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ACTIONS OF INSECT TOXIN AND OTHER TOXINS DERIVED FROM THE VENOM OF THE SCORPION *ANDROCTONUS AUSTRALIS* ON ISOLATED GIANT AXONS OF THE COCKROACH (*PERIPLANETA AMERICANA*)

BY M. PELHATE AND E. ZLOTKIN

Laboratoire de Physiologie, U.E.R. Médecine, Université d'Angers, France and Department of Zoology, The Hebrew University of Jerusalem, Israel

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SUMMARY

1. Insect toxin, mammal toxins I and II and crustacean toxin were obtained from the venom of the scorpion *Androctonus australis*. Their effects on the isolated giant axon of the cockroach *Periplaneta americana* were investigated by current-clamp and voltage-clamp techniques.

2. In current-clamp conditions, mammal toxins and crustacean toxin (1·3–13 μM) induced a large prolongation of the falling phase of the evoked action potentials. Insect toxin (0·13–3·3 μM) induced a progressive slow depolarization of the membrane potential and repetitive firing of action potentials. No changes in the time-course of the action potential were induced by insect toxin.

3. In voltage-clamp conditions, mammal and crustacean toxins induced a slowing of the turn-off of the transient inward sodium current, with either no change or a small increase in the peak sodium current. Insect toxin by contrast induced an increase in the peak sodium current and a slowing of the sodium current turn-off, this effect being greatest at lower values of the clamped membrane voltage.

4. It is concluded that the repetitive activity induced by insect toxin results from a voltage-dependent modulation of sodium inactivation coupled with an increase in both the resting and active sodium permeabilities of the cockroach axonal membrane.

INTRODUCTION

Scorpion venoms are highly toxic to a wide range of vertebrates and invertebrates. The lethal and paralytic effects of scorpion venoms are due to the presence of a series of low-molecular-weight basic neurotoxic proteins (Zlotkin *et al.* 1978). Earlier studies on the chemistry and pharmacology of scorpion venoms, motivated largely by medical requirements, employed vertebrates (mainly mammals) as experimental animals. However, the introduction of arthropods as test animals for monitoring the process of fractionation of the crude venom of the North African scorpion *Androctonus australis* Hector resulted in the discovery of some hitherto undetected proteins. It

emerged that the toxicity of this venom to insects was mainly due to the protein insect toxin IT (Zlotkin *et al.* 1971, 1972), which could be distinguished from the three toxins active on mammals (Mammal toxins MT I, MT II and MT III – Miranda *et al.* 1970) and an additional toxin specifically affecting crustaceans (Crustacean toxin CT – Zlotkin *et al.* 1972, 1975). When compared to other scorpion venom toxins, IT showed the highest degree of specificity and its activity appeared to be confined to insects. This selectivity was demonstrated by (*a*) whole animal toxicity tests (Zlotkin *et al.* 1971*a*, 1972); (*b*) physiological studies on neuromuscular preparations of an insect (Walther, Zlotkin & Rathmayer, 1976), a crustacean (Rathmayer, Walther & Zlotkin, 1977), a spider (Rhuland, Zlotkin & Rathmayer, 1977), and a mammal (Tintpulver, Zerachia & Zlotkin, 1976); (*c*) a series of binding experiments indicating specific and exclusive binding of insect toxin to the nervous tissue of an insect (Teitelbaum, Lazarovici & Zlotkin, 1979). The intact nervous system of insects is practically impermeable to crude venoms and to purified scorpion toxins (Parnas, Avgar & Shulov, 1970; D'Ajello *et al.* 1972) and their paralytic effects are due to actions at the neuromuscular junctions. When applied to the locust hind leg extensor tibiae nerve–muscle preparation, IT has been shown to induce a dramatic stimulation of the skeletal musculature due to a presynaptic excitatory action on the exposed terminal branches of the motor nerve (Walther, Zlotkin & Rathmayer, 1976). So, as previously shown for the scorpion venom mammal toxins (see review Zlotkin, Miranda & Rochat, 1978), the site of action of IT also appears to be the axon membrane.

