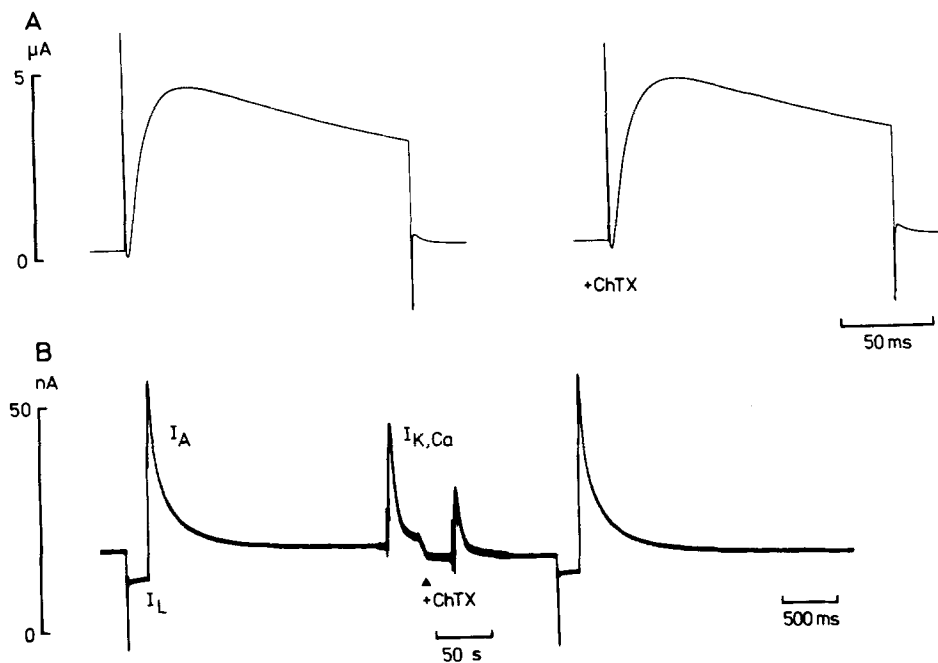


FIGURE 6. Specificity of ChTX action. (A) Effect of ChTX (100 nM) on the voltage-activated, delayed rectifier K current. Membrane currents were recorded in Ca-free ASW containing 10 mM Co and the cell was injected with EGTA (500 nA, 5 min). Holding potential, -40 mV; depolarizing voltage pulses to $+30$ mV. R15 cell. (B) The current trace shows, from left to right, an inward current (I_L) in response to a hyperpolarizing voltage pulse to -80 mV, followed by a transient outward current (I_A) on the repolarizing phase of the voltage jump to -40 mV. The Ca-activated K current ($I_{K,Ca}$) was induced by the injection of Ca ions (200 nA, 2 s). After application of ChTX (50 nM), $I_{K,Ca}$ and I_L were reduced but I_A was not altered. Holding potential, -40 mV. R15 cell.

the characteristics of a ChTX-sensitive channel in *Aplysia* neurons. Ca-activated K channels were recorded in the cell-attached, the inside-out, and the outside-out modes (see Methods). Single channel currents recorded over a range of internal (cytoplasmic side of the patch) Ca concentrations are shown in Fig. 8A. The top recording shows the channel activity in nominally zero Ca solution containing 10 mM EGTA. Gating of the channel was characterized by openings of short duration and a low probability of opening, which suggests that the

channels can be activated either at very low Ca concentrations or that the activity of the channel reflects the voltage-dependent component of channel opening (Barrett et al., 1982; Pallotta, 1985). Increasing the internal Ca concentration increased the number of channels open, the frequency of opening, and the time the channels spent in the open state. In Fig. 8A (middle trace), a substantial increase in channel activity in response to 1 μM Ca can be seen, with a maximum of three channels open (see inset). On decreasing the Ca concentration to 0.3 μM , channel activity decreased within the time resolution of the bath exchange to a lower level (Fig. 8A, bottom trace). The channel open probability of 1–2% in Ca-free, 10 mM EGTA solution increased to 11–19% in 0.3 μM Ca and to 79–93% in 1 μM Ca solution (two patches). The single channel conductance in asymmetrical K solution was 39 pS at 0 mV. The average single channel conductance from cell-attached (2 patches) and from excised patch recordings

