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A mechanistic interpretation of the action of toxin II from *Anemonia sulcata* on the cardiac sodium channel

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Cardiac sodium channels, modified by *Anemonia sulcata* toxin II, have been analyzed by the patch-clamp method. The open state of the modified sodium channels proved to be prolonged highly significantly and reopening from a closed state denoted c*-state frequently occurred, interrupted by silent periods, denoted i*-state. Activation from the c*-state was apparently not affected by toxin action, whereas activation from the i*-state was markedly prolonged. Upon higher depolarizations toxin-induced sodium channels disappeared and this behaviour has been attributed to dissociation of the toxin from the channel by use of a special pulse-protocol. The onset of the toxin effect on the action potential proved to depend on stimulation, and it is concluded that the toxin binds preferentially to the open (o)-state. Taking together the results, a kinetic scheme is suggested for action of the toxin on the cardiac sodium channel.

Introduction

Up to five separate binding sites for biological toxins are known to exist on the electrical excitable sodium-channel protein [1]. Binding studies with radioactive labelled toxins to these receptor domains have provided the first evidence that sodium- and potassium channels are separate entities. Also, estimates of sodium channel density, information about coupling of the activation (m-gate) and inactivation (h-gate) process and about voltage-dependent conformational changes associated with activation have been obtained [2,3]. Allosteric interactions between the different receptor domains are going to be studied and some of these toxins provide valuable tools for the isolation of sodium channels from different sources.

Anemonia sulcata toxin II (ATX-II), a basic polypeptide, 47 amino acid residues long has been shown to bind selectively to receptor domain III of the sodium channel of various sources [4–7], to stimulate $^{22}Na^{+}$-uptake [4,8,10,24] and to slow down inactivation without affecting activation in electrophysiological experiments [9,11–15,17–19].

The electrically gated cardiac sodium channels from mammals that are responsible for initiation and conduction of excitation in heart muscle differ in their pharmacological profile from the sodium channel in nerves. They are relatively insensitive to the classical sodium channel blocker tetrodotoxin (TTX), but their affinity to ATX-II is pronounced [10]. The aim of this study was to analyse the action of ATX-II on single cardiac sodium channels with the patch-clamp method and to design experiments, the results of which, allow the proposition of models for association and dissociation and the action of the toxin on the cardiac sodium channel on the molecular level.

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Materials and Methods

Ventricular cardiomyocytes were isolated from adult rats (180–250 g) of both sexes by perfusion of the Langendorff heart with collagenase-solution (Worthington; type CI-28) as described [20]. The cells could be kept alive up to 5 days after isolation in an incubator (37° C; gassed with 95% O_2, 5% CO_2) in cell culture medium (M 199; Biochrom, Berlin (West)) containing 5% fetal calf serum (Gibco, Uxbridge), penicillin (Sigma; 100 i.u./ml) and streptomycin (Sigma; 100 i.u./ml).

Action potential and single-channel recordings were performed following the method of Hamil et al. [21] using a patch-clamp amplifier (L/M-EPC 7; List Medical, Darmstadt, F.R.G.) and stored on PCM-tape (Sony PCM 501 ES audioprocessor, modified according to Benzanilla [22], and Panasonic video casette recorder NV-430). The extracellular medium had the following composition (mM): 137 NaCl; 5.4 KCl; 2 $mgCl_2$; 10 Hepes/Na^+ buffered to pH: 7.4 and 10 glucose. For action potential recordings the pipette (= intracellular)-solution contained (mM): 140 KCl; 2 $MgCl_2$, 1 $CaCl_2$; 11 EGTA/K^+ and 10 Hepes/K^+ buffered to pH 7.4. After a gigaohm-seal was established the membrane patch under the opening of the pipette was destroyed by suction to gain access to the cell interior. Stimulating pulses to evoke action potentials were supplied by an arbitrary waveform generator (Wavetek 275; San Diego, U.S.A.).

Single-channel currents were measured in the cell-attached configuration, the pipette containing extracellular medium, variable amounts of ATX-II (Sigma; 0.8–200 nM) and 1 mM $BaCl_2$ to block potassium-channels [17].

Single sodium channel currents were elicited by hyperpolarizing the membrane patch for 20 to 50 mV relative to the resting membrane potential and then applying suprathreshold voltage pulses, by applying triangular potential waves to the membrane patch or by setting the membrane potential to the desired value (steady-state recordings).

