Supersensitivity to tetrodotoxin and lignocaine of sea anemone toxin II-treated sodium channel in guinea-pig ventricular muscle

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- 1 Sea anemone toxin II (ATX II, 20–30 nM) doubled the action potential duration in guinea-pig papillary muscles without affecting the maximum rate of rise of the action potential ($\dot{V}_{\max}$) and the resting potential.
- 2 Tetrodotoxin and lignocaine shortened the prolonged action potential in the ATX II-treated papillary muscles in concentrations (30 nM – 3 μ M) at which these drugs did not suppress the $\dot{V}_{\max}$.
- 3 Whole-cell voltage-clamp experiments with single ventricular cells showed that ATX II produced a slowly decaying inward sodium current following a transient sodium current upon depolarization.
- 4 The ATX II-induced slowly decaying current was reduced by tetrodotoxin or lignocaine in concentrations (300 nM–1 μ M for tetrodotoxin, 3–10 μ M for lignocaine) at which these drugs failed to affect the $\dot{V}_{\max}$ in cells not treated with ATX II.
- 5 These results suggest that sodium channel modification by ATX II not only changes its kinetics but also increases the susceptibility of the channel to block by tetrodotoxin and lignocaine.

Keywords: Na channel; supersensitivity; sea anemone toxin II; tetrodotoxin; lignocaine; guinea-pig ventricular muscle

Introduction

Several neurotoxins and local anaesthetics bind with high affinity and specificity to different sites of voltage-sensitive sodium channels and modify their properties. At present, six different receptor sites for toxins and one site for local anaesthetics are recognized (Narahashi, 1974; Hondeghem & Katzung, 1977; Catterall, 1977; 1980; 1984; 1988; Catterall & Beress, 1978; Coraboeuf *et al.*, 1979; Jaimovitch *et al.*, 1982; Barchi, 1988; Trimmer & Agnew, 1989).

Sea anemone toxin II (ATX II) is a polypeptide toxin of 4935 molecular weight isolated from *Anemonia sulcata* (Alsen, 1983). This toxin, like other polypeptide toxins (α -scorpion toxins, anthopleurin A), binds to receptor site 3 among the six distinct sites mentioned above, resulting in a slowing or block of the inactivation process of sodium channels (Romey *et al.*, 1976; Isenberg & Ravens, 1984; Wu & Narahashi, 1988). ATX II has also been reported to enhance the binding and action of lipid-soluble toxins such as batrachotoxin acting at receptor site 2 (Catterall, 1984; Sheldon *et al.*, 1986). More recently, the sensitivity to tetrodotoxin (a site 1 toxin) and lignocaine (a local anaesthetic) of axonal sodium channels has been demonstrated to be enhanced by another site 3 toxin, anthopleurin A (Muramatsu *et al.*, 1990). The evidence suggests that site 3 toxins can interact with other toxins which bind to different receptor sites. The purpose of the present study is to examine the possible interaction of ATX II with tetrodotoxin or lignocaine in the cardiac sodium channels.

Methods

Recording of action potential in guinea-pig papillary muscle

Male guinea-pigs weighing 250–350 g were anaesthetized with sodium pentobarbitone (30 mg kg⁻¹, i.v.) and the hearts were quickly isolated. The papillary muscles were dissected from the ventricles. The preparation was mounted in a tissue bath and perfused continuously with normal external solution of the following composition (mM): NaCl 136.9, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.0, NaHCO₃ 23.8, NaH₂PO₄ 0.21 and glucose 5.6. The bathing solution was gassed with 95% O₂ and 5%

CO₂ at 30°C. Square wave pulses of 1 ms duration were applied every 10 s. The membrane potential and the maximum rate of depolarization ($\dot{V}_{\max}$) of action potential were recorded by a conventional microelectrode technique (Muramatsu *et al.*, 1987), digitized using a PCM recording system (RP-880, NF Circuit Design Block, Japan) and stored on videocassette (NV 730, National, Japan) for later analysis. The membrane potential was simultaneously recorded on a strip chart recorder (RJG-4122, Nihon Kohden, Japan).