In this paper we examine the effects of insect toxin, and other toxins from scorpion venom, upon the giant axon of the cockroach *Periplaneta americana* using current-clamp and voltage-clamp techniques.

MATERIALS AND METHODS

Toxic substances

The crude venom of *A. australis* Hector was obtained by electrical milking followed by lyophilization. The neurotoxic peptides IT, MT I, MT II and CT were purified from the crude venom according to methods described earlier (Zlotkin *et al.* 1971*a*, *b*, 1975; Miranda *et al.* 1970). The MT I and MT II were generously provided by Prof. H. Rochat (Marseille, School of Medicine). 4-aminopyridine (4-AP) was obtained from the Merck Chemical Co., Germany. The synthetic saxitoxin (STX) used in this study was the generous gift of Prof. Y. Kishi (Harvard University, U.S.A.).

4-aminopyridine (4-AP, concentrations indicated in the text) was employed in order to selectively block the potassium current (Pelhate & Pichon, 1974) and synthetic saxitoxin (STX, 2×10^{-7} M) was used for selective, reversible blockage of sodium currents (Sattelle, Pelhate & Hue, 1979). The purified scorpion toxins were lyophilized in the presence of bovine serum albumin (BSA), Fraction V, Armour Co. U.S.A.) in the ratio of 1/10 (w/v).

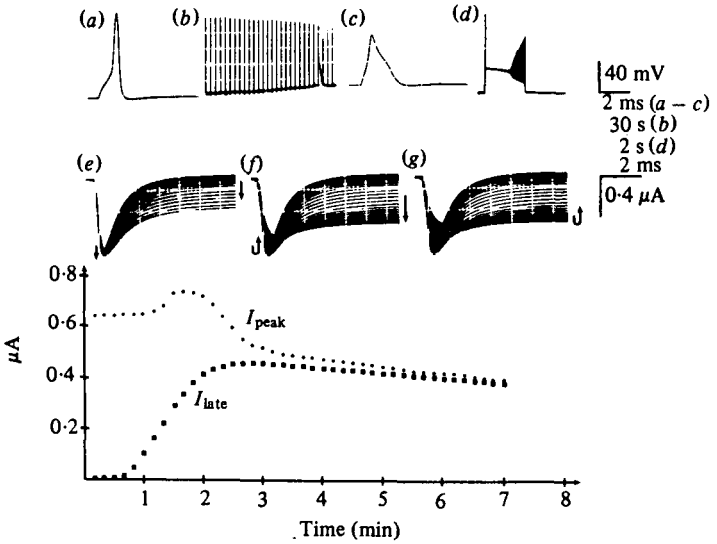

Fig. 1. The effect of the crude venom of *A. australis* (200 μg/ml) on the cockroach axonal preparation.

(*a–d*) Current-clamp experiments. (*a*) control action potential (a.p.) evoked by a current pulse of 10 nA, 0·5 ms. (*b*) 1 min after the application of the crude venom, a series of a.p. evoked by state current pulses are recorded; a continuous depolarization accompanied by the reduction in the amplitude of the a.p. and the occasional appearance of prolonged potentials are indicated leading finally to (*c*) a partial block of the a.p. Following an artificial repolarization by a constant hyperpolarizing current for 2 min (*d*) a.p. with a fast rising phase followed by a prolongation (plateau) with superimposed short bursts of repetitive firing was observed.