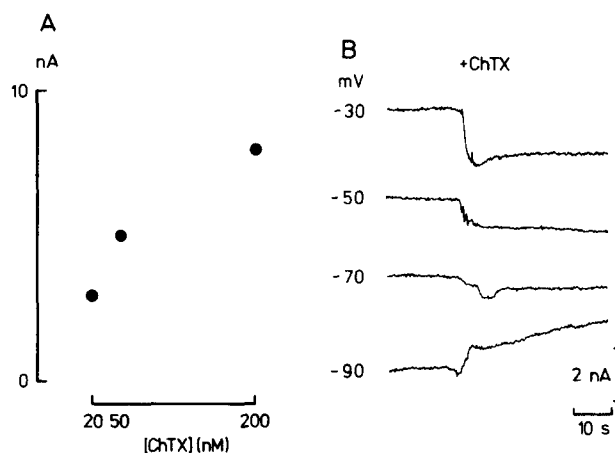


FIGURE 7. ChTX induces an apparent inward current. The application of ChTX (50 nM) activates an inward current whose amplitude is dependent on the toxin concentration (A) and on the membrane potential (B). All data are from an R15 cell at a holding potential of -30 mV in A.

was 35 ± 5 pS at $V = 0$ mV (16 patches). The amplitude of the single channel currents was not dependent on the Ca concentration. ChTX applied at the interior side of the membrane had no blocking effect on Ca-activated K channels (one patch).

The reversal potential of single channel currents extrapolated from current-voltage relationships (see below) was close to the theoretical value of E_K , i.e., -90 mV. E_{Cl} is calculated to be -12 mV in our solutions. Therefore, it appears unlikely that the channels recorded here were Cl channels. The conductance of "S" channels (serotonin-sensitive channels; Siegelbaum et al., 1982) is, on average, 39 pS (at $V = 0$ mV), i.e., ~ 10 – 20% larger compared with Ca-activated K channels. In addition, S channels are neither Ca nor voltage sensitive and therefore can easily be distinguished from Ca-activated K channels (Brezina et al., 1987). It is also unlikely that these channels were Ca-dependent, nonselective

cation channels, i.e., equally permeable to K and to Na ions (Colquhoun et al., 1981; Yellen, 1982), because of their negative reversal potential and their voltage dependence.

As expected from macroscopic current measurements in *Aplysia* neurons (Gorman and Hermann, 1979; Gorman and Thomas, 1980), the Ca-activated K

