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SHORT COMMUNICATIONS

A Novel Mode of Neurotoxin Action

A Polypeptide Toxin Isolated from *Anemonia sulcata* Shifts the Voltage Dependence of the Maximal Rate of Rise of Na^+ Action Potentials in a Mouse Neuronal Clone

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SUMMARY

Mouse neuroblastoma N-18 cells, which evoked a mixed Na^+ and Ca^{2+} action potential under appropriate tissue culture conditions, were used to study the electrophysiological pharmacology of a polypeptide neurotoxin (ATX-II) from a sea anemone. When applied extracellularly, ATX-II in concentrations as low as 10^{-7} M increased reversibly the electrical excitability of N-18 cells, e.g., the toxin caused spontaneous firing in which the duration and the maximal rate of rise of each action potential were increased. A set of results obtained in this work strongly suggests that this effect of the toxin was mainly due to its interaction with the inactivation gate of the Na^+ channel of N-18 cells, i.e., ATX-II inhibited both the time-dependent and the steady-state processes of Na^+ channel inactivation. Accordingly, this toxin is a useful tool for elucidating the molecular structure of the voltage-sensitive inactivation gate of the Na^+ channel.

Various neuroactive substances, including neurotoxins, are highly useful tools for studying the molecular structure of ionic channels underlying an action potential. The idea of separation between Na^+ and K^+ channels, first suggested by Hodgkin and Huxley (1), has been confirmed by pharmacological investigations with the use of the chemical agents that selectively block one type of channel without affecting another type: e.g., tetrodotoxin, a Na^+ channel-blocking agent, and tetraethylammonium, a K^+ channel-blocking agent (2, 3). Furthermore, several polypeptide toxins from scorpions and sea anemones have recently been shown to affect specifically either the activation (4) or the inactivation (5–12) of the Na^+ current, which suggests the existence of two distinct molecular structures controlling the two separate processes, respectively. Among these toxins, ATX-II[2] (13) from *Anemonia sulcata*, a polypeptide (47 amino acids) whose primary structure has now been established (14, 15), is known to prolong markedly the duration of the action potential by slowing the closing process of the inactivation gate [h-gate (7)] of the Na^+ channel in crustacean axons (10), frog myelinated nerve fibers (11), and cultured cardiac cells (16). Biochemical approaches have also been used to examine the interaction of ATX-II with Na^+ channels (16–18). Thus, the toxin inhibits the time-dependent process of the Na^+ channel inactivation. In the present study, we have examined effects of ATX-II on the electrical activity of mammalian neurons (mouse neuroblastoma N-18 cells), and observed that ATX-II releases Na^+ channels from steady-state (voltage-dependent) inactivation in addition to inhibiting the time-dependent inactivation. ATX-II is the first neurotoxin reported to affect the Na^+ channel in this manner, insofar as we are aware.

N-18 cells differentiate with respect to electrical excitability and generate all-or-none action potentials under appropriate tissue culture conditions. Some of these action potentials are composed of both Na^+ spike and Ca^{2+} spike components in a normal salt solution [millimolar: NaCl, 150; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.8; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-NaOH, 5.0 (pH 7.3 ± 0.15)], and the others are almost pure Na^+ action potentials (19). The cultivation procedure for obtaining such electrically differentiated cells was as reported previously (19); N-18 cells were treated with aminopterin (4 μM, for 24 hours) and subsequently maintained in Dulbecco's modified Eagle's medium supplemented with 0.1–0.5% (v/v) newborn calf serum at 37° for at least 2 weeks. Intracellular recordings were done

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[2] The abbreviation used is: ATX-II, toxin II of *Anemonia sulcata*.

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with a single microelectrode (20–50 MΩ filled with 3 M KCl) during constant perfusion with the normal salt solution or that containing ATX-II and/or tetrodotoxin at 35 ± 2°. The preamplifier (M-701, WPI) used was equipped with a bridge circuit in its input stage, so that voltage (V) could be recorded while polarizing current (I) was simultaneously passed across the membrane. The rate of the membrane potential change (dV/dt) was measured by the use of an electronic differentiator. In the present study, we used selected cells with neurites no longer than 150 μm in order to evaluate the current across the soma membrane during an action potential from the value of dV/dt. The insides of the cell body and the neurites of these cells were nearly isopotential (20). ATX-II was obtained from Ferring (Kiel, Germany).

