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DECREASED RATE OF SODIUM CONDUCTANCE INACTIVATION IN THE NODE OF RANVIER INDUCED BY A POLYPEPTIDE TOXIN FROM SEA ANEMONE

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SUMMARY

The effects of two toxins extracted from the tentacles of *Anemonia sulcata* on ionic currents have been tested on the nodal membrane of myelinated nerve fibres from *Rana esculenta*. While external application of Toxin I at 100 μM leaves both specific ionic currents unmodified, Toxin II at 10 μM reacts with a receptor site associated with the sodium conductance inactivation gating. Since internal application by diffusion of Toxin II at a concentration of 700 μM leaves the ionic currents unchanged, the receptor site is most likely located on the external side of the nodal membrane. An equilibrium dissociation constant for the effects of Toxin II was estimated as 20 μM. The on-reaction is fast (rate constant for the on-reaction roughly equal to 3.10^3 $M^{-1} \cdot s^{-1}$) suggesting a readily accesible receptor site for the toxin. The kinetics characteristics of the sodium currents recorded in the presence of Toxin II suggest that there are at least two steps in the reaction leading to Na^+-channels with the inactivation gate completely immobilized. The relatively fast reversibility of the intermediate stage of the reaction and the rather slow but, in the end, complete reversal of the toxin effects suggest that the toxin acts by modifying the energy profile for the transition "inactivation gate in the open configuration to inactivation gate in the closed configuration". Toxin II at higher concentrations (greater than 100 μM) also inhibits the potassium currents but these effects were not studied in any detail.

INTRODUCTION

It has been reported that the process of inactivation of the sodium conductance, g_{Na}, the h-process in Hodgkin-Huxley terminology, in giant fibres from invertebrates can be destroyed by cleaving certain proteins with pronase [1, 2]. Intracellular

Abbreviations: ATX-I (ATX-II), toxin I (toxin II) extracted from the tentacles of *Anemonia sulcata*.

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perfusion with pronase has proven to be a useful tool in the further study of the molecular organization of the inactivation process in these nerves [2]. The number of toxins interacting with the h-system in these unmyelinated nerve fibres is already important [3]. In the case of the myelinated nerve fibres of vertebrates, however, there are fewer toxins interacting specifically with the h-system [4]. The purpose of this paper is to describe the results obtained with one of such toxin extracted from tentacles of *Anemonia sulcata*. The toxin (referred to as ATX-II) is a polypeptide of 4197 molecular weight [5]. If externally applied to the node of Ranvier at a low concentration (10 μM range), it reduces considerably the rate of inactivation of the sodium conductance. At a high concentration (100 μM range), it reduces the potassium conductance g_K without affecting its kinetic properties.

A preliminary note with some of the results has been published [6].

METHODS

Sensory and motor myelinated nerve fibres from the tibial nerve of *Rana esculenta* were used. The methods employed had been described in detail elsewhere [7] and we need only to remind the reader of the nomenclature used. There are four small compartments in the nerve chamber:
C: pool for input electrode and proximal internode;
E: for output electrode and distal internode;
A: for electrode from voltage source and for test node;
B: for ground electrode.
Between pools B and C an air gap was made. In most experiments 120 mM KCl was used in pools C and E but on occasions 120 mM CsCl was also used. Pools A and B contained initially Ringer's solution. Pool A was later perfused with K^+-free Ringer at constant temperature of about 13 °C. Solutions with toxins were always prepared immediately before each experiment. Tetraethyl ammonium was used at a concentration of 10 mM and tetrodotoxin in the range of 100 nM. Internal applications of toxin were achieved by diffusion from pools C and E. For these experiments the internodes were cut shorter than usual, leaving internodal segments of about 0.5 mm in length. Immediately after cutting the fibre the voltage clamp was turned on and the holding potential was adjusted to give a steady state inactivation of the sodium system of 30 % by using variable potential sources in the leads of the electrodes E and A. Under these conditions the holding potential was assumed to be −70 mV. In some experiments a −20 mV holding potential was added. Voltage clamp pulses were applied from this holding level.