(*e–g*) Voltage-clamp experiment demonstrating the effect of the venom on the sodium current. Continuous recording of the changes in I_{Na} associated with a voltage pulse to $E_m = -10$ mV from a holding potential of $E_h = -60$ mV at a frequency of 0·1 Hz after blockage of I_K by a pretreatment of 4 min with 4-AP 2×10^{-4} M. (*e*) During the first 90 s of venom application the peak sodium current (peak I_{Na}) was increased and its turn-off was progressively slowed (arrows). (*f*) During the next 90 s peak I_{Na} began to decrease (arrow) and the slowing of I_{Na} is continued (arrow). (*g*) After 7 min the whole Na current was slowly decreasing. The evolution of the peak and of the late sodium currents versus time corresponding to (*e*)–(*g*) are presented in the lower diagram.

Insect axonal preparation

Voltage-clamp and current-clamp experiments were performed on giant axons dissected from abdominal nerve cords of the cockroach *Periplaneta americana* using the double oil-gap, single-fibre technique (Pichon & Boistel, 1967). Normal physiological saline had the following composition (in mM): NaCl, 200; KCl, 3·1; $CaCl_2$, 5·4; $MgCl_2$, 5·0. The pH was maintained at 7·2 using a phosphate–carbonate buffer ($NaHCO_3$, 2; NaH_2PO_4, 0·1). Current-clamp experiments were performed at 20–22 °C, voltage-clamp experiments were performed at $12 \pm 0{\cdot}5$ °C.

Table 1. *The relative changes in the peak and the late sodium* currents induced by the crude venom* (120 μg/ml), *CT, MT I, MT II* (1·3–3·4 μM) *and the IT* (1·3–2 μM) *in two different voltage steps*

	$E_m = -30$ mV	$E_m = -10$ mV	
	Increase as percentage of the control peak current	Increase as percentage of the control peak current	
	Late †	Peak †	Late †
CV	—	+11 ± 9·2 (n = 6)	+36·6 ± 3·3 (n = 6)
CT	—	+3·3 ± 6·2 (n = 3)	+20·5 ± 8·3 (n = 4)
MT I	—	+1 ± 6 (n = 3)	+18·7 ± 7·2 (n = 3)
MT II	+10·98 ± 6·02 (n = 12)	+5·19 ± 8·07 (n = 12)	+25·18 ± 6·67 (n = 12)
IT	+20·5 ± 4·61 (n = 11)	+17·60 ± 8·08 (n = 11)	+7·60 ± 3·60 (n = 11)

* All preparations were pretreated by 4-AP (2×10^{-4} M) in order to block the potassium current.
† The data are expressed as the percentage increase from the value of the control peak sodium current and include the average, ± standard deviation and number of observations (n).

RESULTS

Effects of the crude venom of the axonal membrane

The crude venom, applied in concentrations ranging between 50 to 300 μg.ml, modified the axonal electrical activity in 0·5–3·0 min. In current-clamp experiments, a gradual depolarization (9·4 mV ± 2·5; 5 experiments) and reduced action potential amplitude were observed (Fig. 1 *a–c*), followed either by short bursts of repetitive activity accompanied by prolonged action potentials, or by prolonged (plateau) potentials with characteristic bursts of repetitive activity (Fig. 1 *d*). Less than 2 min after the application of venom, an artificial repolarization of the resting membrane potential restored the amplitude and shape of evoked action potentials. Two to 10 min after application, repolarization resulted in repetitive activity with, in some cases (Fig. 1 *d*), prolongation of action potentials.

In voltage-clamp experiments, after 6 min of application, the crude venom slightly reduced the potassium current (by 13·3% ± 3·7; three experiments) but more substantially modified the sodium current (Fig. 1 *e–g*; Table 1). The progressive actions of the venom can be divided into three phases: (1) an initial increase in the amplitude of the peak sodium current (cf. Table 1) and the slowing of its turning off; (2) a steep decrease in the peak I_{Na} and a progressive increase in the late Na^+ current (cf. Table 1); and (3) a slow decrease of peak I_{Na} and late I_{Na}. A prolonged application of the crude venom (200 μg/ml – more than 10 min) always resulted in a progressive suppression of sodium and potassium currents.