For computer-aided evaluation, the recordings were replayed from tape and sampled continuously by the analog-to-digital converter of an HP-1000 A-900 minicomputer (Hewlett-Packard) at sampling rates between 10 and 20 kHz and stored on the 132 MByte disc drive. The small capacitance currents that remained after analog capacitance compensation were subtracted from the records by the computer and then open and closed times were evaluated under user control.

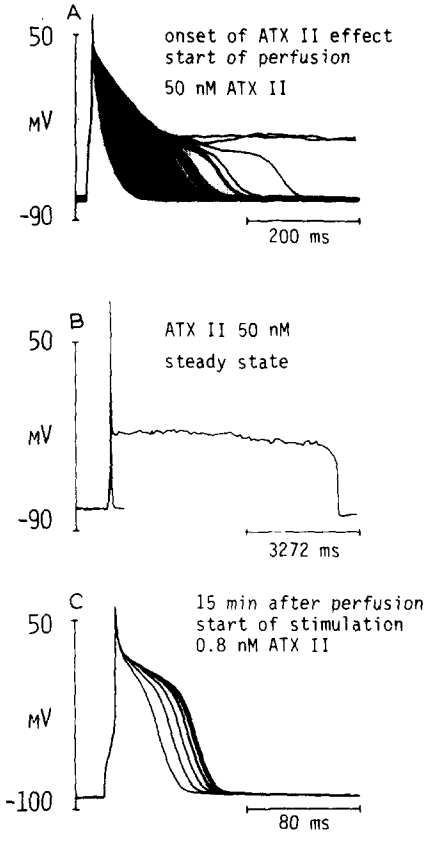

Fig. 1. (A) The time-dependent development of the ATX-II effect on the action potential of an isolated rat ventricular cell under constant stimulation (1 Hz) at 50 nM ATX-II. (B) The effect of 50 nM ATX-II on the identical myocyte as in Fig. 1A under steady-state conditions (3 min after onset of perfusion). An action potential before perfusion with ATX-II is also plotted (note the different time scales in Figs. 1A, 1B and 1C). (C) A rat ventricular cardiomyocyte was superfused with ATX-II-containing solution (0.8 nM) for a time far exceeding the duration necessary to reach equilibration of the bath with toxin solution (15 min). The potential was kept constant at resting membrane potential, without stimulation. After 15 min presence of the toxin the stimulation was started (1 Hz). During the first 4 or 5 action potentials elicited, the duration increased gradually step by step. Shown is the first action potential following 15 min rest and superimposed are the ensuing excitations.

Results

Action potential measurements

Under the given experimental conditions the cells showed a resting membrane potential of -89 ± 4 mV ($n = 15$) and action potentials could be evoked by injecting depolarizing current pulses. Upon perfusion with 50 nM ATX-II and at a stimulus rate of 1 Hz, within 1 to 2 min, a pronounced effect on the plateau phase of the action potential developed (Fig. 1A) that reached steady-state within 3 min, after the onset of perfusion (Fig. 1B). During toxin action additional hyperpolarizing current had to be injected into the cell to keep the resting membrane potential constant. In the equilibrium state of toxin action the stimulus rate of 1 Hz had to be decreased in order to get repolarization and action potentials with a duration of up to 1 min could be observed.

In one experiment the cell-bath was perfused with ATX-II containing solution (0.8 nM) and after solution exchange the cell was kept for 15 min in the ATX-II containing solution at resting membrane potential (RP) without evoking action potentials. Then the cell was stimulated at 1 Hz and the first action potentials evoked are shown in Fig. 1C. The plateau-phase increased markedly after every stimulation.

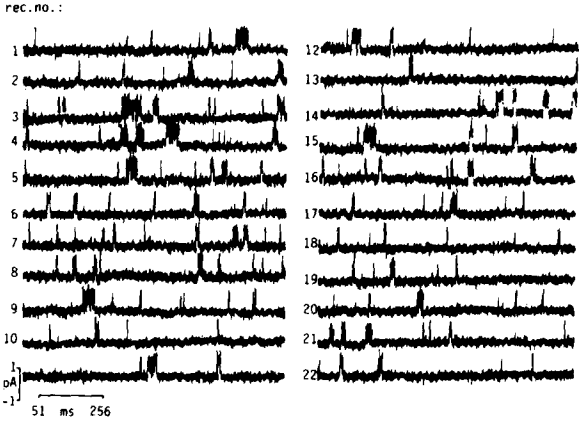

Fig. 2. Steady-state single-channel currents induced by ATX-II at resting membrane potential (RP) + 30 mV; cell-attached recording, pipette solution contained 200 nM ATX-II; digitally low-pass filtered at 1.5 kHz. Rapid flickering of the channel between an open and a closed state is interrupted by longer silent periods.

