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

I. SELECTIVE BLOCK BY SYNTHETIC SAXITOXIN

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SUMMARY

Voltage-clamp experiments on isolated giant axons of the cockroach *Periplaneta americana* L. show that chemically synthesized saxitoxin specifically and reversibly blocks the transient inward sodium current without affecting the steady-state outward potassium current. From the concentration dependence of sodium current suppression it is concluded that individual sodium channels are blocked by single molecules of synthetic saxitoxin which bind reversibly to part of the channel with a dissociation constant of $3{\cdot}0 \times 10^{-9}$ M. Synthetic saxitoxin blocks sodium channels in cockroach axons at a lower concentration than tetrodotoxin. Sodium channel block by synthetic saxitoxin is more readily reversed than tetrodotoxin-induced block.

INTRODUCTION

Neurotoxins are essential tools in the analysis of the molecular events underlying axonal conduction and synaptic transmission (see Narahashi, 1974, 1975). Saxitoxin (STX) is one of the most toxic low molecular weight compounds known and in its natural form is extracted from the clam *Saxidomus giganteus*, the mussel *Mytilus californianus* and axenic cultures of the dinoflagellate *Gonyaulax catenella* (Ghazarossian *et al.* 1974). As is the case for the neuropoison tetrodotoxin (TTX) extracted from the puffer fish (*Tetraodon lineatus*), natural saxitoxin (STX) selectively blocks transient sodium currents in nerve membranes (Narahashi, Haas & Therrien, 1967; Evans, 1969). However, post-toxification recovery of sodium channel function is more rapid after exposure to STX than following TTX application (Narahashi *et al.* 1967; Hille, 1975). In addition, the dissociation of toxin from a component of solubilized garfish olfactory nerve membranes is almost four times faster for STX (Henderson, Ritchie & Strichartz, 1973) than for TTX (Henderson & Wang, 1972). Clearly, STX would be the chemical probe of choice for many experiments on membrane ionic permeability if it were more widely available. The structure of

natural STX has now been established by X-ray crystallography (Schantz *et al.* 1975; Bordner *et al.* 1975) and the recent total synthesis of the toxin (Tanino *et al.* 1977) makes the more widespread use of this molecule in membrane studies a practical possibility. In an earlier, brief communication, giant axons of the cockroach (*Periplaneta americana*) were shown to be sensitive to synthetic STX (Pelhate & Sattelle, 1978). Here we report the first detailed account of the actions of synthetic STX on axonal membrane currents.

Giant axons of the cockroach (*P. americana*), which are used in the present study, closely resemble other unmyelinated axons in that they exhibit, under voltage-clamp conditions, transient sodium currents and slower, steady-state potassium currents (cf. Pichon, 1974). In this study, synthetic STX, natural STX and TTX are applied to isolated, voltage-clamped, cockroach axons. The aims of the investigation are as follows: (1) to assess the usefulness of synthetic STX as a specific inhibitor of sodium channel function; (2) to quantitatively compare the affinity for insect axonal sodium channels of various blocking agents (natural and synthetic STX and natural TTX).

MATERIALS AND METHODS

Adult male cockroaches (*Periplaneta americana*) were used throughout these experiments. An isolated (2–3 mm) length of one giant axon was dissected from a connective linking the fourth and fifth abdominal ganglia and cleaned of adhering fibres. The preparation was transferred to an experimental chamber in which two lateral Ag–AgCl electrodes were in contact with the severed ends of the axon and a central Ag–AgCl electrode was in contact through the external bathing solution with a 100 μm length of dissected axon. The preparation was immersed in paraffin oil and the 'artificial node' created by this non-electrolyte (see Pichon & Boistel, 1967) was voltage-clamped as described in detail earlier (Pelhate, Hue & Chanelet, 1978). This space-clamped region of the axon was superfused by either normal saline or test solutions. Analogue compensation for leakage and capacity currents, by the method of Hille & Campbell (1976), ensured that currents recorded during the depolarizing clamp pulses were specific ionic currents. Most experiments were performed using a step depolarization to a membrane potential (E_m) of -10 mV from a holding potential (E_h) of -60 mV. The leak correction was adjusted for this value of E_m. No leakage rectification is normally detected in cockroach axons until E_m values of $\simeq +40$ mV are reached (Pichon, 1969*a*).

