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PHARMACOLOGICAL PROPERTIES OF AXONAL SODIUM CHANNELS IN THE COCKROACH *PERIPLANETA AMERICANA* L.

II. SLOWING OF SODIUM CURRENT TURN-OFF BY *CONDYLACTIS* TOXIN

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Condylactis toxin induces a prolonged falling phase in action potentials recorded from giant axons of the cockroach *Periplaneta americana* L. A distinct plateau potential can be recorded in the presence of *Condylactis* toxin at concentrations of 0·5 mg/ml and above. In cockroach axons, voltage-clamp experiments show that *Condylactis* toxin acts primarily by slowing the sodium current turn-off without affecting either the time-course of the sodium conductance increase or the peak amplitude of the transient sodium current.

INTRODUCTION

It is established that tetrodotoxin (Pichon, 1969*a*, *b*, 1974, 1976) and saxitoxin (Pelhate & Sattelle, 1978; Sattelle, Pelhate & Hue, 1979) specifically block the sodium channels in giant axons of the ventral nerve cord of the cockroach (*Periplaneta americana*). No chemical probe specific for the sodium channel inactivation (closing) mechanism of insect axons is currently available. In the present study, extract from the tentacles of the marine coelenterate *Condylactis gigantea*, containing *Condylactis* toxin (CTX), a neurotoxic polypeptide (Shapiro, 1968*a*), is tested on the axonal membrane of the cockroach (*P. americana*).

When applied to nerve bundles of a crayfish (*Oronectes virilis*), CTX produces repetitive firing of action potentials (Shapiro, 1968*b*). In addition, action potentials recorded from giant axons of the lobster (*Homarus americanus*) and from the stretch receptor cells of the crayfish (*O. virilis*) exhibit a prolonged falling phase at CTX concentrations of 0·2 mg/ml and above (Shapiro & Lilleheil, 1969). Voltage-clamp experiments on giant axons of another crayfish (*Procambarus clarkii*) indicate that these effects of CTX are due to a slowing of the turn-off of the transient sodium conductance (Narahashi, Moore & Shapiro, 1969). Subsequent analysis of the kinetics of sodium conductance changes in toxified crayfish (*P. clarkii*) axons under voltage-clamp have confirmed this view and show that the steady-state sodium inactivation curve is shifted in the direction of hyperpolarization (Murayama *et al.*

1972). Another polypeptide toxin (ATX_{II}) has been purified by Béress *et al.* (1975) from a coelenterate (*Anemonia sulcata*). This toxin also prolongs action potential duration in giant axons of a crayfish (*Astacus leptodactylus*) by selectively slowing the sodium inactivation mechanism (Romey *et al.* 1976). ATX_{II} does not affect either the sodium activation or the steady-state potassium conductance (Romey *et al.* 1976). Prolonged exposure of crustacean axons to these anemone toxins results in conduction block with no more than a few millivolts depolarization of the axonal membrane (Shapiro & Lilleheil, 1969; Romey *et al.* 1976). Anemone toxins CTX and ATX_{II} therefore act on the mechanism by which the sodium channels of crustacean nerve membranes are closed.

The sensitivity of crustacean neurones to CTX and ATX_{II} contrasts sharply with the reported insensitivity to these toxins of molluscan neurones. For example, the giant axon of the squid is unaffected by CTX applied either externally (Narahashi *et al.* 1969) or internally (T. Narahashi, personal communication). In the same way, ATX_{II} is without effect on the giant axon of the cuttlefish (*Sepia officinalis*) when applied externally (Romey *et al.* 1976). It is of interest therefore to discover whether axons of arthropods other than crustaceans are also sensitive to CTX. In this study CTX is applied to giant axons of the cockroach (*P. americana*) which closely resemble other unmyelinated axons in both the passive permeability characteristics of their membranes and the mechanism of excitability (see Pichon, 1974). In particular, the application of voltage-clamp techniques to isolated cockroach giant axons has resulted in the demonstration of a transient, inward sodium current and a late, outward, potassium current during a depolarizing voltage-clamp pulse (Pichon, 1969*a*, 1974, 1975). We have therefore examined the actions of CTX on isolated giant axons of the cockroach (*P. americana*) in order (*a*) to further assess the specificity of this toxin within the Arthropoda and (*b*) to attempt to extend the range of currently available specific chemical probes of the molecular mechanisms underlying excitation in insect axonal membranes.