Analogue compensation for leakage, fast and slow capacity currents was used throughout. The compensation was achieved by means of a function generator producing three signals in phase with the rectangular voltage-clamp pulse applied. Each signal was a declining exponential voltage with adjustable initial value and relaxation time constant. They were summed and used to compensate the membrane current transients for a hyperpolarizing pulse of 100 mV. This was done by feeding the signal from the voltage-clamp amplifier to one input of the differential-passive filter amplifier of the recording oscilloscope. By feeding the signal from the function generator to the other input, at least three components were balanced, the leakage, the fast and the slow capacity currents (Bergman, C. and Rojas, E., unpublished).

Therefore, most of the membrane currents recorded during the depolarizing pulses represent specific ionic currents, I_{Na+} and I_{K+}.

RESULTS

Effects of toxin II on sodium and potassium currents

Experiments with two of the three toxins extracted from the tentacles of *Anemonia sulcata* have shown that toxin I (referred to as ATX-I) externally applied at a concentration of 100 μM for nearly 30 min had no effect on either sodium or potassium currents whereas toxin II (referred to as ATX-II) externally applied at a concentration of 10 μM rapidly reduced the rate of sodium conductance inactivation without affecting much potassium currents. If higher concentrations of ATX-II are used the potassium current, I_K, is partially blocked without appreciable changes in its kinetics.

The possibility of internal receptors was examined in one experiment. The toxin was added to the KCl solution in pools C and E (the internodal segments were cut shorter than 0.5 mm). With a concentration of ATX-II estimated as 700 μM the shape of the currents did not change for nearly 30 min.

Fig. 1 depicts two I_K records made in Ringer's solution without (a) and with (b) 380 μM ATX-II. For this run tetrodotoxin was used to eliminate sodium currents. The results of two experiments on motor fibres may be summarized by saying that ATX-II at high concentrations (greater than 100 μM) reduces the potassium conductance. Thus, neither the shape of curve potassium permeability P_{K+} versus membrane potential [8] is appreciably changed nor its position in the voltage axis shifted but 50 % reduction in its maximum value is obtained with roughly 350 μM ATX-II,

Time course of the on-effect

Having available for experimentation only 10 mg of ATX-II we decided to study first the effects on the Na-system. It was obvious from the start that the effects of

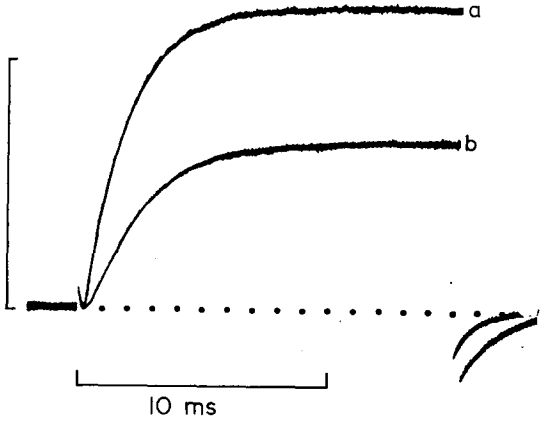

Fig. 1. Effects of 380 μM ATX-II on $I_K{}^+$. Motor fibre in Ringer's solution plus 10 nM tetrodotoxin (a) plus 380 μM ATX-II (b) 120 mM KCl in pools C and E. Temperature 13.5 °C. Holding potential −20 mV (absolute level assumed to be equal to −90 mV. See Ref. 7). Pulse size 140 mV (absolute potential level during the pulse was 50 mV).