Single-channel recordings

ATX-II modified sodium channels were recorded in ten different experiments and all of them gave similar results. Under steady-state conditions (membrane potential kept constant for several minutes) at moderate depolarizations non-inactivating single-channel currents could be observed (Fig. 2) whereas at higher depolarizations single-channel currents inactivated. To elucidate further this voltage-dependent behaviour and for

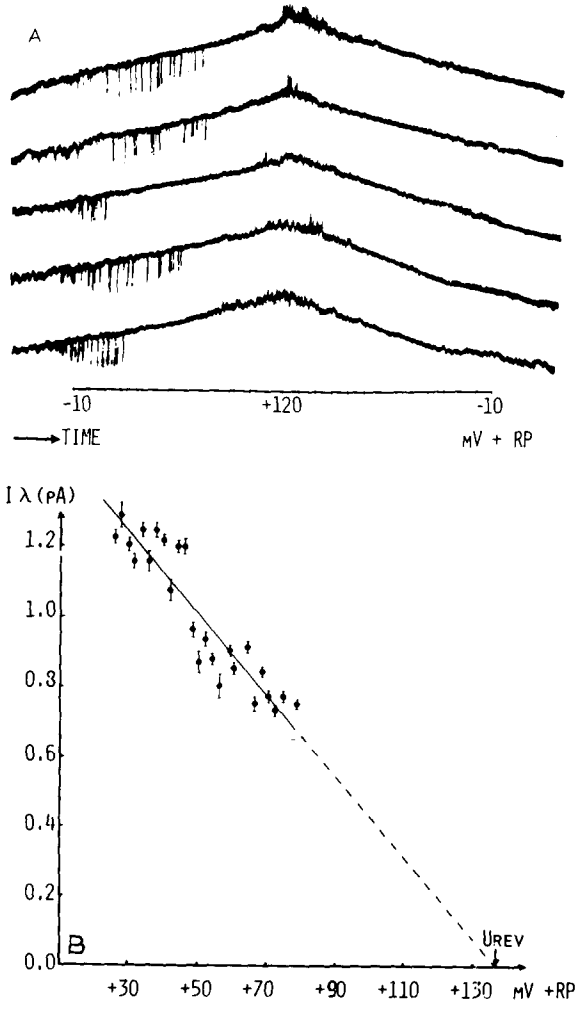

Fig. 3. (A) Single, ATX-II-induced, channel openings as a result of triangular waves of voltage across a cell-attached membrane patch. Pipette solution contained 200 nM ATX-II; analog low-pass filtering at 1 kHz. (B) Single-channel current (I_λ) plotted against patch potential. 200 nM ATX-II, cell-attached membrane potential. Bars indicate standard deviations of mean values. $U_{Rev} = 138$ mV + RP.