External application of ATX-II reversibly enhanced the electrical excitability of all of the cells examined. The toxin at concentrations between 10^{-8} and 10^{-7} M decreased the minimal intensity of the depolarization for spike initiation (data not shown). At concentrations higher than 10^{-7} M, spontaneous, slow oscillations (up to 4 mV peak to peak) were observed in a voltage record and often resulted in spontaneous firings, as shown in Fig. 1. Each toxin-induced action potential in Fig. 1 consists of a fast peak and a following plateau (80–500 msec in duration), whereas such plateaus cannot be found in the action potentials evoked by current stimulation in the absence of ATX-II. The toxin also increased the peak height and the maximal rate of rise of the action potentials. These effects of ATX-II were accompanied with no or little change in the resting potential level.

A similar ATX-II-induced prolongation of action potentials has previously been reported for crayfish and frog axons (10–12) and cardiac cells (16). Voltage-clamp analyses with these axons have shown that ATX-II slows down the time-dependent inactivation process of the transient Na^+ current, presumably by immobilizing the h-gate (inactivation gate) of Na^+ channels (10, 11). If this is applicable to mammalian neurons, the prolongation of the action potentials observed with N-18 cells is ascribable to this mode of action of ATX-II. This notion is supported by the finding that the toxin (10^{-6} M) did not change any electrophysiological properties of the cells in the presence of 2 μM tetrodotoxin where both K^+ and Ca^{2+} channels were able to be activated by current stimulation.[3] Thus, the toxin treatment at concentrations lower than 10^{-6} M affected neither K^+ nor Ca^{2+} channels. This fact suggests that the effects of ATX-II were mediated mainly by Na^+ channels.

In spite of the above suggestion, neither the spontaneous firing nor the increase in the maximal rate of rise of the action potential can be explained by this mode of ATX-II action alone. Na^+ channels in various excitable tissues show consistently a steady-state inactivation. The degree of the steady-state inactivation can be conveniently estimated from a change in the maximal rate of rise of Na^+ action potentials evoked at different membrane potentials[4] in some excitable tissues (21). As Fig. 2A and B shows, N-18 cells elicit anode break action potentials after hyperpolarizations exceeding a certain magnitude. In Fig. 2C, the values of the maximal rate of rise of the anode break-action potentials thus obtained are plotted as a function of a conditioning membrane potential at the end of the preceding hyperpolarization.

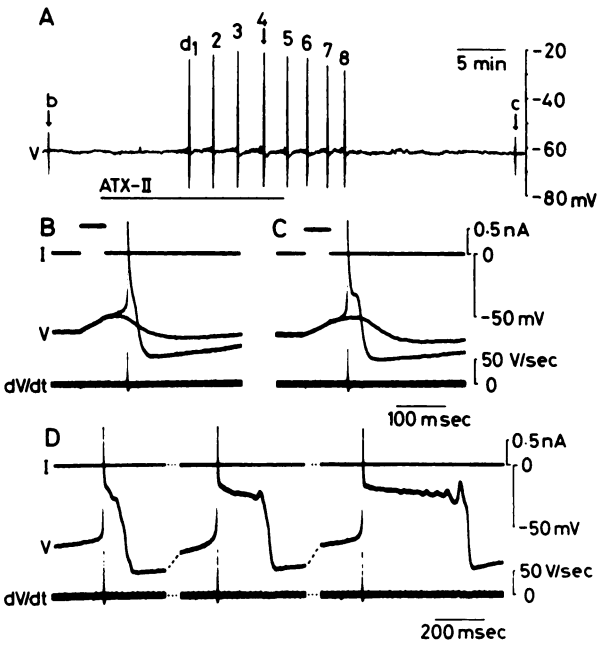

FIG. 1. *Effects of ATX-II on the electrical activity of mouse neuroblastoma N-18 cells*

A. A pen-writing record of the membrane potential before and after the external application of 10^{-7} M ATX-II and after its washout. A *bar* below the *V*-record indicates the duration of the toxin application. In the experimental apparatus used here, the bath solution was almost completely exchanged within 2 min after the exchange of the perfusion solution. The action potentials are represented on a small magnitude because of the low-frequency response of the pen-writing recorder used. The two action potentials indicated by *arrows b* and *c* were evoked by means of depolarizing current pulses (50 msec), but the other activities (d_{1-8}) occurred spontaneously. Note that each deflection of d_{1-8} consists of three action potentials.

B and C. Oscilloscope records of action potentials before the ATX-II application (B) and after its washout (C). An action potential and a subthreshold membrane response are superimposed in B and C. The two families of the records (B and C) were obtained at the times indicated by *arrows b* and *c* in A, respectively.

D. The representative oscilloscope record of the ATX-II-induced train composed of three action potentials. This record was obtained at the time indicated by *arrow* d_4 in A.

[3] M. Miyake and S. Shibata, unpublished data.