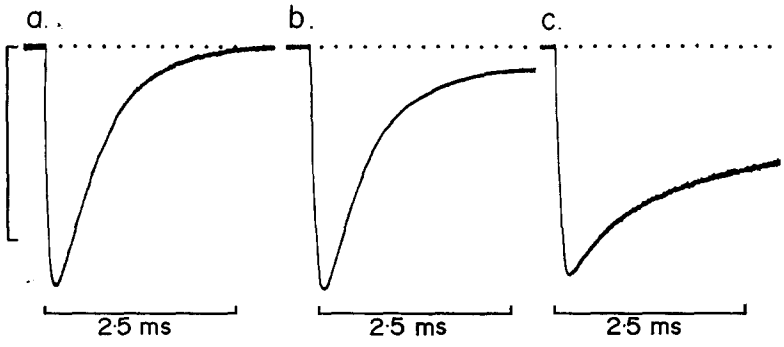

Fig. 2. Time course of the effect of ATX-II on inward sodium currents. Motor fibre in K^+-free Ringer with 120 mM CsCl in pools C and E. Holding potential −20 mV (absolute level equal −90 mV). Pulse size 90 mV (absolute level during the pulse equal 0 mV). (a) Record made just before the Ringer's solution with 36 μM ATX-II arrived to the chamber. (b) Record made 15 s after exposure of the test node to the toxin. (c) Record made 30 s after. Calibration = 100 mV.

ATX-II occur after only a few seconds of exposure of the test node to the toxin. Fig. 2 shows part of a set of records made immediately after switching from Ringer without to Ringer with ATX-II. Three sodium current records made every 15 s starting from the estimated time of arrival of the toxin to the test pool are shown. Already in the second record there is a clear change in the inactivation of I_{Na+}. The change is obvious in record c made 30 s after the arrival of ATX-II to the pool. Leaving the test node exposed to the toxin for longer times did not change considerably the shape of record c. Therefore, the last record in Fig. 2 may be taken as the steady-state condition in a fibre treated with 21 μM ATX-II.

ATX-II reduces the rate of sodium conductance inactivation

Fig. 3 shows four I_{Na+} records made in Ringer's solution plus ATX-II at various sweep speeds. It may be seen that even after 400 ms there is a small inward current. These late currents were blocked by external application of tetrodotoxin. Although the fibre used for the experiment illustrated in the figure was a sensory fibre normally exhibiting a small fraction of channels without inactivation process [9], sustained inward currents like those shown in Fig. 3a were also seen in motor fibres treated with ATX-II. Since the inward current shown in 3a was blocked by tetrodotoxin, we consider it to represent I_{Na+}.

From records like those shown in Fig. 3 we analysed the effects of ATX-II on the rate of inactivation of the sodium system following the arbitrary scheme illustrated in Fig. 4. The figure depicts only one record showing the rapidly changing part of the current. The analysis of this current during ATX-II assumes that there is no change in the maximum sodium conductance induced by the toxin and that the driving force for I_{Na+} does not change during the sustained depolarizing pulse. The figure shows that the falling phase of I_{Na+} may be described in terms of three time constants.

After the sodium conductance has been fully activated by a sudden displacement of the membrane potential we may consider that

$$I_{Na+}(t) = I_{\tau_0} + I_{\tau_1} + I_{\tau_2}, \quad t > t_p \tag{1}$$

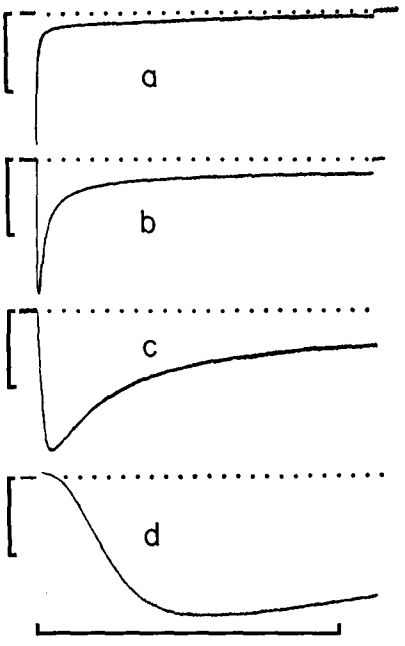

Fig. 3. Sodium current in 14 μM ATX-II. Holding potential −20 mV; Absolute membrane potential during the pulse 0 mV. Temperature 14 °C. Sensory fibre in K^+-free Ringer plus 10 mM tetraethylammonium plus 14 μM ATX-II with 120 mM KCl in pools C and E. Calibrations: vertical = 100 mV; horizontal = 400 ms for a, 40 ms for b, 4 ms for c, and 0.4 ms for d. Record a has been used to estimate the extra-entry of sodium during the slowly declining current as follows