MATERIALS AND METHODS

Using the double oil-gap, single-fibre technique (Pichon & Boistel, 1967), voltage-clamp and current-clamp experiments were performed on giant axons dissected from abdominal nerve cords of the cockroach (*Periplaneta americana*). The methods and recording techniques were as previously described (Pelhate, Hue & Chanelet, 1978; Sattelle *et al.* 1979). The ionic composition of normal saline was as described in the preceding paper (Sattelle *et al.* 1979). Voltage-clamp experiments were performed at $12 \pm 0{\cdot}5$ °C. For current-clamp experiments the temperature was in the range 20–22 °C. *Condylactis* toxin (CTX) was obtained from Sigma Chemical Co., Poole, Dorset U.K. (batch no. 126C–0277). The synthetic saxitoxin hydrochloride (STX) used in this study was the generous gift of Prof. Y. Kishi (Harvard University).

RESULTS

Current-clamp experiments

No effect was detected on either the resting potential or the action potential of an isolated giant axon when CTX was applied at concentrations of 0·1–0·3 mg/ml for 10 min. At 0·5 mg/ml and higher concentrations, CTX did not affect either the resting potential or the time-course and amplitude of the rising phase of the action potential, but the falling phase of the action potential was prolonged. Within 3–10 min of toxin application at a concentration of 0·5 mg/ml action potentials with an extended plateau in the falling phase were evoked (Fig. 1). The effects on the action potential of a 10 min exposure to CTX were reversed by washing the axon with normal saline for 30 min (Fig. 1).

The duration of the toxin-induced plateau in the falling phase of the action potential was prolonged by longer exposure to CTX (0·5 mg/ml) but was shortened when the stimulus frequency was increased. For example, in all axons tested increasing the stimulus frequency from 0·1 to 1·0 Hz attenuated the duration of the plateau potential (Fig. 2). When axons were stimulated at very low frequencies (0·01 Hz) plateau potentials of up to 0·8 s were recorded. Repetitive firing was not observed in CTX-treated axons when short (0·5 ms) depolarizing pulses were applied to the axonal membrane but 2–4 action potentials were sometimes observed during longer (50·0 ms) depolarizing pulses.

By the application of a series of depolarizing and hyperpolarizing pulses across the axon membrane it was possible to determine the current–voltage (I–V) relations for a single axon in normal saline and after a 5 min exposure to 0·5 mg/ml CTX (Fig. 3). A sharp break in the I–V curve was always noted in the presence of CTX. The CTX-induced changes in the I–V curve and the repetitive activity sometimes observed during long depolarizing pulses could be the result of a prolonged sodium current. To test the hypothesis that exposure of cockroach axons to CTX resulted in a prolonged sodium current, voltage-clamp experiments were performed.

Voltage-clamp experiments

The actions of CTX (0·5 mg/ml) on cockroach giant axons were tested under voltage-clamp conditions. Membrane currents were recorded in response to 4 ms step depolarizations to membrane potentials (E_m) of -10 mV (first pulse) and $+40$ mV (second pulse) from a holding potential (E_h) of -60 mV. The twin pulse regime was repeated every two seconds. By this mean the actions of CTX on the total membrane currents and the late outward, potassium current were continuously monitored. As shown in Fig. 4 neither the amplitude nor the time to peak of the inward current was affected by CTX. The late current was however decreased by 19% when $E_m = +40$ mV. Moreover when $E_m = -10$ mV the late current, normally outward, became inwardly directed. The late inward current recorded when $E_m = -10$ mV was maintained even when a longer depolarizing pulse (10 ms duration) was applied (Fig. 4*c*). So although CTX weakly inhibited the late, potassium, outward current its major effect appeared to be a slowing of the sodium channel inactivation mechanism. To test this directly, the actions of CTX on the sodium current alone were examined.