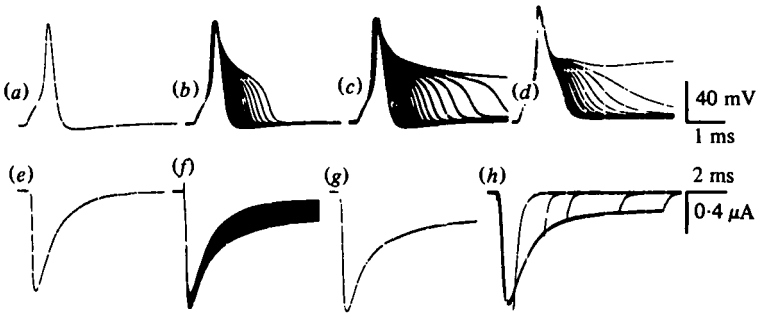

Fig. 2. The effect of the MT II and the CT on the cockroach axonal preparation.
(*a–d*) Current-clamp experiments. (*a*) Control action potential evoked by a current pulse of 10 nA 0·5 ms. (*b*) Application of 6·9 μM of MT II has slowed the rising phase and repolarization phase of the a.p. (*c*) After 60 s of application a very slight decrease of the resting potential has slowly developed and long 'plateau' potentials were recorded. (*d*) In an other axon during the action of CT (5·5 μM) a similar drastic prolongation of the AP was recorded.
(*e–h*) Voltage-clamp experiments demonstrating the effect of the MT II (3·4 μM) on the sodium current associated to a voltage pulse to $E_m = -10$ mV from $E_h = -60$ mV after blockage of I_K by 4-AP 2×10^{-4} M. (*e*) Before toxin. (*f*) Continuous recording after the application of the toxin: an increase in the peak Na current and a progressive slowing of its turning off are seen. (*g*) 3 min after toxin application. (*h*) Superimposed records of sodium current in response to pulses of different duration showed that, in the MT II treated axon, repolarization to the holding potential ($E_h = -60$ mV) rapidly terminated the sodium current.

Effects of the mammal (MT I, MT II) and crustacean (CT) toxins on the axonal membrane

In current-clamp experiments, mammal and crustacean toxins, applied at concentrations of 1·3–13 μM, greatly prolonged the evoked action potentials (Fig. 2*a–d*) within 0·5–3 min of application. Concentrations below 10^{-6} M were usually ineffective. Washing with normal saline greatly attenuated the 'plateau' response, resulting in an action potential with a residual 'shoulder' of duration of 3–5 ms. A small depolarization of the axonal membrane (2–7 mV) was noted only after about 6 min (Fig. 2*c*).

Turn-off of the sodium current was slowed (Fig. 2*e–g*), and peak sodium current was not clearly modified (cf. Table 1). The potassium current was decreased by 12·6 ± 4·2% by MT II (five experiments). The action of MT I and CT (Fig. 5*d–f*) was studied in two experiments and results indicate that a large potassium current persisted in the presence of these toxins. The effects of MT II on the sodium current were systematically demonstrated in the membrane current recorded in response to a series of step depolarizations (Fig. 6*d–e*), and the resulting *I–V* curves (e.g. Fig. 7). The relative changes induced by crustacean and mammal toxins in the peak I_{Na} and I_{Na} are presented in Table 1. In addition the prolonged Na current was rapidly terminated upon repolarization to a holding potential of −60 mV (Fig. 2*b*).