In all experiments the preparation chamber was maintained at $12 \pm 0{\cdot}5$ °C. Normal physiological saline had the following composition (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. Synthetic saxitoxin dihydrochloride was diluted in this saline from a stock solution (1·0 mg/ml toxin) generously provided by Prof. Y. Kishi (Harvard University). Natural saxitoxin was also diluted in saline from a stock solution (0·1 mg/ml toxin) kindly donated by Dr M. H. Evans (Agricultural Research Council Institute of Animal Physiology, Babraham, U.K.). Crystalline tetrodotoxin (natural) from Calbiochem was also prepared in physiological saline.

RESULTS

Effects of synthetic STX on axonal membrane currents

Membrane currents were measured under voltage-clamp conditions in response to a step depolarization from a holding potential (E_h) of -60 mV to a membrane potential (E_m) of -10 mV. When synthetic STX was externally applied to the axon at concentrations in the range 10^{-7} to 5×10^{-7} M the inward sodium current was rapidly blocked without affecting the steady-state outward potassium current (Fig. 1). Rebathing the axon in normal saline resulted in recovery of the inward sodium current (Fig. 1). An outward sodium current, generated by clamping the membrane at $E_m = +60$ mV, was also blocked by 10^{-7} M synthetic STX indicating that the toxin prevented ion movements through the sodium channels in both directions.

To measure directly the actions of synthetic saxitoxin on the inward sodium current alone, the axon was first pretreated with 4-aminopyridine (4-AP). It has been established that 4-AP specifically reduces potassium currents in the cockroach axon (Pelhate & Pichon, 1974), the squid axon (Meves & Pichon, 1977) and the frog node of Ranvier (Ulbricht & Wagner, 1976). Following suppression of the outward potassium current by 5×10^{-4} M 4-AP, synthetic STX at 10^{-7} M completely blocked the remaining inward (sodium) current (Fig. 2). We have therefore demonstrated total