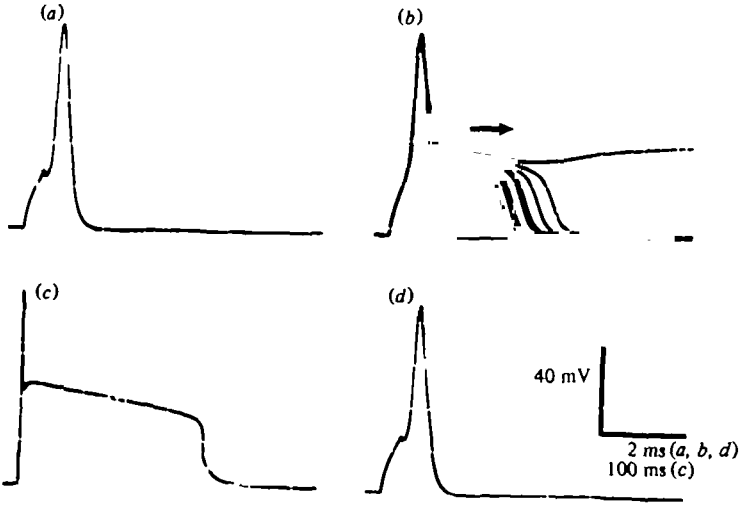

Fig. 1. Effects of CTX on action potentials recorded under current-clamp conditions from a cockroach giant axon. Action potentials were evoked by a depolarizing current pulse (0·5 ms duration) applied at a frequency of 0·2 Hz. (*a*) Action potential recorded in normal saline. (*b*) Superimposed action potentials recorded continuously during the external application of 0·5 mg/ml CTX (arrow indicates progressive action of toxin). (*c*) Action potential recorded 7 min after exposure of axon to CTX showing plateau potential. (*d*) Action potential recorded 30 min after washing the axon with normal saline. Temperature 20 °C.

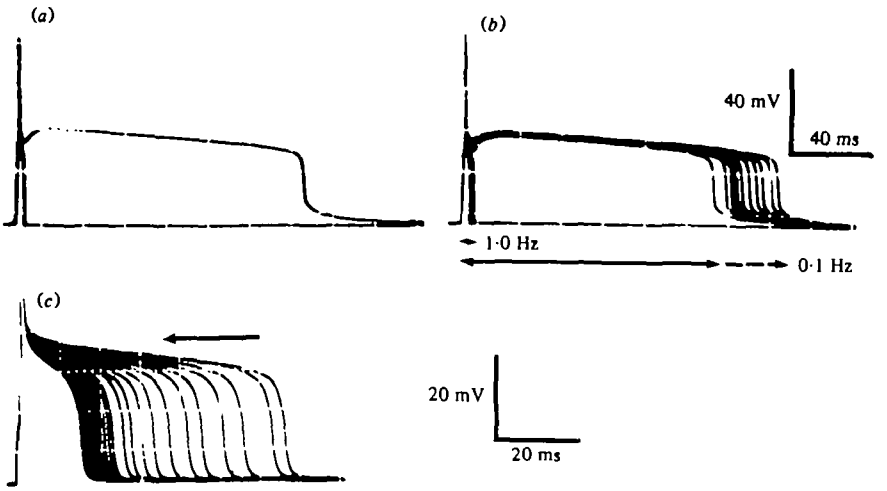

Fig. 2. Dependence on the duration of plateau potentials on stimulus frequency in current-clamped giant axons exposed to CTX (0·5 mg/ml) for 5 min. (*a*) Depolarizing pulses (0·5 ms duration) applied at a frequency of 0·2 Hz evoked action potentials with an extended falling phase and intermittently action potentials with a distinct plateau phase. (*b*) Twelve depolarizing pulses were then applied at a frequency of 0·1 Hz to the same axon. All the evoked action potentials exhibited plateau potentials varying somewhat in duration. A burst of ten pulses at a frequency of 1·0 Hz was then applied to the same axon resulting in action potentials without plateau potentials. Horizontal bars indicate action potential duration at different stimulus frequencies. (*c*) In a separate axon, the frequency of applied depolarizing pulses (0·5 ms duration) was progressively increased from 0·1 Hz to 1·0 Hz. Arrow shows direction of change in duration of plateau potential during the increase in stimulus frequency. Only the lower part of the initial spike is shown.