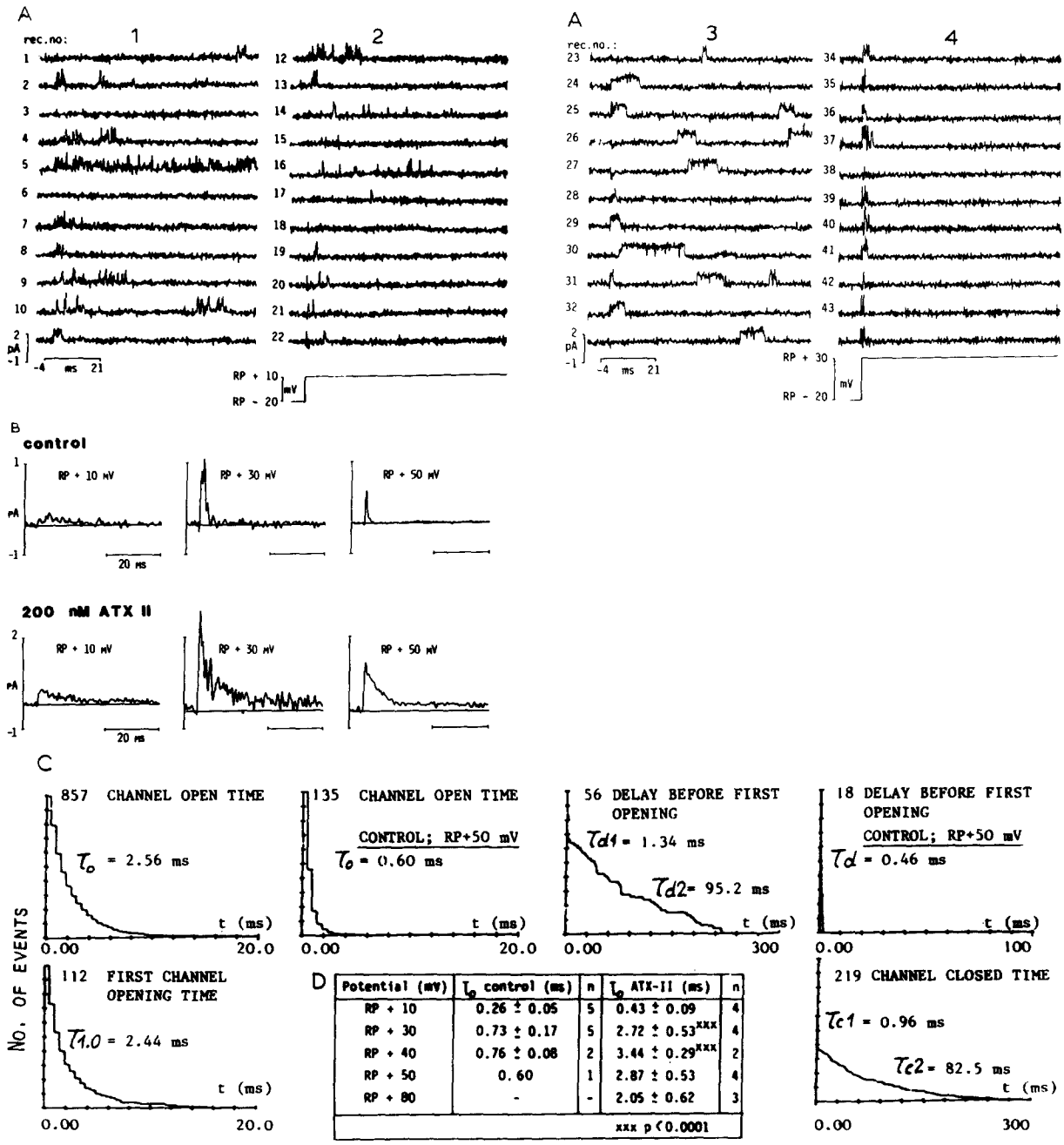

Fig. 4. (A) Activation of ATX-II-induced single-channel openings, following suprathreshold voltage pulses (columns 1 and 3), compared to control sodium-channel recordings (columns 2 and 4). Cell-attached membrane patches; 200 nM ATX-II; analog low-pass filtering at 3 kHz. RP = +10 mV (columns 1 and 2); RP = +30 mV (columns 3 and 4). (B) Averaged single-channel traces from original data such as shown in Fig. 4A. Lower row: cell-attached membrane patch, 200 nM ATX-II; number or records for calculation of average: 135, 159, 114 (from left to right). Upper row: cell-attached membrane patch, control recordings; number of records for calculation of average: 46, 22, 27 (from left to right). (C) Open time distributions (τ_o), delay time distributions (τ_{d1}, τ_{d2}) and closed time distributions (τ_{c1}, τ_{c2}) for ATX-II-induced single-channel events and under control conditions (200 mM ATX-II; RP = +50 mW). (D) (table) Average durations of open state (τ_o) for ATX-II-modified sodium channels and for control sodium channels at different potentials. Mean values ± S.D.; n, number of experiments; *** mean values differ with a statistical error probability of less than 0.1%.

the estimation of the reversal potential of single-channel current, we applied slow triangular waves of potential (70 s peak to peak duration; from values 50 mV more negative than the resting membrane potential (RP = −50 mV) to RP = +120 mV and back. As can be seen from Fig. 3A, frequent channel openings and closings occur when the wave is starting from negative potentials and the first channel opening in such a record could be seen at RP = −2.4 mV ± 2.4 mV in average (mean ± S.D., $n = 11$), the last at RP = +51.7 mV ± 9.5 mV ($n = 10$). Once the single-channel activity had disappeared and the potential was stepping back from RP = +120 mV to RP = −50 mV we were never able to detect single-channel activity (Fig. 3A). In membrane patches not treated with the toxin, sodium channel openings are almost undetectable using this ramp protocol. From those experiments, with ATX-II, the single-channel current was measured and plotted vs. membrane potential (Fig. 3B). The reversal potential of single-channel currents was estimated to be RP = +138 mV, which is close to the Nernst potential for sodium ions. The slope conductance of the ATX-II-induced sodium channels was estimated to be 11.5 pS.