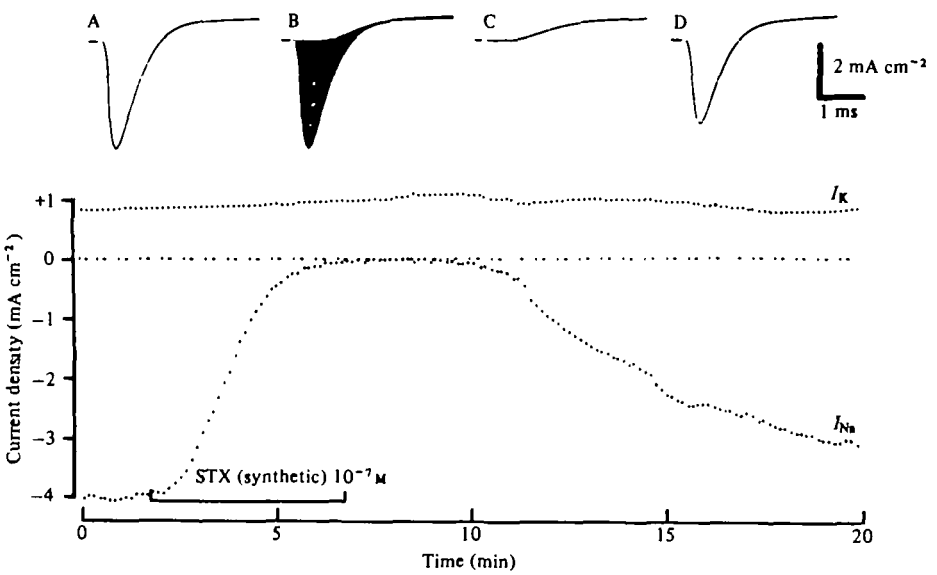

Fig. 1. Voltage-clamp experiment showing the effects of synthetic saxitoxin on the isolated giant axon of *Periplaneta americana*. Membrane currents are recorded in response to step depolarizations to a membrane potential (E_m) of -10 mV from a holding potential (E_h) of -60 mV. Plots of the changes in amplitude of peak inward sodium (I_{NA}) and outward potassium (I_K) current densities during exposure to and recovery from a five-minute application of 10^{-7} M synthetic saxitoxin. Outward currents are depicted as positive and inward currents are shown as negative. Period of STX application is indicated by horizontal bar. Inserts (A–D) show membrane currents recorded at different times (t) after the beginning of the experiment. (A) normal saline, $t = 0$ min; (B) superimposed voltage-clamp records during progressive selective block of transient inward current by STX, $t = 0$–8 min; (C) normal saline, $t = 10$ min; (D) normal saline, $t = 25$ min.

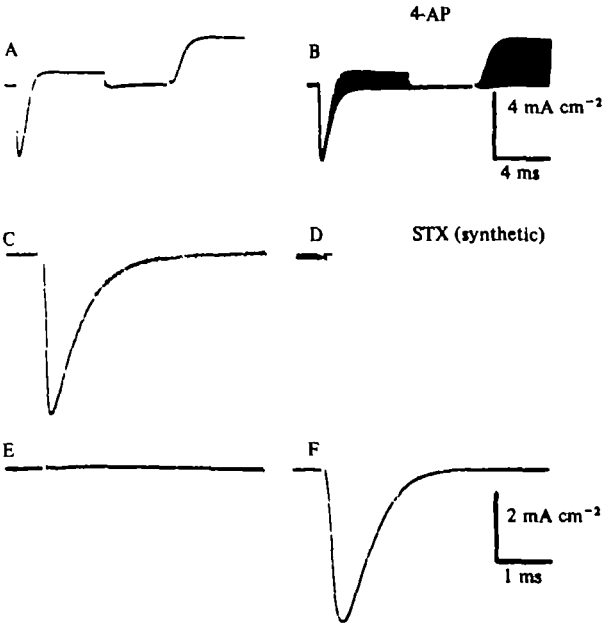

Fig. 2. Complete block of all membrane currents by 4-aminopyridine (4-AP) and synthetic saxitoxin. (A, B) Twin voltage-clamp pulses applied to the axon to show total ionic currents (first pulse, $E_m = -10$ mV) and the potassium current alone (second pulse, $E_m = +40$ mV) both before (A) and during (B) the action of 4-AP (5×10^{-4} M). (C, D, E, F) the effects of synthetic STX on inward sodium current alone. (C) pure sodium current from 4-AP (5×10^{-4} M) treated axon; (D) progressive block of sodium current by synthetic STX (5×10^{-7} M); (E) abolition of sodium current after 5 min exposure to synthetic STX; (F) recovery after 20 min wash in saline containing 4-AP (5×10^{-4} M). $E_h = -60$ mV.

elimination of sodium and potassium currents using only synthetic, selective, channel-blocking agents.

Axons were exposed to various concentrations of synthetic STX and the data plotted as the dose-dependence of the percentage inhibition of the peak transient inward sodium current (Fig. 3*a*). STX binding to the axon membrane is considered as a simple case of reversible adsorption to surface sites (receptors), in which case we can write:

$$\log\left(\frac{R}{100-R}\right) = \log\frac{1}{k_D} + \log\,[\mathrm{STX}],$$

where R is the percentage inhibition of the peak transient sodium current and k_D is the dissociation constant. As shown in Fig. 3(*b*) $\log\,(R/100-R)$ was plotted against the logarithm of the concentration of synthetic STX bathing the axon membrane. The result expected if one molecule of synthetic STX binds reversibly to each receptor site with a dissociation constant of $3{\cdot}0 \times 10^{-9}$ M is shown by the straight line in Fig. 3(*b*). The data points are close to the line. The simplest interpretation of these data is that individual sodium channels are blocked by single molecules of synthetic STX which bind reversibly to part of the channel with a dissociation constant of $3{\cdot}0 \times 10^{-9}$ M.