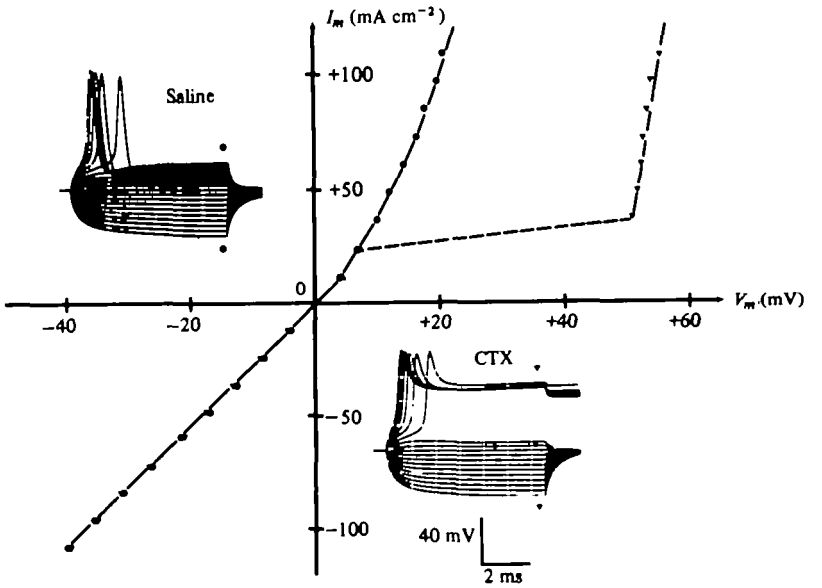

Fig. 3. Current–voltage relations for a cockroach giant axon in normal saline (circles) and in saline containing 0·5 mg/ml CTX (triangles). Insets show effects of square pulses of hyperpolarizing and depolarizing current of increasing intensity on the membrane potential of the axon in current-clamp conditions. Values of V_m were obtained at the end of a 7 ms pulse at the points indicated by the symbols (circles, triangles) on the insets.

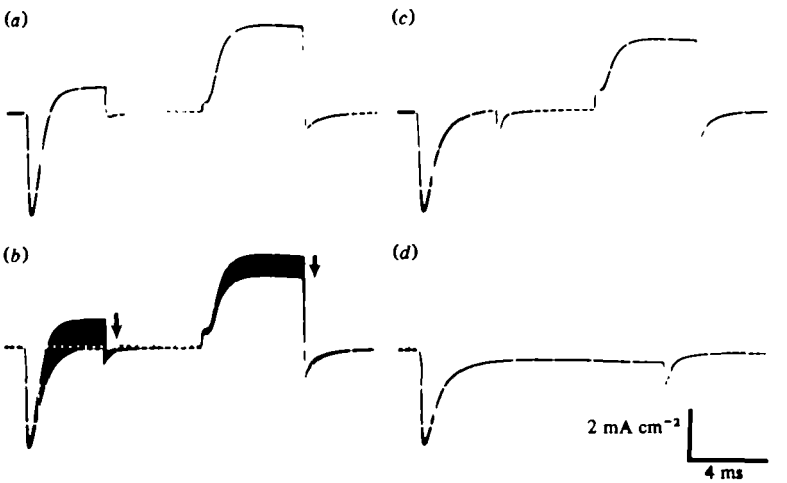

Fig. 4. Voltage-clamp experiment showing the effects of CTX (0·5 mg/ml) on total membrane currents recorded from a cockroach giant axon. Membrane currents are recorded in response to step depolarizations to membrane potentials (E_m) of −10 mV (first pulse) and +40 mV (second pulse) from a holding potential (E_h) of −60 mV. The twin pulse regime was repeated every 2 seconds. (*a*) Normal saline. (*b*) Superimposed voltage-clamp records during the progressive action of CTX (indicated by the direction of the arrows). The peak inward current was not affected by CTX but the late current was completely abolished when $E_m = -10$ mV (first pulse) and decreased by 19 % when $E_m = +40$ mV (second pulse). (*c*) Membrane currents after 5 min application of CTX. (*d*) Following 10 min exposure to CTX, the late current for a longer pulse to $E_m = -10$ mV remained inward throughout the depolarizing pulse.