The dynamic aspects of channel gating were explored by using the following experimental protocol: The membrane potential was stepped to RP = −20 mV for 500 ms and then depolarizing steps with a duration of 500 ms were applied to the membrane patch in order to activate sodium channels.

Original registrations from such experiments compared to recordings, where ATX-II was not present in the pipette solution at two different clamp-potentials are shown in Fig. 4A. The macroscopic sodium current can be reconstructed by an averaging these single-channel traces and it can be seen from Fig. 4B that first, macroscopic activation of the ATX-II modified sodium current greatly resembles that of normal sodium current but secondly, inactivation is delayed and not complete, i.e. a small sodium inward current persists after the initial decay, that is slowed down considerably by the action of the toxin. The decay of open times was analyzed and showed a monoexponential decline for all potentials that have been tested (Fig. 4C) and the time constant for channel closing was prolonged highly significantly ($P < 0.0001$) at intermediate depolarizing potentials (table, Fig. 4D).

To test whether the observed fast onset of macroscopic sodium currents under toxin action shown in Fig. 4B is a result of unmodified channels present in the patch, the time constant for channel closing was estimated separately for the first observed channel opening after the voltage jump ('fast channels') and for all subsequent channel openings. As can be seen from Fig. 4C the time constants for channel closing were equal in both cases within experimental accuracy.

In the original registrations (refer to Fig. 4A) it could be seen, that the toxin-modified sodium channels opened very rapidly after the onset of the voltage jump in one part of the recordings, whereas in the other it took considerable time for them to activate.

A measure for the time constant for activation of the single sodium channel was obtained from the delay after the onset of the voltage pulse to the first channel opening. The analysis of this delay time proved, that two time constants are involved in activation of ATX-II-modified sodium channels (Fig. 4C). A fast one, that resembles the time constant of activation for unmodified sodium channels, and a slow time constant, that is induced by the toxin. The results were qualitatively identical for all performed experiments, but quantitatively differed by a factor of about 4 for the different experiments.

In order to investigate whether the observed, complete inactivation of ATX-II-induced single-channel currents at higher depolarizations (refer to Fig. 3A) is due to gating properties of the toxin-bound channel or to dissociation of the toxin-channel complex, the following experimental protocol was used: In cell-attached membrane patches (200 nM ATX-II in pipette-solution), the membrane potential was first stepped to RP = +115 mV for 30 s in order to inactivate ATX-II-induced channels.

Then the potential was changed back to −25 mV negative to RP for 500 ms and sodium channels were activated by suprathreshold pulses to RP = +30 mV (duration: 500 ms). This sequence of hyperpolarizing and suprathreshold pulses was repeated for 1 min and then the potential was

stepped again to RP = +115 mV for 30 s to start the next cycle (see Fig. 5A for voltage-jump protocol).

The corresponding single-channel records were corrected for capacitance and leakage current and first were divided by simple inspection by eye into records containing ATX-II induced channels and records containing unmodified sodium-channel openings. 18 complete cycles were recorded and only in one cycle were we able to observe ATX-II-induced channels during the first suprathreshold pulse after the 30 s depolarization pulse. During four such cycles ATX-II-induced channels did not appear, but unmodified sodium channel openings could be observed throughout the cycle. The residual 12 cycles had the appearance of the original single-channel recordings shown in Fig. 5A, i.e. unmodified sodium channel openings were present in the recordings until ATX-II-modified channels appeared and then unmodified sodium channels disappeared virtually. The recordings were analyzed for single-channel open time (Fig. 5B) and the result was again, that open time was markedly prolonged under the action of the toxin and that open-time distributions relaxed in a mono exponential manner.

It had been shown that ATX-II-induced channels exhibit frequent reopenings upon depolarization, whereas unmodified sodium channels do not. Therefore, the open time distribution for the first channel openings in the given records were analyzed. Here modified and unmodified channels should contribute equally to this time distribution (Fig. 5B). This open-time distribution was almost identical with the open-time distribution of all ATX-II-induced channels and only one time constant could be observed. The development of ATX-II modification of single sodium channels was measured, and the result is shown in Fig. 5C. This was done by counting the relative frequencies of the event that still unmodified Na^+-channels