Effects of insect toxin on the axonal membrane

In current-clamp experiments, IT (0·13–3 μM) induced two types of responses. First, a slow, progressive depolarization was noted; this response was antagonized by TX (Fig. 3*d*) and was accompanied either by repetitive activity at lower (< 10^{-6} M)

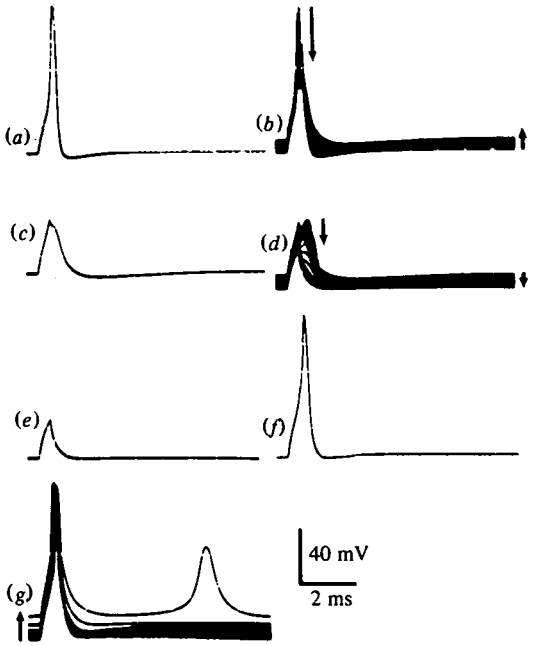

Fig. 3. The effect of IT on the cockroach axonal preparation in current-clamp conditions. (*a*) Control a.p. evoked by a current pulse of 10 nA 0·5 ms. (*b*) Application of 3·3 μM of IT. A series of superimposed recordings indicating a progressive depolarization of the resting potential and a decrease in the amplitude of the evoked a.p. (arrows) leading (*c*) to a partial block 2 min after the application of the IT. (*d*) Application of STX (2×10^{-7} M) repolarized the membrane (arrow) and completely suppressed the evoked partial response. (*e*) 1 min after STX application only a passive response to the current pulse was recorded. (*f*) After 15 min of washing with normal saline and artificial maintenance (by constant current) of normal resting potential, evoked subnormal a.p.s were recorded. (*g*) An additional application of IT (3·3 μM) resulted in a progressive depolarization and repetitive activity which was maintained during several minutes.

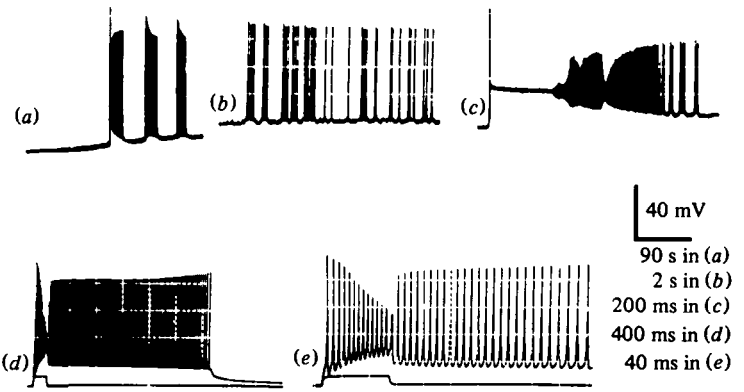

Fig. 4. The effect of the IT on the cockroach axonal preparation in current-clamp conditions. (*a*), (*b*). Records of two examples of spontaneous repetitive activity obtained 5 min after the application of 1·3 μM of IT. (*c*) The same preparation partially repolarized by a constant hyperpolarizing current was stimulated by a short current pulse (10 nA 0·5 ms) responding by a transient phase followed by a 'plateau' of oscillations of a progressively increasing amplitude up to final a.p.s. (*d*) and (*e*) In the same axon during continuous washing repetitive firing is obtained during and after depolarizing pulses.

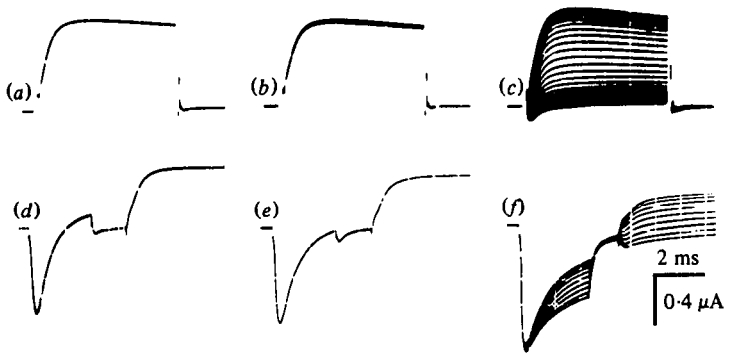

Fig. 5. The effects of the IT (*a–c*) and CT (*d–f*) on the K^+ current in the cockroach axonal preparation in voltage-clamp conditions.