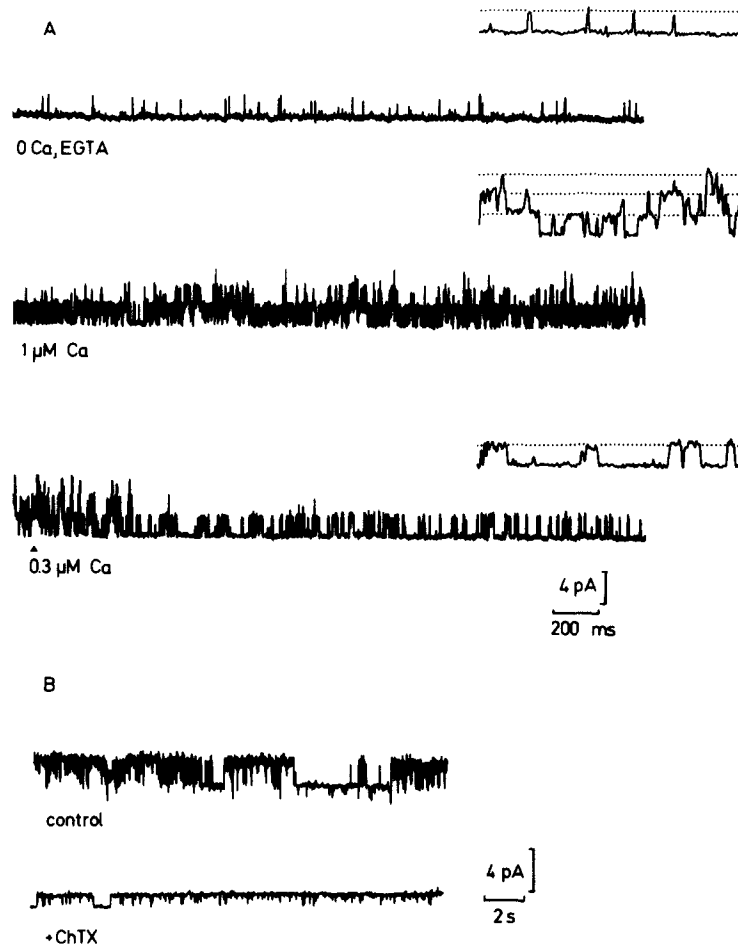


FIGURE 8. ChTX effect on single Ca-activated K channels. (A) Recording from an inside-out patch in 0 Ca, 10 mM EGTA, 1 μ M Ca, and in 0.3 μ M Ca (arrowhead) internal solution. The insets show single channel recordings on an extended time scale (100-ms traces). (B) Outside-out patch recording before (control) and after application of ChTX (200 nM) to the bath solution. The patch pipette contained 1 μ M Ca. The membrane potential in A and B was 0 mV.

channels are voltage dependent. Fig. 9A shows recordings from an inside-out patch at membrane potentials between -20 and $+60$ mV at a bath Ca concentration of 0.3 μ M. The amplitude of the unitary currents as well as the probability of channel opening increased with more positive membrane potentials (Fig. 9C).

The channel opening probability changed e-fold per 44-mV change in membrane potential. In a different patch, the open probability changed e-fold for a 56-mV change in membrane voltage. The current-voltage relationships obtained from the single channel measurements exhibit outward rectification, as expected from

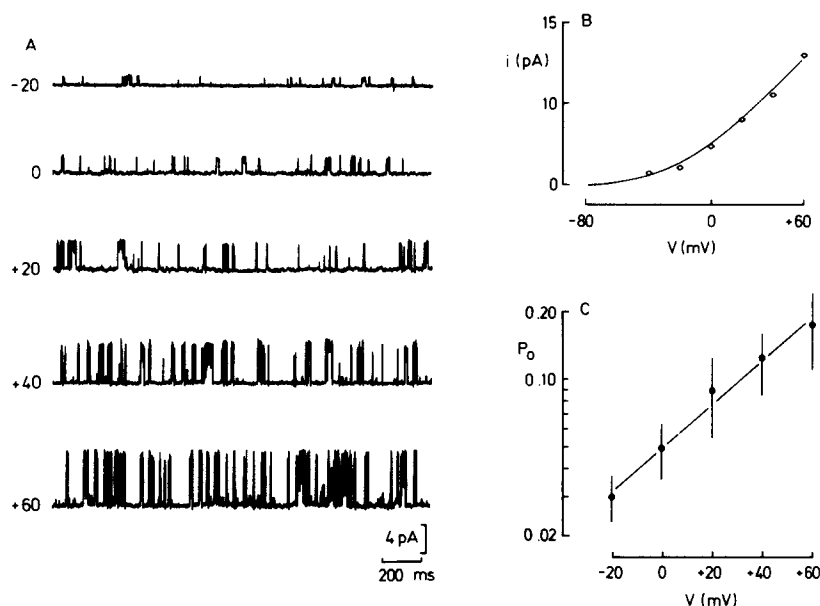


FIGURE 9. Voltage dependence of the Ca-activated K channel. (A) Recording from an inside-out patch at membrane potentials from -20 to $+60$ mV. The bath medium contained $0.3 \mu\text{M}$ Ca. (B) Current-voltage relation of the Ca-activated K channel. The data points are averages of ~ 100 channels measured at each voltage. The standard deviation is within the size of the symbols. The line drawn to the experimental points was calculated using the constant field equation: $\gamma_{\text{K,Ca}} = \pi_{\text{K,Ca}} E[\text{K}]_o / E - E_{\text{K}} F^2 / RT \exp(E_{\text{K}} F / RT) - \exp(EF / RT) / \exp(E / RT) - 1$, where $\gamma_{\text{K,Ca}}$ is the unit conductance of a single Ca-activated K channel, $\pi_{\text{K,Ca}}$ is the channel permeability, with a value of $8 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$, E is the membrane potential, E_{K} is the K equilibrium potential, $[\text{K}]_o$ is the external K concentration, and R , T , and F have their usual meanings. (C) Plot of the open probability of a single channel, P_o (mean \pm SD), measured from an inside-out patch (bath Ca concentration, $0.1 \mu\text{M}$), vs. membrane potential. To estimate P_o , single channel currents were integrated over 11–16 records of 1.024 s duration, and divided by the record length, by the unitary current, and by 3, the number of channels in the patch. The linear least-squares fit to the experimental points indicates an e-fold change of P_o per 44-mV change in potential; $r = 0.98$.