$$\frac{1}{F}\int_0^{0.5s} I_{\tau_2} dt = 0.4 \cdot 10^{-14} \text{ Mol of } Na^+,$$

where F is the Faraday's number. The initial value of $I_{\tau_2}(0)$ was estimated as $16 \cdot 10^{-10}$ A assuming that the resistance of the measuring internodal segment was 10 MΩ. For a cylindrical node (1 μm height and 4 μm in diameter) the maximum change in concentration of sodium would be roughly 80 mM.

TABLE I

ANALYSIS OF THE EFFECTS OF ATX-II IN TERMS OF A REACTION IN TWO STEPS

$I_{\tau_i}(0)$ = Initial value of the component of the current inactivating with a time constant τ_i. The analysis of experiment 242 in the case of ATX-II = 26 μM is shown in Fig. 4.

Exp.	ATX-II (μM)	$(I_{Na^+peak})_{before}$ (mV)	$I_{\tau_0}(0)$ (mV)	$I_{\tau_1}(0)$ (mV)	$I_{\tau_2}(0)$ (mV)	τ_0 (ms)	τ_1 (ms)	τ_2 (ms)	$\Sigma_{I_{Na,i}}$ (mV)
249–1	0.15	245	300	—	—	0.85	—	—	300
	0.48	245	300	—	—	0.85	—	—	300
242	7	220	250	41	23	0.86	3.6	>50	314
	14	220	175	48	25	0.86	3.5	>50	248
	26	220	137.5	85	28	0.80	2.9	>50	250.5
249–2	21	250	145	107	47	0.85	3.2	>50	299
259	21	125	80	39	32	0.90	3.8	>50	151
192	71	125	29	42	40	0.80	3.25	>50	111
	71	125	6	66	13	0.80	3.8	>50	85

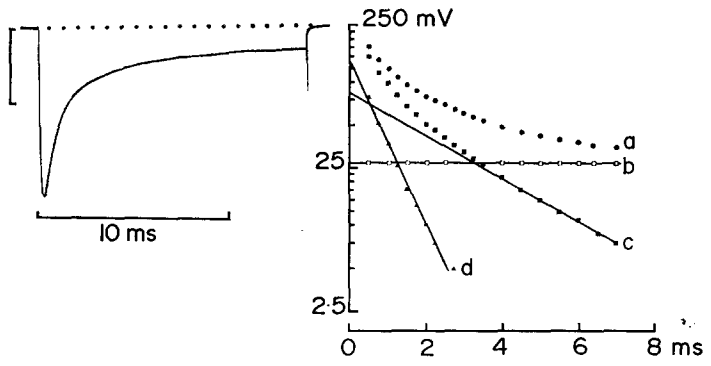

Fig. 4. Analysis of the effect of ATX-II on the inactivation of the sodium current. Sensory fibre in K^+-free Ringer solution plus 10 mM tetraethylammonium and 26 μM ATX-II. 120 mM KCl in pools C and E. Holding potential −20 mV; pulse size 90 mV. Temperature 13 °C. (a) Sodium current samples (●) taken from the current trace shown on the left side of the figure. (b) Current values representing the fraction of Na^+-channels with τ_2 greater than 50 ms obtained by extrapolation of I_{τ_2}; initial value 28 mV. (c) Represents the difference a–b (■); initial value 85 mV; time constant τ_1 = 2.9 ms. (d) Difference between the values in c (■) from 0 to 2.5 ms and the corresponding values on the straight line τ_1 = 2.9 ms shown in c. Initial value 137.5 mV; Time constant τ_1 = 0.8 ms.

where t_p is the time at which I_{Na+} reaches its maximum value and I_{τ_1} represents the sodium current characterized by a rate of inactivation τ_i^{-1}. From data like that illustrated in Fig. 4 we obtained rough estimates of τ_i and $I_{\tau_1}(0)$. To a first approximation this arbitrary method gave quantitative estimates of the various parameters which have been summarized in Table I.