(*a–c*) I_K associated with a voltage step from $E_h = -60$ mV to $E_m = +40$ mV in a preparation pretreated with STX 2×10^{-7} M. (*a*) Control, (*b*) continuous recording of I_K during 10 min of superfusion with IT (3·3 μM): the I_K was only slightly decreased. (*c*) Superimposed records during the progressive action of 2×10^{-4} M 4-AP completely suppressing the I_K in less than 3 min.

(*d–f*) Membrane currents recorded in response to a twin pulse to membrane potentials $E_m = -10$ mV (first pulse) and $E_m = +40$ mV (second pulse) from a holding potential $E_h = -60$ mV. The twin pulse regime was repeated every 5 s. (*d*) Normal saline. (*e*) Membrane currents after 5 min application of CT (5·5 μM). (*f*) After 20 min of perfusion with CT, a postreatment with 2×10^{-4} M of 4-AP reveals the persistent presence of a potassium current progressively blocked by 4-AP.

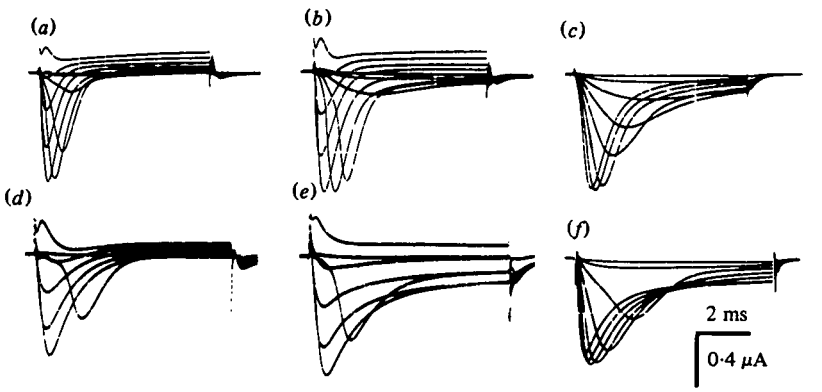

Fig. 6. The effects of IT and MT II on the sodium currents in the cockroach axonal preparation under voltage-clamp. Membrane currents associated with step depolarizations were recorded from giant axons pretreated with either 1×10^{-4} M 4-AP (*a*, *b*), partially blocking I_K, or 2×10^{-4} M 4-AP (*c,d,e,f*), almost completely blocking I_K. (*a*) Normal saline. The pulses were from $E_h = -60$ mV to $E_m = +60$, $+40$, $+20$, 0, -20, -25, -30, -35, -40 mV. (*b*) The same series of pulses as in (*a*) was applied after treatment of the axon with 1·3 μM IT for 6 min. (*c*) A family of currents in another axon treated with 1·3 μM IT for pulses $E_m = -10$ to -45 MV in steps of 5 mV. (*d*) Another axon, normal saline. Voltage steps to $E_m = +60$, $+40$, $+20$, 0, -20, -30, -40 mV. (*e*) The same program as in (*d*) after treatment with MT II ($3{\cdot}4 \times 10^{-6}$ M). (*f*) In another axon treated by 3·4 μM MT II. This family of currents was associated with the same series of pulses as in (*c*).