the asymmetric K distribution on either side of the patch ($\text{K}_i = \sim 360 \text{ mM}$, $\text{K}_o = 10 \text{ mM}$). The line drawn to the experimental points was calculated from the constant field equation (Hodgkin and Katz, 1949) and follows the prediction for a K-selective channel.

In recordings from outside-out patches containing 5×10^{-7} – 10^{-7} M Ca in the pipette, voltage-sensitive K channels were observed that had current amplitudes of 2.5–3 pA, corresponding to a conductance of 32 ± 6 pS at $V = 0$ mV (six patches). Channel activity was reduced by external TEA at submillimolar concentrations and was completely blocked by 2–5 mM TEA (four patches). The onset of the block was fast, i.e., within the limits of the bath exchange rate of a few seconds, and the block was fully reversible. 4-AP had no blocking effect on K channels at concentrations up to 10 mM (one patch). The channels therefore

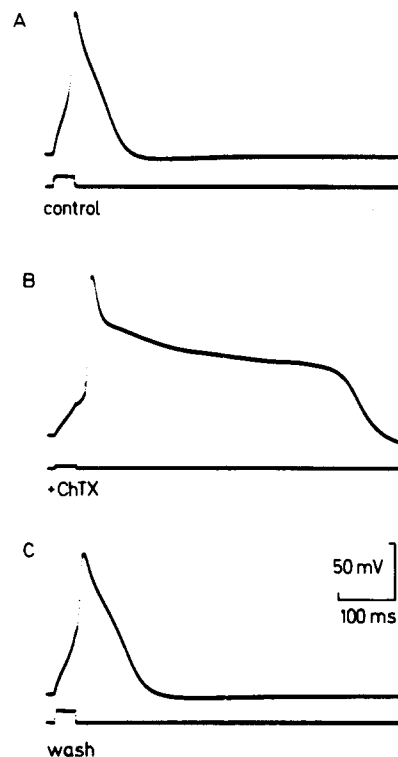


FIGURE 10. Effect of ChTX on action potentials. The cell (R15) was held in ASW containing 10 mM 4-AP. The action potentials were elicited by brief, 30-ms intracellular current injections (traces beneath action potentials). Action potentials are shown before application (A), in ChTX-containing solution (B), and after washout of the toxin (C). The membrane potential was kept constant at -40 mV by current injection.

show pharmacological properties unique to macroscopic Ca-activated K currents (Hermann and Gorman, 1981*a, b*). These features, together with a single channel conductance similar to that obtained from inside-out patches, identifies these channels as Ca-activated K channels. Fig. 8*B* shows single channel recordings (previously identified as Ca-activated K channels) from an outside-out patch, before and after the addition of ChTX (200 nM) to the external solution. ChTX

completely blocked the Ca-activated K channels (larger current amplitudes) but had no effect on a channel of smaller current amplitude (two experiments).

Effects of ChTX on Action Potentials and Bursting Pacemaker Activity

Various cells in the *Aplysia* abdominal ganglion discharge action potentials in a bursting or beating manner or are silent (Kandel, 1976; Gorman and Hermann, 1982). ChTX affects different types of action potentials differently. It prolonged the duration of action potentials from bursting cells (four experiments) but had no effect on action potentials of beating cells or silent cells after direct stimulation (three experiments). The effect of the toxin on the action potential of bursting R15 cells is shown in Fig. 10. The action potentials were elicited by 30-ms intracellular current injections into the cells bathed in ASW containing 10 mM 4-AP to suppress voltage-dependent K conductances. After the application of ChTX, the duration of the action potential was prolonged seven- to eightfold (measured at 40% amplitude), but its rising phase was not altered, which indicates that under these conditions, a Ca-activated K conductance contributes to the repolarization of the action potential. The effect was reversible after washout of the toxin.