[4] The maximal rate of rise of the action potentials is strictly proportional to the maximal total inward current but not to maximal inward Na^+ current. However, we can safely conclude that the magnitude of the maximal rate of rise is practically a good measurement of the maximal inward Na^+ current on the following three bases. (a) The maximal rate of rise of pure Ca^{2+} action potentials, which were evoked in the presence of 10^{-6} M TTX, was mostly less than 2% of that of the original mixed Na^+ and Ca^{2+} action potentials at every conditioning potential. (b) The activation of the delayed K^+ current also did not practically contribute to the maximal rate of rise of the action potentials, since 10 mM TEA did not affect the magnitude of the maximal rate of rise. (c) The specific membrane resistance (8–20 KΩ cm^2) of the cells used was approximately 10 times larger than that of the squid giant axons, suggesting that the leak current of the N-18 cells was relatively small.

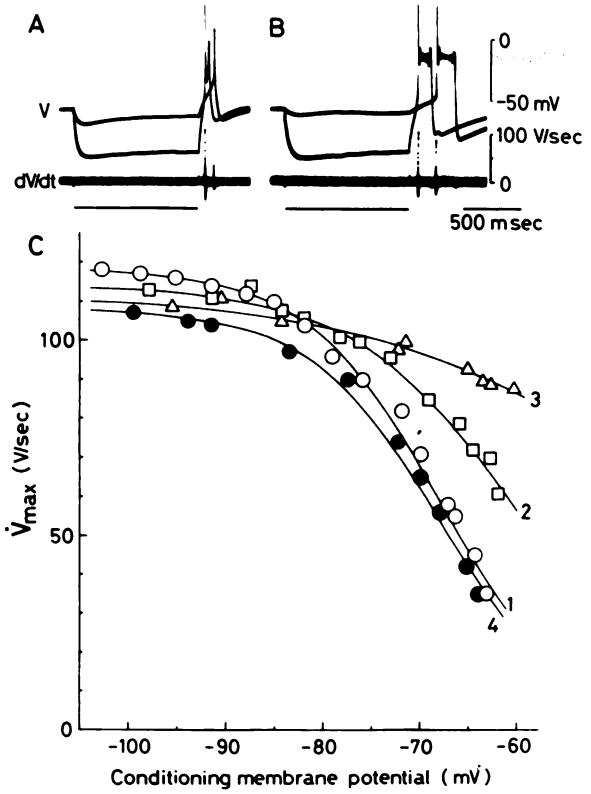

FIG. 2. *The reversible removal of the steady-state Na^+ channel inactivation by external ATX-II*

A and B show the anode break-action potentials after the end of hyperpolarization in the normal salt solution with (B) and without (A) 10^{-7} M ATX-II. Each *bar* at the bottom represents the period when hyperpolarizing current pulses (A, 0.45 and 1.56 namp; B, 0.1 and 1.2 namp) were applied. Two anode break-action potentials evoked at different conditioning potentials are superimposed in A and B. Note that in the normal solution the maximal rate of rise of the action potentials is significantly increased with an increase in the conditioning potential. The polarization from the resting potential for nearly 1 sec is sufficient for Na^+ channels to reach the new degree of their steady-state inactivation at the conditioning membrane potential (22). This was confirmed by the finding that the results obtained by use of 0.5-, 1-, and 5-sec pulses were identical in both the presence and absence of the toxin (10^{-7} M).

C represents plots of maximal rate of rise ($\dot{V}_{max}$) as a function of the conditioning potential in the normal solution (○, control; ●, 45 min after the washout of the toxin) and in the presence of ATX-II (□, 3×10^{-8} M; △, 10^{-7} M). The data points in the presence of the toxin were taken after the previous perfusion for longer than 20 min with the solutions containing the toxin at concentrations indicated. All data were obtained with a single cell, and the experiments were carried out in the following sequence; ○ → □ → △ → ●. The anode break stimulations were applied at time intervals of approximately 30 sec. Slight differences in the maximal level of the maximal rate of rise may be due to the gradual injury of the membrane by means of the microelectrode impalement. Curves were drawn according to Eq. 1 in the text, where the values of the parameters used are as follows: $V_{0.5}$, −68.0 (ATX-II-free), −60 (3×10^{-8} M), and −41 mV (10^{-7} M); k, 6.7 (ATX-II-free), 8.5 (3×10^{-8} M), and 15.5 mV (10^{-7} M); $\dot{V}_{max}°$, 109 (control), 107 (3×10^{-8} M), 106 (3×10^{-8} M), and 104 V/sec (washout).