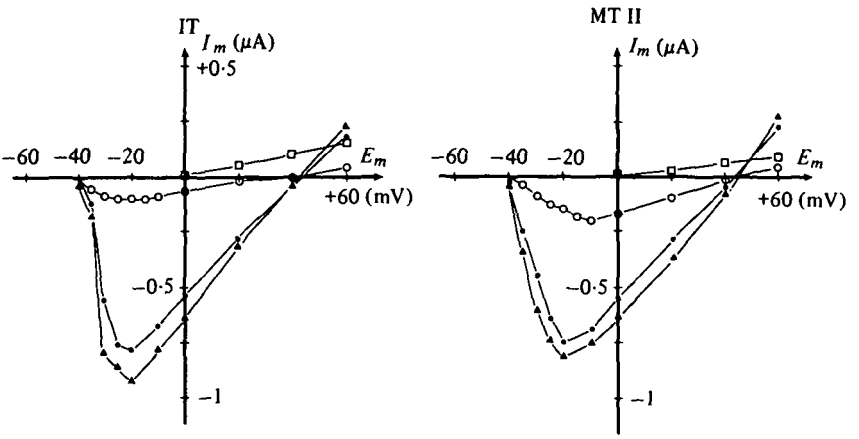

Fig. 7. The effects of the IT (1·3 μM) and MT II (3·4 μM) on the sodium currents in the cockroach axon: current–voltage relations. The axons were pretreated with 2×10^{-4} M 4-AP in order to block most of the potassium current. Plots of membrane current intensity (I_m) measured in μA against membrane potential (E_m) measured in mV are indicated. ●, Normal peak I_{Na}. △, Peak I_{Na} following toxin application. ○, Late I_{Na} (corrected from the remaining I_K) following toxin application. □, Late membrane current, largely the remaining K^+ currents.

concentrations of the toxin or by a partial (Fig. 3*b*, *c*) or even complete block of the evoked action potentials with higher ($> 10^{-6}$ M) concentrations. The blocking effect was overcome by artificial repolarization of the membrane (Fig. 3*f*). Second, induction of repetitive firing was observed. Two forms of repetitive firing were noted: (*a*) induced repetitive activity, either by short depolarization evoking a single action potential followed by a series of oscillations of increasing amplitude (Figs. 3*g*, 4*c*), or by a longer depolarization inducing long trains of impulses (Fig. 4*d*, *e*); (*b*) spontaneous repetitive activity, independent of previous stimulation, occurring in bursts of action potentials (Fig. 4*a*, *b*). It should be emphasized that the repetitive activity of action potentials of normal time course (short in duration) was the dominant effect of IT at low concentration (10^{-6} M). This repetitive firing, however, was always preceded or at least accompanied by an instability and a slow progressive depolarization (up to 10–15 mV with 3 μM of IT) of the resting potential. All the above effects of IT were not reversed by washing in normal saline.

It was also shown in voltage-clamp experiments that IT had no effect on the potassium current (Fig. 5*a–c*), unlike the mammal and crustacean toxins (Fig. 5*a–f*). Effects of IT on the sodium current were demonstrated under voltage-clamp by examining a series of membrane currents each associated with step depolarizations of different amplitude (Fig. 6*a–c*). *I–V* curves were drawn from these and similar records (Fig. 7, IT). An increase (of up to 17%) of the peak sodium current was noted (Fig. 6*a*, *b*, Table 1). In addition, a slowing of the sodium current turn-off as expressed in the increase of the late Na^+ current was detected (Figs. 6*c*; Fig. 7, IT). This later effect, however, appeared to be strongly related to the voltage of the membrane potential (E_m), reaching maximal values at $E_m = -30$ mV (Table 1, Fig. 6*c*).