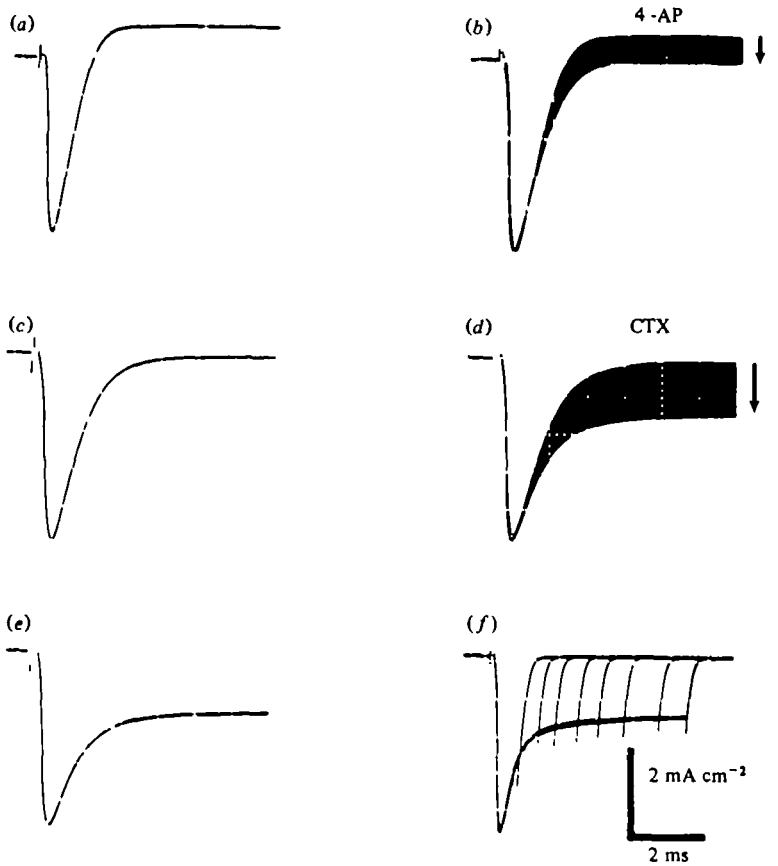

Fig. 5. Actions of CTX on the inward sodium current of a cockroach giant axon. (*a*) Ionic currents recorded in normal saline for step depolarization to $E_m = -10$ mV from $E_h = -60$ mV. (*b*) Superimposed voltage-clamp record during the progressive block (arrow) of the late, outward, potassium current by 10^{-4} M 4-aminopyridine. (*c*) The remaining inward sodium current after a 5 min exposure to 4-aminopyridine (4-AP). (*d*) Superimposed voltage-clamp records during the progressive action (arrow) of CTX (0·5 mg/ml) in the presence of 10^{-4} M 4-aminopyridine. (*e*) 5 min after commencing exposure to CTX an inward (sodium) current was maintained throughout the depolarizing pulse. (*f*) Superimposed records of sodium current in response to pulses of different duration showed that repolarization to E_h (−60 mV) rapidly terminated the sodium current.

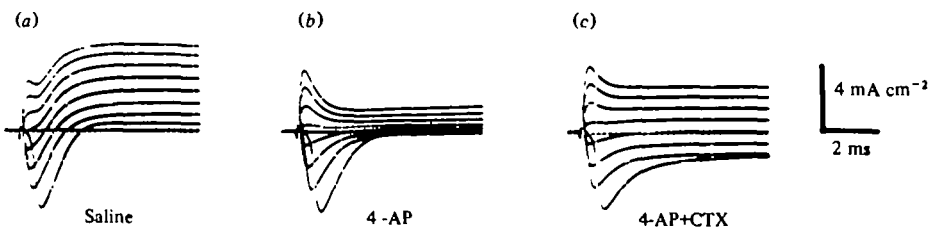

Fig. 6. Membrane currents in a cockroach giant axon associated with step depolarizations from $E_h = -60$ mV to $E_m = +120$ mV in 20 mV steps. (*a*) Normal saline, (*b*) after 5 min exposure to 4-aminopyridine (10^{-4} M), (*c*) after 5 min exposure to CTX (0·5 mg/ml) in the continued presence of 4-aminopyridine, both inward and outward sodium currents are prolonged.