Preparation of single ventricular cells

Guinea-pig heart was dissected and mounted on the Langendorff apparatus to perfuse the coronary artery with oxygenated and pre-warmed (37°C) normal external solution of the following composition (mM): NaCl 136.9, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.5, glucose 10 and HEPES 5. The pH of the external solution was adjusted to 7.3 with NaOH. After the heart had been cleared of blood, the perfusate was changed to Ca-free external solution containing 0.4 mg ml⁻¹ collagenase (Type 1, Sigma, St. Louis, U.S.A.) and the solution was recirculated by use of a peristaltic pump for 25 min. Thereafter, the collagenase was washed by 100 ml of high K-low Cl solution (storage solution). The composition of the storage solution was (mM): taurine 10, oxalic acid 14, glutamic acid 80, KCl 25, KH₂PO₄ 10, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulphonic acid (HEPES) 10, glucose 11, ethylene glycol bis (β -amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA) 0.5, pH was adjusted to 7.2 by adding KOH (Isenberg & Klöckner 1982). The preparation was stored in this solution at 4°C for the experiments.

Whole-cell clamp experiments

A piece of tissue was dissected from the ventricle mentioned above and the cells were dispersed in the experimental chamber (0.5 ml in volume) filled with the storage solution. After the cells had settled on the floor of the chamber, the perfusion with normal external solution was started at a rate of 2–3 ml min⁻¹. The temperature of the perfusate in the experimental chamber was maintained at 33°C. Patch electrodes were made from glass capillaries of 1.5 mm o.d. with a double step microelectrode puller (PP-83, Narishige, Japan). After the tip had been polished by heat, the electrode was filled with internal solution of following composition (mM): potassium aspartate 100, KCl 20, MgCl₂ 2.5, HEPES 5, EGTA 5, adenosine-5'-triphosphate 5 and phosphocreatine 5.

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The pH was adjusted to 7.3 with KOH. Outer diameter of the electrode tip was 2–5 μm and the electrode resistance ranged between 1 and 3 M Ω . Patch clamp amplifier (S-3666, Nihon Kohden) was used to record the whole-cell membrane currents. After the electrode tip was touched to the cell surface, a negative pressure of 20–40 cmH₂O was applied to the interior of the electrode. Within a few seconds, a giga-ohm seal was established between the electrode tip and the cell surface. Then the membrane patch at the tip of the electrode was ruptured by applying further suction of –50 to –100 cmH₂O. Rupture was indicated by a large increase in the capacitive current. The current signals and membrane potentials were displayed on an oscilloscope (VC-10, Nihon Kohden) and stored by the same recording system as that used in the action potential experiments.

Drugs used were sea anemone toxin II (Ferring, Kiel, Germany), tetrodotoxin (Sankyo, Tokyo, Japan) and lignocaine (Sigma).

Experimental values are given as mean $\pm$ s.e.mean.

Results

Effects of tetrodotoxin and lignocaine on membrane potential in the presence or absence of sea anemone toxin (ATX II)

Guinea-pig papillary muscle had a resting potential of -84 ± 1 mV in amplitude and produced action potentials of 359 ± 15 ms in duration at 90% repolarization (APD₉₀; $n = 12$). ATX II (30 nM) gradually prolonged the action potential (Figure 1b left). The effect reached its maximum within 30 min and was observed for more than 30 min. ATX II at concentrations of 20–30 nM doubled the action potential duration (approximately 700 ms at APD₉₀). At such concentrations, ATX II failed to affect the maximum rate of rise of action potential ($\dot{V}_{\text{max}}$) and the resting potential. At higher concentrations (100 nM and higher), ATX II prolonged the action potential remarkably (more than 1 s) but variably (data not shown); therefore, constant prolongation could not be elicited by electrical stimulation at intervals of 10 s. ATX II at 20–30 nM was used in the subsequent experiments.

The prolonged action potential under treatment with ATX II was shortened by the addition of 100 nM tetrodotoxin (Figure 1b middle). However, in muscles not treated with ATX

II, tetrodotoxin at 100 nM had no effect on the action potential duration, and a higher concentration of tetrodotoxin (10 μM) was required to suppress the action potential duration (Figure 1a). $\dot{V}_{\text{max}}$ was also suppressed by high concentrations of tetrodotoxin (such as 10 μM) both in the ATX II-treated and -untreated muscles.