DISCUSSION

The characteristic prolongation of the evoked action potentials elicited by the mammal and crustacaean toxins in the present study has been previously demonstrated with scorpion crude venom as well as purified toxins on isolated axons of frog (Adam *et al.* 1966; Kopenhoffer & Schmidt, 1968), squid (Narahashi *et al.* 1972) and crustacean (Romey *et al.* 1975; Rathmayer *et al.* 1977). In the above studies the prolongation of the action potentials was attributed, mainly, to the partial blockage of the sodium current inactivation as demonstrated in voltage-clamp experiments (Kopenhoffer & Schmidt, 1968; Narahashi *et al.* 1972; Romey *et al.* 1975). In the present study it has been shown that the mammal and crustacean toxins slow the sodium current turn-off at all levels of the induced membrane potentials (Fig. 7*a*) without affecting the time course of the sodium current increase. The peak amplitude of this current (Table 1, Figs. 6*c*, *d*, 7*a*) is only slightly affected. These data are consistent with the hypothesis that these toxins act by modifying the sodium inactivation mechanism.

It is useful to compare the action of insect toxin with the effects of MT II, which is the most potent toxin to mammals that has been isolated from scorpion venoms (Zlotkin, Miranda & Lissitzky, 1978) and is more active on the cockroach axon than MT I or CT.

In current-clamp conditions IT shows completely different characteristics from MT II. It does not prolong the action potentials. Its main effect is to generate repetitive activity of normally shaped action potentials (Fig. 4) accompanied by a small and progressive depolarization of the resting membrane potential (Figs. 3*b*, *g*). A gradual depolarization of the resting membrane was also previously demonstrated with crude scorpion venoms when studied on the axonal preparation of a frog (Adam *et al.* 1966) and squid (Narahashi *et al.* 1972). Unlike the effects of MT II, the effects of IT are not reversed by washing and strongly resemble the persistent irreversible repetitive activity induced by IT on the locust *extensor tibiae* motor nerve (Walther *et al.* 1976). IT was also about one order of magnitude more potent than MT II.

At first glance, IT and MT II appear to show a similar pattern of activity in voltage-clamp conditions (Figs. 2*f*, *g*, Fig. 6*b*). Both toxins prolong the sodium current, increase the peak current, and have practically no effect on the potassium current. However, in the presence of IT the relative increase in the peak current was not proportional to the increase of the late sodium current, in distinct contrast to data obtained for MT II (Fig. 7, Table 1). It may be assumed that this difference between the two toxins represents either a certain degree of suppression of the sodium activation by MT II or, more reasonably, an increase in sodium activation by IT. Voltage-dependent modulation of sodium inactivation (Fig. 7, IT) and the possible enhancement of sodium activation may account for the ability of IT to induce repetitive firing of normal action potentials as demonstrated in current-clamp experiments (Fig. 4*a–b*).

The action of the crude venom demonstrates characteristics of both the IT and the mammal and crustacean toxins. The progressive depolarization of the resting membrane potential (Fig. 1*b–c*) and the superimposed oscillations and repetitive activity (Fig. 1*d*) in current-clamp experiments, together with the relative increase

in the peak sodium current in the voltage-clamp experiments, can be attributed to IT. The occasional 'plateau' potentials (Fig. 1*d*) and the enhanced slowing of the sodium inactivation (Fig. 1*f*, *g*) are due to the mammal and crustacean toxins. The final decrease in the peak sodium current (Fig. 1, diagram) may be attributed either to the combined action of the mammal and crustacean toxins or to additional factors in the crude venom.

To summarize, it appears that IT irreversibly affects sodium conductance in the isolated cockroach axon through (1) a progressive increase of sodium permeability at the resting potential, (2) a specific voltage-dependent modulation of the sodium inactivation mechanism, (3) a possible stimulation of the sodium activation mechanism as expressed by the increase of the sodium peak current. A combined effect of all these three elements may be responsible for the prominent axonal repetitive activity induced by IT.

IT has unique neuropharmacological characteristics different from those previously reported for scorpion venoms and their toxins. Due to its binding selectivity to insect neuronal tissues (Teitelbaum *et al.* 1979) and its effects upon sodium conductance, IT may provide a valuable pharmacological tool in insect neurobiology and neurochemistry.

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