In the normal solution, the maximal rate of rise increases from 35 to 100 V/sec with a hyperpolarization of the conditioning potential from −70 to −85 mV, and the increased magnitude of the maximal rate of rise is not apparently changed (approximately 100 V/sec) in the conditioning potential range more negative than −85 mV (*line 1* in Fig. 2C). If the maximal rate of rise is a good index of the early Na^+ current of action potentials[4] and its voltage dependence serves as a conventional measurement of the steady-state inactivation curve (22) in N-18 cells, the relationships in Fig. 2C may be described by

$$\dot{V}_{max} = \dot{V}_{max}° / \left(1 + \exp \frac{V - V_{0.5}}{k}\right) \qquad (1)$$

where $\dot{V}_{max}$ represents the maximal rate of rise, $\dot{V}_{max}°$ is the saturated value of $\dot{V}_{max}$, V is the conditioning membrane potential, $V_{0.5}$ is a half-inactivation potential, and k is a slope constant. *Lines 1* and *4* in Fig. 2C clearly show that data points obtained in the normal solution satisfy the above equation. The values of $V_{0.5}$ and k are estimated to be −68.0 mV and 6.7 mV, respectively. These magnitudes are in fairly good agreement with those [$V_{0.5}$, −65 mV; k, 9.5 mV (23)] obtained with a mouse neuroblastoma N1E-115 clone under voltage-clamp conditions. As *lines 2* and *3* in Fig. 2C show, ATX-II decreases in a dose-dependent manner the voltage dependence of the Na^+ channel inactivation without affecting the saturated level of the maximal rate of rise at sufficiently large negative conditioning potentials (more negative than −85 mV in this case). These results strongly suggest that $V_{0.5}$ is depolarized and k is increased by the external treatment with ATX-II.[5] An increase in k represents a decrease in the slope of the steady-state inactivation curve. In contrast, voltage-clamp experiments on frog node of *Ranvier* showed that ATX-II brought about a slightly reduced slope but no shift along voltage axis in its steady-state inactivation curve of the Na^+ channel (11). The difference in the results for the mouse neuroblastoma and the frog node of *Ranvier* may stem from the difference in molecular structure of the Na^+ channel (h-gate) between these preparations.

In this way, we can conclude that ATX-II at a concentration less than 10^{-7} M removes the steady-state inactivation of the Na^+ channel at resting potential level (around −60 mV), presumably by interacting with the h-gate. This mode of ATX-II action can reasonably and directly interpret the toxin-induced increase in the maximal rate of rise of the action potentials generated at the resting potential. Furthermore, the removal of the steady-state Na^+ channel inactivation may contribute, at least in part, to the generation of the toxin-induced spontaneous firing. The small and slow depolarization, which causes the further voltage-dependent inactivation of the Na^+ channel without ATX-II and thereby fail to elicit an action potential, is sufficient to generate an action potential in the presence of the toxin at appropriate concentrations. Here one may raise the question of whether the spontaneous firing during the ATX-II treatment, apparently analogous to that caused by a decrease in external Ca^{2+} concentration (24–26), could be due to the hyperpolarizing shift of the critical membrane potential for the spike initiation. However, this possibility is unlikely since ATX-II at a concentration of 3×10^{-7} M

[5] This conclusion should be confirmed by the voltage-clamp measurements, and this is in progress.

did not affect the threshold potential for the spike initiation within errors of a few millivolts when action potentials were evoked by the anode-break stimulation after the hyperpolarizations more negative than −85 mV. Under these experimental conditions, the steady-state inactivation must be almost completely removed in both the presence and absence of the external toxin.

Na^+ channels in excitable membranes have the unique property that the decrease in the voltage difference (inside negative) across the membrane triggers a transient increase in their permeability to Na^+. The molecular mechanisms underlying the above permeability change, as yet to be elucidated, may involve charge movements in response to changes in membrane potential (1), suggesting that the voltage-sensitive structures in the Na^+ channel consist of charges and/or dipoles. In fact, current (the gating current) presumably due to the rearrangement of the fixed charges and/or dipoles in Na^+ channels has been observed during the opening process of the activation gate (m-gate) of this channel in squid giant axons (27, 28). However, the current component due to the closing process of the h-gate has not yet been detected. Furthermore, little is known about the molecular structure of the h-gate except that this gate seems to be composed of some proteinous component (29). The present results show that ATX-II applied externally inhibited the voltage dependence of the steady-state Na^+ channel inactivation, probably by interacting with the h-gate. This polypeptide toxin is therefore a useful tool for further studies elucidating the voltage-sensitive mechanisms and the molecular structure of the h-gate in electrophysiological and pharmacological aspects.

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