Record a in Fig. 3 clearly shows that if the depolarizing pulse is kept during 400 ms the current I_{τ_2} decreases towards the base line with a time constant which changed with the concentration of toxin assayed. We think that this slow decay of I_{Na+} may be associated with a drop in the driving force due to a rise in the concentration of sodium inside rather than a true slow inactivation [10]. Assuming a nodal area of 25 μm^2 corresponding to a cylinder of 4 μm in radius and a height equal to 1 μm, with the values of I_{Na+} at 20 ms and at 420 ms, we estimate that the internal sodium concentration could have increased by as much as 80 mM at the end of the inward current shown in Fig. 3a. This supports the idea that I_{τ_2} decays as a result of an internal accumulation of sodium. τ_2 (see Table I) may be greater than estimated, thus lending support to the notion that the reaction toxin receptor leads to an "effective removal" of the h-process. From Table I we may see that the fraction of Na-channels having reached the postulated end point of the reaction is rather small, less than 10 % at ATX-II close to K_d.

Effects of ATX-II on the voltage dependence of the inactivation h_∞

The insert in Fig. 5 illustrates the effects of a 50 ms conditioning pre-pulse (taking the membrane potential from its holding value of −90 mV to −50 mV absolute potential) on I_{Na+} due to a test pulse to 0 mV. It may be seen that the peak value has been reduced by nearly 90 % leaving a component affected to a lesser extent. The most reproducible effect of ATX-II on the $h_\infty - V$ curve was (as shown in Fig. 5) a clear decrease in the slope of the curve.

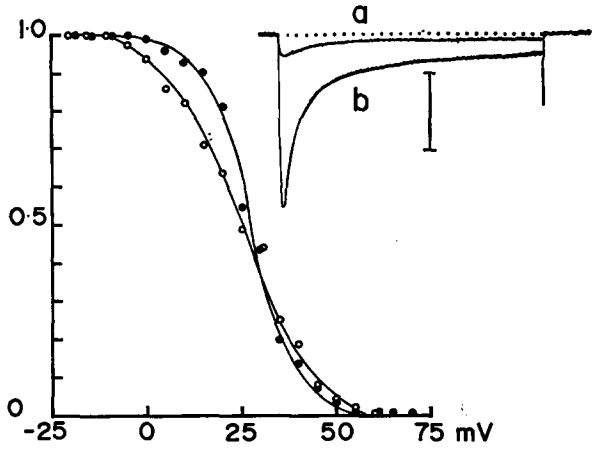

Fig. 5. Steady-state sodium conductance inactivation curve before and during external application of 14 μM ATX-II. Experiment on a sensory fibre in K^+-free Ringer plus 10 mM tetraethylammonium and isosmotic KCl in pools C and E. Temperature 14 °C; holding potential −20 mV (corresponding to 0 on the abscissa). Vertical axis represents the fraction of sodium channels affected by a 50 ms conditioning pre-pulse. Insert shows two examples of membrane current records obtained during a 90 mV pulse lasting 14 ms (a) with and (b) without pre-pulse equal to 40 mV. The base line is indicated by the series of points 500 μs apart. Vertical calibration = 100 mV. Abscissa represents the size of the conditioning pre-pulse. ●—●, before toxin; ○—○, during toxin.

The rate of activation of the sodium conductance is not affected by ATX-II

Shown in Fig. 6 are two sets of superimposed I_{Na+} records made before (a) and during (b) the treatment with ATX-II. A number of records like these were used to calculate the time constants for the activation of the sodium system from negative holding potentials. To evaluate the possible effects on the activation of I_{Na+} the records were fitted with

$$I_{Na+}(t) = \begin{cases} 0, & 0 \leqslant t \leqslant \delta t \\ I'_{Na+} m^2 h, & \delta t < t \end{cases} \qquad (2)$$

where δt has been introduced to take into account the effects of negative holding potentials (initial delay in the activation of I_{Na+}), m and h have their usual meanings in the Hodgkin-Huxley terminology, namely, m represents a first order activation