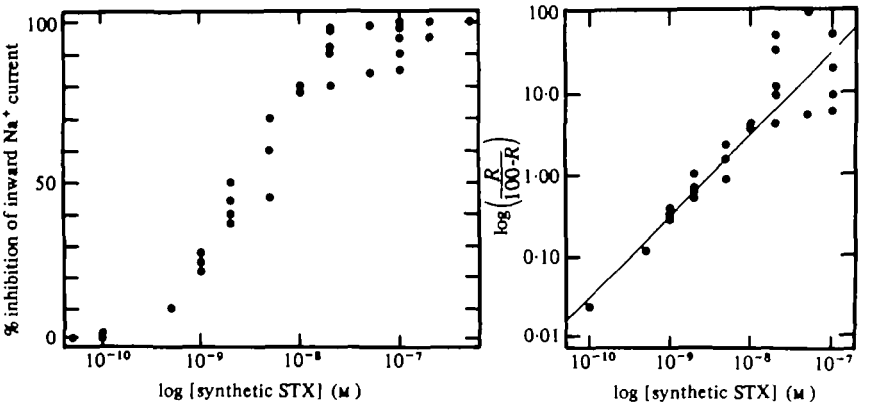

Fig. 3. (*a*) Dose–response curve for the suppression of inward sodium current by synthetic STX. Peak inward sodium current after 10 min exposure to test solution is expressed as percentage of initial peak inward current measured in normal physiological saline. Results of experiments on 31 axons of *Periplaneta americana* are shown. In all cases sodium currents are recorded in response to step depolarizations to $E_m = -10$ mV from $E_h = -60$ mV.

(*b*) The logarithm of (R/100-R) is plotted against log STX (synthetic) concentration using data from Fig. 3(*a*) for toxin concentrations in the range (10^{-10} to 10^{-7} M). R = the percentage inhibition of the amplitude of the peak transient sodium current. The straight line is the expected result if one molecule of synthetic STX binds reversibly to each receptor site with a dissociation constant of $3{\cdot}0 \times 10^{-9}$ M.

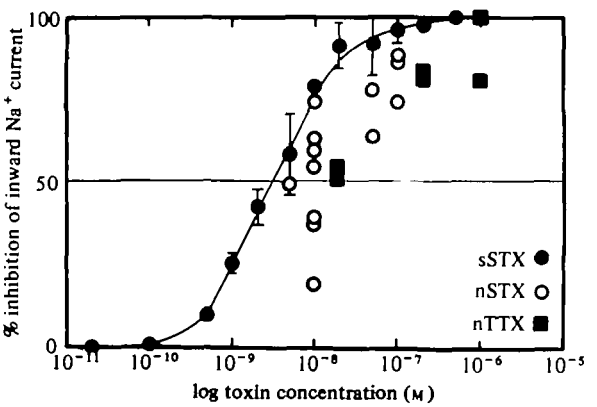

Fig. 4. A comparison of synthetic saxitoxin (sSTX), natural saxitoxin (nSTX) and natural tetrodotoxin (nTTX) as axonal sodium channel blocking agents. Data for synthetic STX is derived from Fig. 3(*a*) and plotted as the mean percentage inhibition of inward sodium current. Vertical bars indicate twice the standard deviation of the mean. In the case of the natural toxins, data points for individual experiments are shown.