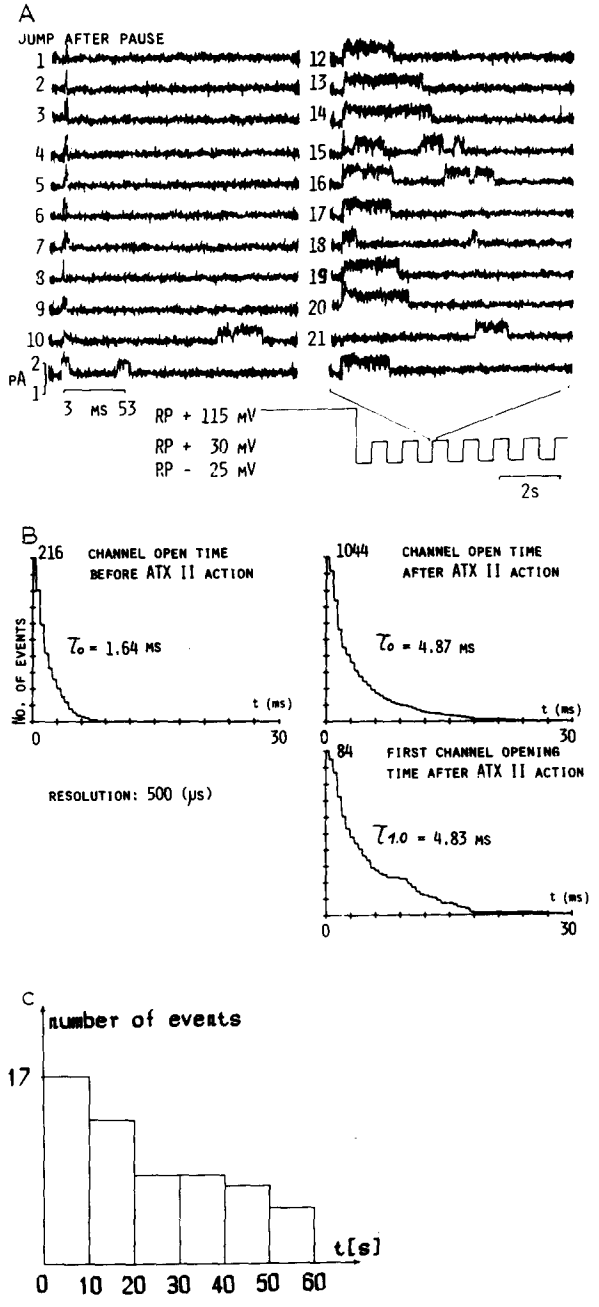

Fig. 5. (A) reactivation of ATX-II-induced channel openings following complete inactivation at higher depolarizing potentials for 30 s. Cell-attached membrane patch, 50 nM ATX-II; RP = +50 mV; digitally filtered at 2 kHz. (The stimulation protocol is shown at the lower right part). (B) Open time distributions (τ_o) for single channel openings before onset of ATX-II modification (upper, left), after onset of ATX-II modification (upper, right) and distribution of open time for the first channel opening observed after onset of the voltage pulse (after onset of ATX-II modification; $\tau_{1.0}$). (C) Time course for the onset of ATX-II modification of single sodium channel currents. The relative frequency of the event that, (given that no ATX-II-modified channels could be observed in the first suprathreshold voltage pulse after complete inactivation) no ATX-II-modified single-channel openings could be detected after the indicated time interval.

were present after increasing time intervals. ATX-II-modified channels (recognized by rapid flickering and/or prolonged open states) appear with increasing probability in correlation to the duration of the sequence of hyperpolarizing and suprathreshold pulses (see Fig. 5A, inset).

Discussion

The results presented in this study confirm previous studies and lead us to novel conclusions upon ATX-II action on the cardiac sodium channel in the following aspects.

Effects on the action potential

In this study it has been shown, that ATX-II prolongs markedly the duration of action potentials in isolated rat ventricular cells. This is in good accordance with findings of other groups on nerve [9,13,15,23], on skeletal muscle [14,16] and on cardiac muscle [17,24], that have been obtained from both whole tissue and single cells.

The experiment in Fig. 1C shows, that excitation of rat ventricular cells is necessary to reach the full effect of ATX-II action. These data indicate that the open sodium-channel conformation has a greater affinity for ATX-II than the closed conformation, that predominates at the normal resting potential of these cells. Romey et al. [9] have shown for crayfish axons, that TTX, which blocks opening of the sodium channel, thus stabilizing the closed conformation, prevents sodium channels from being modified by ATX-II. From their results, however, allosteric interactions between the TTX and ATX-II-binding site could not be excluded.