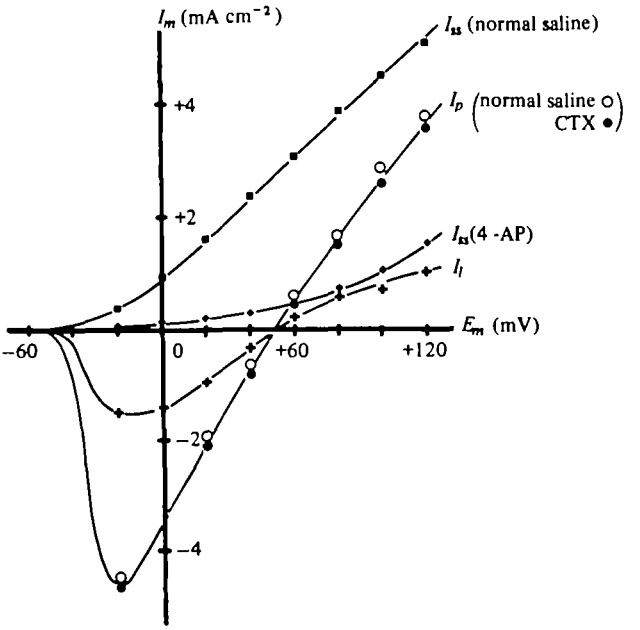

Fig. 7. Current–voltage relations in a cockroach giant axon. Plots of membrane current density (I_m) measured in mA cm^{-2} against membrane potential (E_m) measured in mV for: the peak transient (inward) current (I_p) in normal saline and in the presence of *Condylactis* toxin (CTX); the late steady-state current (I_{ss}) in normal saline and in the presence of 4-aminopyridine (4-AP). The late current in the presence of CTX and 4-AP at the end of a 7 ms voltage pulse is calculated as follows: $[I_{ss}(\text{CTX}+4\text{-AP})-I_{ss}(4\text{-AP})]$ and is labelled I_l. I_{ss} (4-AP) increases when E_m becomes more positive which is characteristic of the voltage-dependent block of the potassium current previously noted for 4-AP (Pelhate *et al.* 1976).

Axons were first exposed to 10^{-4} M 4-aminopyridine for 5 min which suppressed almost all of the potassium current (cf. Pelhate & Pichon, 1974). The subsequent application of CTX (0·5 mg/ml) in the continued presence of 4-aminopyridine prolonged the sodium inward current without affecting the time required to reach peak inward current (Fig. 5).

It was also found that step depolarizations to E_m values more positive than the equilibrium potential for sodium ions elicited outward sodium currents which in the presence of CTX (0·5 mg/ml) remained outwardly directed throughout the duration of the depolarizing pulse (Figs 6, 7). Thus CTX was able to slow the closing of sodium channels of cockroach axons irrespective of the direction in which the sodium current was flowing through the membrane. After pretreatment of axons with CTX (0·5 mg/ml) resulting in prolonged inward sodium currents, the application at 10^{-6} M concentrations of the specific sodium channel inhibitor synthetic saxitoxin (Pelhate & Sattelle, 1978; Sattelle *et al.* 1979) in the continued presence of CTX, suppressed completely both the transient and the CTX-induced sodium currents. In two CTX-treated axons 10^{-7} M synthetic STX blocked respectively 80% and 90% of the peak inward sodium current. In one CTX-treated axon exposed to $2{\cdot}10^{-8}$ M synthetic STX, 80% of the peak inward sodium current was blocked. These effects of synthetic STX were not significantly different from those reported previously

for synthetic STX in the absence of CTX (Sattelle *et al.* 1979). The sensitivity of the cockroach axon to synthetic STX was not, therefore, affected by pretreatment with CTX.