In addition to the effect on action potentials, ChTX reduced the membrane resting potential. In bursting pacemakers, the depolarization increased the burst frequency and eventually caused repetitive discharge of action potentials. Upon repolarization by current injection to a potential level where the first action potentials of the bursts were superimposed, bursting activity was re-established, with some changes in the burst parameters. (a) The number of action potentials was increased by 20–25%. (b) The post-burst hyperpolarization was diminished by 2–6 mV. (c) Although the first action potentials from the burst before and after the application of ChTX were identical, the final action potentials of the burst after toxin application were prolonged (three cells). These effects were completely reversible after removal of the toxin. In beating or silent cells, ChTX induced a higher frequency of repetitive discharge of action potentials or initiated irregular discharge activity.

DISCUSSION

The major conclusion from the experiments reported here is that ChTX is a high-affinity, specific blocker of the Ca-activated K conductance in *Aplysia* neurons. The results confirm and extend previous findings on the mechanism of ChTX action in Ca-activated K channels from mammalian skeletal muscle (Miller et al., 1985).

ChTX reversibly suppresses the Ca-activated K current elicited by either depolarizing voltage pulses or by ionophoretic Ca injections into the cells in a dose-dependent manner. The apparent dissociation constant for the toxin-receptor complex is ~30 nM at $V = -30$ mV and 170 nM at $V = +20$ mV. Compared with TEA, another blocker of the Ca-activated K current in *Aplysia* neurons, which has a K_D of 0.5 mM at $V = +20$ mV (Hermann and Gorman, 1981b), ChTX is ~3,000-fold more effective. The toxin is even more effective at negative membrane potentials, and is so far the most powerful agent known to block Ca-activated K channels in these cells.

From the fast onset of action, as well as the fast recovery after washout, we conclude that ChTX is not inserted into the lipid phase of the membrane, nor does it pass through the membrane. From its specificity of action, it also appears less likely that the toxin exerts its effect via a nonspecific target such as the membrane lipid phase. The voltage dependence of the block produced by ChTX further indicates that the molecule is sensitive to ~35% of the membrane electric field.

In addition to its effectiveness in blocking the Ca-activated K current, ChTX is also highly specific in its action. Other K currents, such as the delayed rectifier current ($I_{K,v}$) or the transient K current (I_A), were unaffected at ChTX concentrations that substantially reduce the Ca-activated K current. Furthermore, ChTX had no effect on the Na current (I_{Na}) or the Ca current (I_{Ca}). Application of the toxin, however, produces an apparent inward displacement of the holding current, whose negative reversal potential, TEA sensitivity, dependence on external Ca, and insensitivity to external 4-AP suggest that it is caused by the blockade of a resting K conductance, which is activated by internal Ca ions. This agrees with findings that in many *Aplysia* neurons, a significant Ca-dependent K conductance contributes to the membrane resting conductance (Gorman and Hermann, 1982; Johnson and Thompson, 1983), and explains the depolarizing action of ChTX on the membrane resting potential. Since a fraction of the ChTX-induced inward current displacement persists in EGTA-injected cells, it is possible that EGTA buffering of Ca ions was not sufficient (Marty and Neher, 1985) and therefore a component of the Ca-dependent K current remained.

It should be noted that apamin, a toxic component from bee venom, which blocks the Ca-activated K current in a variety of tissues (cf. Lazdunski, 1983; Cook and Haylett, 1985), was relatively ineffective in blocking the Ca-activated K current in *Aplysia* neurons (Hermann and Hartung, 1983).

Recordings from Ca-activated single K channels revealed that ChTX applied to the external membrane surface of outside-out patches blocks their activity. As expected from macroscopic current recordings (Hermann and Gorman, 1981*a*, *b*), the channels were also sensitive to submillimolar concentrations of external TEA but were not blocked by external 4-AP.