On the other hand, Beress et al. [25] found on guinea-pig papillary muscle, that increasing stimulus frequency decreased the action potential duration under ATX-II action. From their data they suggested, that opening of the sodium channel is not required for ATX-II binding. The duration of the plateau phase in cardiac muscle, however, is thought to be the result of net inward and outward currents, and extracellular increase of potassium and incomplete inactivation of the time-dependent outward current i_{x1} [26] could as well be responsible for this frequency-dependent effect.

Ion-selectivity of the ATX-II induced channels

Direct evidence for the action of ATX-II on the fast sodium system has been obtained so far by measurement of sodium uptake [4,10,24] or by direct voltage-clamp analysis [11,15,17–19]. Our analysis, on the single cardiac sodium channel level, has demonstrated, that ATX-II-induced channel currents reverse at the sodium Nernst equilibrium potential (see Fig. 3B).

When applying triangular waves of potential to a cell-attached membrane patch (Fig. 3A) at potentials around the cell's resting membrane potential, ATX-II-induced channel openings could be detected, that had a smaller unitary conductivity than channel openings at higher depolarizations, that were plotted in Fig. 3B. This could be due to the limited time resolution of our experimental setup, (open times are very short at these potentials, refer to Fig. 4D (table)) but also correspond to sodium channel openings with reduced conductivity, as have been observed by Cachelin et al. [27] in cultured cardiac cells. As channel substates are not within the scope of this study, this effect has not been investigated further.

Activation of ATX-II modified sodium channels

From averaging single-channel currents resulting from depolarizing voltage jumps and thus reconstructing macroscopic sodium currents (Fig. 4B), it can be seen, that activation of the fast sodium current is not significantly affected by ATX-II action. This is supplementing other macroscopic studies on several preparations [9,11,15, 17]. When analyzing individual single-channel records, however, and taking the time-delay from onset of the voltage jump to the first channel opening as a measure for activation of ATX-II-induced channels, a fast and a slow time constant appear (Fig. 4C). Two possibilities exist to account for this behaviour: (i) ATX-II slows down the activation process of sodium channels, but sodium channels in a given patch are not modified by the toxin all the time and the fast activation process observed is the result of unmodified channels. (ii) Under the action of the toxin two populations of closed sodium channels, available for opening, exist, one that activates very fast, comparable to normal activation, and a second with delayed activation. To test whether this biphasic process is

due to unmodified channels in the patch, the time constant for inactivation was measured separately for the first channel opening after onset of depolarization and for all subsequent channel openings (Fig. 4C).

This time constant is markedly prolonged for toxin-modified channels (see below) and proved to be identical for the first channel opening and for all subsequent channel openings. Therefore we conclude, that in the given experiment, sodium channels were modified by the toxin all the time and that under toxin action two pathways for activation exist. This result is in accordance with the study of gating currents by Neumcke et al. [18] on frog myelinated nerve, who found, that the total amount of fast, 'on'-charge displacement is reduced under ATX-II action. Additionally this group found that the time constant for fast 'on'-charge displacement is slightly accelerated by the toxin. This supports our conclusion drawn from action potential measurements (Fig. 1C), that the affinity of the sodium channel for ATX-II is greatest for the open state. In our study comparison of delay-time constants with and without ATX-II present in the extracellular solution is possible only in qualitative terms, as this time constant depends on the number of channels present in a given patch, this number can vary from patch to patch and is not known.

Inactivation of ATX-II modified sodium channels

Several possibilities exist to account for the prolonged sodium inward currents into several cell and tissue preparations under toxin action observed with $^{22}Na^{+}$-uptake studies and voltage-clamp experiments. (i) Single channel open time can be prolonged under toxin action, (ii) previously silent channels can be activated, or (iii) sodium channels can reopen several times under ATX-II action.

Our study shows, that both inactivation of the cardiac sodium channel is slowed down highly significantly under the action of the toxin and the channel is able to reopen several times (Fig. 2, Figs. 4A and 4C). Sodium channel reopenings are grouped into bursts that are interrupted by longer silent periods, representing a second non conducting state of the channel with slow activation. When channel closed times are analysed in steady-state recordings (Fig. 4C) these two time constants can be quantitated and reflect at least qualitatively the time constants for activation. This indicates that the $c^* \leftrightarrow i^*$ interconversion is not, or only slightly voltage dependent.