Comparison of saxitoxin and tetrodotoxin as axonal sodium channel blocking agents

Saxitoxin (STX) and tetrodotoxin (TTX) from natural sources were applied to the axonal membrane of the cockroach and their effects on sodium currents compared to results obtained with synthetic STX. Both natural toxins were able to block axonal inward sodium currents completely without affecting outward potassium currents. Nevertheless to achieve the same percentage inhibition of the peak inward

sodium current as that induced by synthetic STX it was necessary to elevate the concentration of natural STX by 2- to 5-fold and that of TTX by about an order of magnitude (Fig. 4). It was also noted that the actions of TTX on cockroach axons were much less readily reversed than the effects of saxitoxin (natural and synthetic). For sixteen axons which were blocked by 10^{-6} M synthetic STX, the mean time required for 50% recovery of the peak inward sodium current following re-exposure to normal saline was 7·38 (S.D. ± 2·12) min. By contrast in two out of five axons blocked by 10^{-6} M TTX, recovery of 50% of the peak inward sodium current required rebathing in normal saline for 45 and 25 min respectively. In the three other TTX-treated axons only 25% of the initial peak inward sodium current had returned after a 30 min wash in normal saline.

DISCUSSION

We have shown that chemically synthesized saxitoxin (STX) is a highly specific sodium channel blocking agent when applied to axonal membranes of the cockroach (*Periplaneta americana*). Since insect axonal sodium channels share many common properties with the sodium channels of other invertebrates and vertebrate unmyelinated axons (cf. Pichon, 1974, 1976), synthetic STX should prove to be widely applicable in studies of axonal ion permeability mechanisms.

Using voltage-clamp data on the concentration-dependence of the suppression of the peak inward sodium current by synthetic STX, it is estimated that one molecule of synthetic STX binds reversibly to each receptor site with a dissociation constant of $3{\cdot}0 \times 10^{-9}$ M. This value is close to the dissociation constant estimates for the binding of natural STX to other axonal membranes. For example, using voltage-clamp data, a value of $1{\cdot}2 \times 10^{-9}$ M has been estimated by Hille (1968) for the binding of natural STX, to the frog node of Ranvier. Also, from studies of the binding of tritiated natural STX to solubilized garfish olfactory nerve membranes, a dissociation constant of $6{\cdot}7 \times 10^{-9}$ has been obtained (Henderson *et al.* 1973). The simplest interpretation of the voltage-clamp, dose–response data is that each sodium channel in the cockroach axonal membrane contains a single receptor site for an STX molecule which when occupied by the toxin prevents the movement of sodium ions through the channel.

Synthetic STX has been shown to be identical to purified natural STX with respect to NMR spectrum, silica-gel TLC and whole animal toxicity (Tanino *et al.* 1977). When applied to the cockroach axonal membrane the chemically synthesized STX was always slightly more active than the natural toxin. A concentration of natural STX approximately 2–5 times greater than that used for synthetic STX was required to produce the same percentage suppression of the inward sodium current. Without detailed chemical analyses of the STX stock solutions it is not possible to account for these differences between the axonal actions of synthetic and natural STX, which may for example derive from trace impurities in the sample of natural toxin. More striking differences are noted between the concentrations of TTX and synthetic STX required to produce the equivalent reduction of the inward sodium current. Our observations on TTX are consistent with the results of earlier voltage-clamp experiments by Pichon (1969*a*, *b*) which showed that TTX at micromolar concentrations blocks cockroach axonal sodium channels. In the present study it has been shown that

concentrations of TTX about 10-fold higher than those of synthetic STX are needed to produce a 50% suppression of the inward sodium current. Also, the actions of TTX are less readily reversed than the effects of STX (synthetic or natural). It therefore emerges that synthetic STX is the most useful of the sodium channel blocking agents tested on the cockroach axon. The development of a synthetic form of saxitoxin has provided the first chemically synthesized probe suitable for investigating the molecular pharmacology of sodium channels in cell membranes.

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