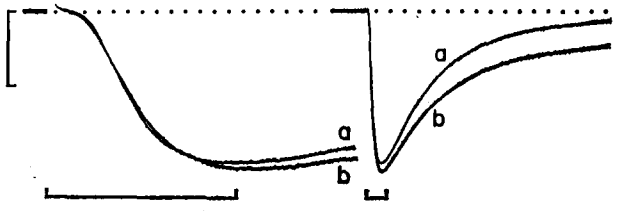

Fig. 6. Activation of the sodium current in the absence (a) and in the presence (b) of 14 μM ATX-II. Fibre with the internodes cut in 120 mM KCl. Holding potential −20 mV. Superimposed current records obtained with a 90 mV depolarizing pulse (a) in K^+-free Ringer solution plus 10 mM tetraethylammonium (b) in K^+-free Ringer plus 10 mM triethanolamine with 14 μM ATX-II 3 min after introducing the toxin in the pool A. Calibrations: vertical = 100 mV; horizontal = 250 μs. Temperature 13 °C.

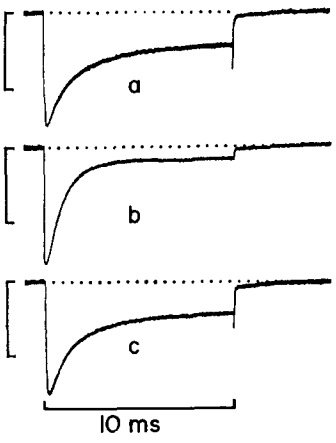

Fig. 7. Recovery from 36 μM ATX-II. Pools C and E with 120 mM CsCl; K^+-free Ringer solution was perfused through pool A. Holding potential −20 mV; test pulse size was 90 mV for the three records shown. (a) Record made 1 min after external application of toxin; (b) Record made 9 min after the removal of the toxin. (c) Record made 2 min after a second exposure to the toxin at the same concentration. Vertical calibration = 100 mV. Temperature 13 °C.

process and h a first order inactivation process assumed to be independent of m [11]. For a pulse taking the membrane potential to an absolute level of 0 mV τ_m before the toxin was 63 μs and it was 60 μs during 26 μM ATX-II.

Reversibility of the toxin effects

Further support for the idea of a reaction in steps (see Discussion) is presented in Fig. 7. Here the test node was exposed to the same concentration of ATX-II twice. During the first exposure record a was made. Record b was made 9 min after the removal of the toxin. During the second exposure record c was made. Although the recovery of $I_{\tau_0}(0)$ is rather slow, the rate of recovery of I_{τ_0} from I_{τ_1} is faster than that of I_{τ_1} from I_{τ_2}. This means that there are two populations of channels recovering at different rates. Although this point could not be studied in any detail, it was clear from several experiments that some time after the removal of ATX-II although I_{τ_1} is practically zero, I_{τ_2} is quite substantial (as illustrated in Fig. 7b).

Equilibrium dissociation constant K_d

The observed reversibility suggested the possibility of estimating the equilibrium dissociation constant, K_d, of the toxin. Fig. 8 presents the results of 9 determinations carried out in 5 different fibres. In two other fibres (results not shown in the figure) we applied 380 μM ATX-II and the fraction of channels unaffected by the toxin was reduced to values too small to be measurable in terms of $I_{\tau_0}(0)$. Only one aberrant value has been ommited on the grounds that it was the only fibre in which there was practically no recovery from the effects of the toxin. It may be seen that as the concentration of ATX-II is increased the fraction of channels unaffected by the toxin decreases, 50 % reduction being obtained at about 20 μM.