Complete inactivation of ATX-II modified sodium channels upon depolarization

When triangular waves of voltage are applied to a given membrane patch containing ATX-II-modified sodium channels (Fig. 3A), complete inactivation can be observed at higher depolarizations that cannot be reversed at moderate depolarizations. Only after the membrane patch has been at hyperpolarizing potentials, that are usually required to reactivate unmodificated sodium channels, channel opening can be detected again. Two possibilities exist to account for this behaviour: (i) The drug dissociates from the channel at higher depolarization or (ii) ATX-II-modified channels inactivate completely upon higher depolarizations and reactivate again only at hyperpolarizing potentials.

Conflicting results about ATX-II binding to depolarized membranes have been obtained so far from different preparations. In rat muscle cell cultures Lawrence and Catterall [7] found that depolarization affects binding of radiolabelled ATX-II and Strichartz and Wang [19] demonstrated with voltage-clamp experiments on amphibian myelinated nerve, that ATX-II looses its toxicity for sodium channels at higher depolarizations. Vincent et al. [6] found, that binding of radiolabelled ATX-II to synaptic nerve endings was not potential dependent and Isenberg and Ravens [17] conclude from their voltage-clamp experiments on isolated guinea pig ventricular cells, that ATX-II remains bound to the cardiac sodium channel even after complete inactivation of ATX-II-induced sodium currents. Barhanin et al. [28] demonstrated with chemically modified ATX-II, that binding of the toxin to the channel and influence on channel gating are governed by two separate processes.

In order to elucidate further this phenomenon on the single-channel level, we used a pulse protocol involving complete inactivation of ATX-II-induced single-channel currents following repetitive stimulation of sodium channels (Fig. 5A). In this

experiment it has been shown, that following complete inactivation first, quasi unmodified sodium channels appear during the suprathreshold pulses, that are substituted by ATX-II-modified channel openings with a relatively slow time course (Fig. 5C). The open time of the first channel opening after onset of the depolarizing voltage pulse was analyzed for current traces that showed ATX-II-modified channel openings, as the ATX-II-modified and normal sodium channels should contribute equally to this probability function if activation is not or only slightly effected (Fig. 5B). This function showed a single-exponential decay due only to channels affected by the toxin. Additionally, we never were able to observe overlapping sodium channel openings at the begin of the depolarizing voltage pulse, that would indicate modified and unmodified channels present simultaneously in the patch (see Fig. 5A). From these findings we conclude, that after complete inactivation at higher depolarizing potentials the sodium channels are functionally in a normal state, that is converted into the toxin-modified state again after return to hyperpolarizing potentials. We must note, however, that due to experimental limitations of the patch-clamp technique, we cannot exclude the possibility that the toxic effect of ATX-II on sodium channels is released at higher depolarizations without dissociation of the drug.

Our findings are in contrast with those from Isenberg and Ravens (1984) obtained with voltage-clamp experiments on guinea pig ventricular cells. A possible explanation could be, that these authors have not been able to measure sodium currents within the first 5 ms after onset of the voltage-jump and therefore missed unmodified sodium currents.

Kinetic scheme of ATX-II action on cardiac sodium channels

Taking together the findings of this study we suggest the following scheme of ATX-II action on cardiac sodium channels (Fig. 6): (i) ATX-II binds preferentially to the open state of the sodium channel. (ii) Quasi-normal macroscopic activation of sodium currents under toxin action is due to the transition of the channel-population beeing in the c*-state to the o*-state. The voltage dependence of this transition is not markedly changed.

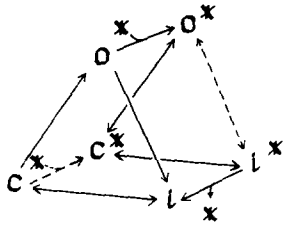

Fig. 6. Proposed model for action of ATX-II on the cardiac sodium channel. States designated with (*) indicate toxin bound states.

(iii) Delayed inactivation of macroscopic sodium currents under toxin action is due to prolonged open time of the o*-state and to repetitive interconversions between the c*- and o*-state and to a final transition from the c*- to the i*-state. (iv) Macroscopic persisting sodium inward currents, following prolonged depolarization, under toxin action are due to channel transitions from the i*- to the c*-state and show markedly delayed activation after the onset of the suprathreshold voltage pulse. (v) The toxin dissociates at higher depolarizing potentials from the channel (i*-state) that is converted thereby to its normal inactivated (i)-state.

The mode of action of another type of sodium-channel gating modifying drug has been analyzed recently by us [29] on the single-channel level. We hope that studies of this kind will contribute to an understanding of the molecular function of the cardiac sodium channel and its modification by natural toxins and synthetic drugs.

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