The conductance of Ca-activated K channels in asymmetric K solution is ~35 pS at $V = 0$ mV. A similar value for the single channel conductance of 40–60 pS in symmetrical K solution has been found in *Helix* neurons (Ewald et al., 1985) and from current fluctuation measurements in *Aplysia* cells (Hartung and Hermann, 1987). These values are larger but of the same order of magnitude as the single channel conductance of ~20 pS (in asymmetrical K solution) derived previously from current noise measurements (Hermann and Hartung, 1982*b*) and from single channel measurements in the cell-attached configuration from *Helix* neurons (Lux et al., 1981). The difference in single channel conductances can be explained by the nonlinear current-voltage relation in asymmetrical K solutions and the difference in the reversal potential in these experiments (Hartung and Hermann, 1987).

These and previous results substantiate the notion that in various types of cells at least two distinct classes of Ca-activated K channels exist: (*a*) channels with a

small conductance in the range of 20–60 pS (small K, or SK), found in molluscan neurons, and (b) channels with a large conductance of 100–300 pS (big K, or BK), found in vertebrate cells (for review, see Marty, 1983; Latorre and Miller, 1983). Although these channels differ considerably in their conductance, they exhibit similarities in their voltage dependence, selectivity (see in Blatz and Magleby, 1984), and pharmacology, i.e., their sensitivity to TEA and to ChTX (cf. Hermann and Hartung, 1983; Latorre and Miller, 1983; this article). From these considerations, it appears that the external mouths of these channels are quite similar but there are some structural differences in their conduction pathways.

Various types of channels also appear to differ in their sensitivity to internal Ca ions. The Ca-activated K channels in *Aplysia* neurons, with an open probability of ~86% at 1 μ M Ca and $V = 0$ mV, are more sensitive to internal Ca as compared with channels from cultured rat muscle (Barrett et al., 1982), where the percent of time open under similar conditions is ~3–4%. The *Aplysia* Ca-activated K channels therefore appear more similar to those of acinar cells from pig pancreas (Maruyama et al., 1983), with an open probability of ~97% at 1 μ M Ca and $V = 0$ mV.

ChTX generally had a small effect on the waveform of action potentials. The reasons are probably that in some cells the Ca-activated K current is small and does not contribute to the action potential, or that other Ca-independent K currents are the predominant factors in shaping its form (Gorman and Hermann, 1982). In the presence of 4-AP, which suppresses the voltage-dependent K conductance but not the Ca-activated K conductance, ChTX prolonged the duration of the action potential considerably. In vertebrate nerve terminals, ChTX added to normal solution has also been found to prolong the duration of action potentials and to eliminate the Ca-dependent afterhyperpolarization (Obaid and Salzberg, 1985).

In *Aplysia* bursting pacemaker cells, ChTX increased the number of action potentials per burst and prolonged the duration of the last action potentials in bursts, which indicates that a Ca-activated K conductance is involved in the termination of bursts and of action potentials. The toxin also somewhat diminished the hyperpolarization following the burst, but did not abolish it. This was expected since the number of action potentials per burst and the duration of action potentials increased, and therefore the amount of internal Ca accumulation is expected to increase, giving rise to an augmented Ca-activated K current. The persistence of the post-burst hyperpolarization may also be due to the fact that a maximum dose of ChTX, to completely eliminate the Ca-activated K conductance, was not used. However, high concentrations of the toxin may, because of its hydrophobic nature, exert nonspecific effects on other current components. Nevertheless, 100 nM ChTX at a membrane potential of -40 to -50 mV (membrane potential obtained during the post-burst hyperpolarization) should block at least 80% of the Ca-activated K current. The effect of the toxin on the post-burst hyperpolarization was small, however, which indicates that this current component may contribute only in part to the termination of the burst. This interpretation of our data is in accord with a previous hypothesis on the

mechanism of bursting pacemaker activity and allows us to unite an earlier hypothesis based on two reciprocating currents, an inward current carried by Ca ions and a Ca-activated outward K current (see Gorman et al., 1981, 1982), and a hypothesis that relies upon the Ca-dependent inactivation of a persistent inward Ca current (Adams and Levitan, 1985; Kramer and Zucker, 1985). Future experiments will have to clarify the quantitative aspects of the contribution of the different mechanisms to the generation of the pacemaker oscillations.

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