DISCUSSION

The present study has established that *Condylactis* toxin (CTX) induces a prolonged negative after-potential in action potentials recorded from giant axons of the cockroach *Periplaneta americana*. Action potentials with a distinct plateau potential (up to 0·8 s duration when the axon is stimulated at 0·01 Hz) can be recorded in the presence of CTX at 0·5 mg/ml and higher concentrations. In cockroach axons CTX acts primarily by slowing the turning-off of the sodium current, without affecting either the time-course of sodium activation or the peak amplitude of the transient sodium current. An ~ 20% decrease in the late outward potassium current of cockroach axons is also detected. The toxin therefore acts in a similar manner on the membranes of cockroach and crustacean axons (cf. Shapiro, 1968*b*; Shapiro & Lilleheil, 1969; Narahashi *et al.* 1969; Murayama *et al.* 1972). One difference has emerged between the actions of CTX on cockroach and other arthropod axons. The actions of CTX can be partly reversed in the case of cockroach axons whereas this is not the case for lobster (*Homarus americanus*) axons (Shapiro & Lilleheil, 1969). The sensitivity of arthropod axonal membranes to the anemone toxins CTX (Shapiro, 1968*b*; Shapiro & Lilleheil, 1969; Narahashi *et al.* 1969; Murayama *et al.* 1972) and ATX_{II} (Romey *et al.* 1976) and the insensitivity of cephalopod axons to the same toxins (Narahashi *et al.* 1969; Romey *et al.* 1976) remains to be explained. These contrasting results may reflect a restricted access of the toxins to the sodium channels of cephalopod axons. Another possible explanation is that there exist differences in the axonal sodium channel closing mechanisms between arthropods and molluscs.

Condylactis toxin provides a useful probe for the molecular mechanisms of sodium channel closing in arthropod axons. The MW of the toxin has been estimated to be in the range ~ 10000–15000 daltons (Shapiro, 1968*a*). A recent investigation (Yost & O'Brien, 1978) has separated two components each of MW ~ 5000 daltons both of which are toxic when injected into a terrestrial crustacean (*Armadillidium vulgare*). The large size and the rapid action of CTX strongly suggest that it acts on the external surface of arthropod axonal membranes. Support for this view is provided by the observation that another anemone toxin (ATX_{II}) of MW ~ 5000 isolated from *Anemonia sulcata* (Wunderer, Machleidt & Wachter, 1976; Abita *et al.* 1977) rapidly produces plateau potentials when iontophoretically applied to the external surface of a crayfish (*Astacus leptodactylus*) axon but fails to produce any effect when applied in the same manner to the cytoplasmic membrane surface (Romey *et al.* 1976).

The present study has established that exposure of the cockroach axonal membrane to CTX does not prevent access of saxitoxin (STX) to the sodium channel. Whether or not the two toxins act at different sites on the axonal sodium channel remains to be determined. The observed attenuation of the plateau potentials in response to an increased frequency of stimulation could be explained in several ways. For example, it may indicate that axonal activity inhibits either the binding of CTX to its receptor or the ability of the CTX-receptor complex to prolong the opening of the sodium

channel. It is also possible that alterations in the ionic gradients across the membrane, slight changes in the resting potential, or even changes in the degree of inhibition of the potassium current could account for the observed effects of stimulation on the duration of plateau potentials.

A number of other chemicals have also been shown to prolong inactivation in cockroach axons. For example, Pichon (1969*b*, 1976) has demonstrated that DDT induces complex permeability changes including a slowing of sodium inactivation. In addition it has recently been observed that allethrin prolongs sodium inactivation in cockroach axons (M. Pelhate, unpublished observations). CTX is nevertheless the most specific probe to date of the mechanism by which the sodium current of cockroach axons is turned off. Suitably radiolabelled it could provide a biochemical approach to the study of the inactivation mechanism of insect axonal membranes. The actions of CTX on cockroach axons described in this paper closely resemble the findings for all other arthropod axons tested. This correspondence points to a similar molecular mechanism underlying the closing of the axonal sodium channels in a variety of arthropod species.

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