Although we did not study the kinetics of the reaction between the toxin and the receptor in any detail (see Eqn. 4), in some voltage clamp experiments (like the one

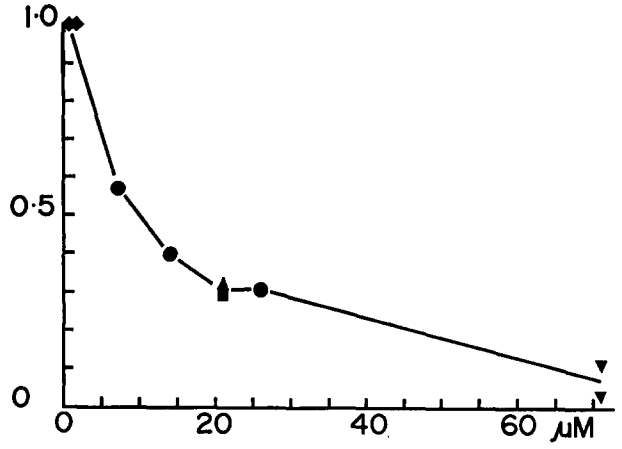

Fig. 8. Fraction of Na^+-channels with unmodified inactivation kinetics as a function of concentration of toxin in the Ringer's solution. Each symbol represents a different fibre.

illustrated in part in Fig. 2) the pulses were applied every 15 s and the toxin was kept in pool A until no further reduction of I_{τ_0} could be detected. In experiments with 21 and 36 μM ATX-II the I_{τ_1} values plotted against time showed a clear increase in the rate of action with increasing ATX-II concentration. Assuming that the onset and the offset of the toxin effect are determined by the rate of reaction with the membrane receptors (as in the case of tetrodotoxin, discussed in ref. 12) and that $\tau_{on} \ll \tau_{off}$ where τ_{off}^{-1} is the rate of recovery and τ_{on}^{-1} is the rate of appearance of the component I_{τ_1}, we may write (after ref. 12)

$$k_1 = \frac{1/\tau_{on} - 1/\tau_{off}}{[\text{ATX-II}]} \simeq \frac{1}{/\tau_{on}\,(\text{ATX-II})} \tag{3}$$

With the data obtained from the experiment illustrated in part in Fig. 2 we estimated τ_{on} as smaller than 10 s making the value of k_1 equal to $3 \cdot 10^3\ M^{-1} \cdot s^{-1}$.

Further support for the concept of separated Na^+ and K-channels

Assuming that the process of activation of I_{Na+} is independent of the process of inactivation, a decrease in the rate of inactivation, such as the one induced by ATX-II, would in principle lead to an increase of I_{Na+}. Thus, assuming m^2h kinetics [13], taking the time constant of inactivation τ_h as 0.8 ms (see Table I) and the time to peak of I_{Na+} as 0.2 ms (see Fig. 3) the maximum I_{Na+} value should increase 1.28 times (I'_{Na+} in eqn. 2).

In 11 of the 12 fibres examined for the effects of these toxins, we observed an increase in the maximum I_{Na+} amounting to no more than 10 % of its control value. Shown in Fig. 9 are the results of one such experiment in terms of current voltage relationships. To maximise the effects we used 380 μM ATX-II. Four curves are shown, two representing peak values of early inward currents (before and during the application of the toxin) and two representing the currents measured at the end of the pulse (lasting 15 ms). It may be seen that the early inward currents (representing $I_{Na+} + I_{K+}$) increase by a factor of 1.15 after treatment with ATX-II. Taking into account

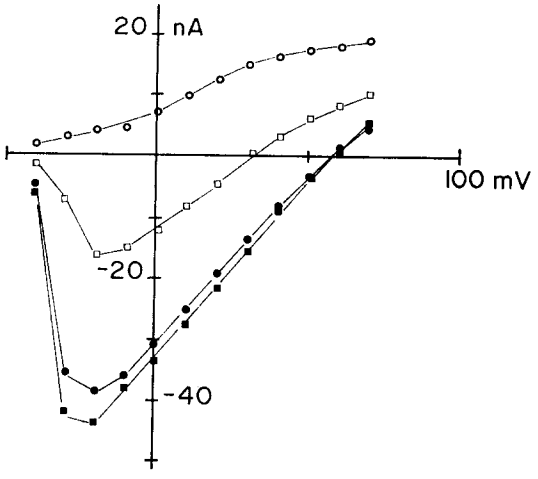

Fig. 9. Increase in sodium currents after treatment with 380 μM ATX-II. Motor fibre in Ringer's solution with 120 mM KCl in pools C and E. ●, peak inward current (control run); (○), outward current measured after 15 ms (control run); ■, peak inward current measured in the presence of toxin; □, current measured at 15 ms in the presence of the toxin. The current (vertical axis) was calculated assuming that the resistance of the axoplasm in the internode extending from pool E to pool A was 10 MΩ [15].

the effects of ATX-II on I_{K+} does not change this estimate because the peak of I_{Na+} occurs when I_{K+} is starting to rise. These measurements in fibres treated with ATX-II confirm in the case of the nodal membrane the results obtained in squid giant axons treated with pronase [1] that $I_{Na+}+I_{K+}$ is not constant. These results do not support the notion that the ionic channels undergo a conformational transition leading to a transient state that accepts Na^+ (activation of the sodium system) and steady state conformation that accepts K^+ (activation of the potassium system and inactivation of the sodium system). Thus, currents measured at the end of the 15 ms pulse in the experiment of Fig. 9, for pulses beyond the reversal potential of I_{Na+}, for example at 60 mV, were always greater than I_{K+} in the presence of ATX-II. This observation suggests two systems operating in parallel rather than in series by interconversion of one into the other as postulated by Mullins [14].

DISCUSSION

We have shown that it is possible to describe the time course of the inactivation of I_{Na+} in terms of at least three time constants. This observation led us to propose that the reaction between the toxin and the receptor sites on the external surface of the nodal membrane may be expressed as

$$S_0+[\text{ATX-II}] \underset{k-1}{\overset{k_1}{\rightleftharpoons}} [S_1\text{-ATX-II}] \underset{k-2}{\overset{k_2}{\rightleftharpoons}} [S_2\text{-ATX-II}] \qquad (4)$$

where S_0 represents the concentration of sites (associated with Na-channels with inactivation process intact) available for the reaction, S_1 concentration associated with Na^+ channels with the inactivation partially affected and S_2 the concentration of sites from channels with the inactivation fully immobilized.

The amino acid composition for ATX-I and ATX-II has been determined [5]. These mini-proteins contain substantial proportions of amino acid residues that have distinctly non-polar chains (alanine, valine, leucine, isoleucine and proline) and relatively non-polar groups (tryptophan and cysteine). On the basis of these data we have been able to compare ATX-I and ATX-II with albumin of known amino acid composition. Thus, attributing weight factors allowing for the measured energy change to transfer the corresponding amino acid to polar solvents [16] we have that, for albumin with 598 amino acids the ratio "weighted number of apolar amino acids/ weighted number of polar plus apolar amino acids" is 0.40 whereas ATX-II with 47 acids gives a ratio of 0.57. This simple calculation shows that the relative proportion of apolar amino acids in these mini-proteins is greater than in a common protein and suggests that the toxins may interact with the gating mechanism in the hydrophobic region of the membrane.

One very important application of ATX-II is in the study of steady state processes in the Na^+ system (like Na^+ channel noise) which require sodium current records of several hundred milliseconds and that normally could not be studied in motor fibres because of the inactivation process of the Na^+ conductance [17].

In the case of the pronase experiments, where the Na^+ channels either inactivate at normal rate or fail to inactivate, Goldman [18] indicated that assuming coupled activation-inactivation it is possible to describe the average kinetic properties of the Na^+ channels, i.e. I_{Na+}, before and after pronase treatment. This result is not surprising, however, since in this limiting case (only two types of channels) the second order variable postulated by Goldman can be expressed in terms of a pair of independent first order variables. Therefore, giant axons treated with pronase represent a limiting case. The case of the node of Ranvier treated with ATX-II in which the rate of sodium conductance inactivation is greatly reduced and the rate of activation is not affected, cannot be described by a second order variable only. That a drastic change in the rate constants of inactivation could take place leaving unmodified the rate constants for the activation of the conductance indicates that the two processs are independent.

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