Figure 2 shows the effects of lignocaine on the ATX II-untreated and -treated papillary muscles. Lignocaine at 100 nM shortened the action potentials prolonged by ATX II (Figure 2b middle). In the muscles not treated with ATX II, however, application of 100 nM lignocaine had no effect on the action potential duration (Figure 2a middle). Lignocaine at 10 μM also shortened the action potentials of ATX II-treated muscles more potently than those of ATX II-untreated muscles. (Figure 2a right and 2b right). $\dot{V}_{\text{max}}$ was suppressed

Figure 2 Effects of lignocaine on action potential and dV/dt in the absence (a) or presence (b) of 30 nM sea anemone toxin (ATX II). Experimental conditions are the same as those in Figure 1.

Figure 1 Effects of tetrodotoxin on action potential and rate of rise of action potential (dV/dt) in guinea-pig isolated papillary muscles in the absence (a) or presence (b) of 30 nM sea anemone toxin II (ATX II). Records (a) and (b) were obtained from two different cells. The muscles were stimulated every 10 s. Microelectrode experiments at 30°C.

by lignocaine at a high concentration ($10\mu\text{M}$) and the effect was not affected by ATX II treatment (Figure 2a right and 2b right).

Concentration-inhibition curves for tetrodotoxin and lignocaine on the action potential duration and $\dot{V}_{\text{max}}$ in the ATX II-treated papillary muscles are shown in Figure 3. Relative decreases in prolongation of APD_{90} and in $\dot{V}_{\text{max}}$ were plotted against the concentrations of tetrodotoxin or lignocaine. As shown in the insets of Figure 3, the difference in the APD_{90} before and after application of each drug in the ATX II-treated muscles was measured and its ratio to the prolonged portion of APD_{90} by ATX II was expressed as a percentage (a% in the inset of Figure 3). Inhibition of $\dot{V}_{\text{max}}$ by treatment with tetrodotoxin or lignocaine was expressed as a percentage of the control $\dot{V}_{\text{max}}$ which was measured in the presence of ATX II before treatment with each drug (b% in the inset of Figure 3). Tetrodotoxin and lignocaine shortened the action potential duration in the ATX II-treated papillary muscles in concentrations at which these drugs did not suppress the $\dot{V}_{\text{max}}$ (30 nM – $1\mu\text{M}$). In contrast to the curves for $\dot{V}_{\text{max}}$, the concentration-inhibition curves for action potential duration have a plateau phase ranging from 30 to 300 nM tetrodotoxin or from 100 nM to $1\mu\text{M}$ lignocaine, suggesting a biphasic inhibition.

Effects of tetrodotoxin and lignocaine on sea anemone toxin (ATX II)-modified ionic currents

In the presence of 2 mM CoCl_2 , sodium inward currents of the single ventricular cells were produced by depolarizing steps to 0 mV from the resting potential (-80 mV) at intervals of 10 s . Figure 4a shows representative recordings before and after ATX II application. Before treatment with ATX II, only a transient inward sodium current flowed. Within 2 min after perfusion with 30 nM ATX II, the transient sodium current was followed by a slowly decaying inward current which was not present in the control (Figure 4a). The slow component developed its maximum within 5 min after the application of ATX II and was observed constantly for more than 30 min .

The slow component developed under treatment with ATX II was inhibited either by tetrodotoxin or lignocaine in concentrations at which these drugs failed to affect the $\dot{V}_{\text{max}}$ in cells not treated with ATX II. Figure 4b and c show the representative results where 300 nM tetrodotoxin or $3\mu\text{M}$ lignocaine was added after treatment with 30 nM ATX II for 10 min . Both the transient and slow components were markedly reduced or not observed after application of tetrodotoxin at high concentrations ($10\mu\text{M}$ and higher, data not shown).

Figure 3 Concentration-inhibition curves for tetrodotoxin (a) and lignocaine (b) of action potential duration and maximal rate of rise of the action potential ($\dot{V}_{\text{max}}$) in the sea anemone toxin (ATX II)-treated papillary muscle. Percentage decreases in prolongation of action potential duration at 90% repolarization (APD_{90} , ●) and in $\dot{V}_{\text{max}}$ (○) were plotted against the concentration of tetrodotoxin (a) or lignocaine (b). Concentration of ATX II at which it doubled action potential duration (20 – 30 nM) was used. Insets show how the percentage decrease in prolongation of action potential duration (a%) and in $\dot{V}_{\text{max}}$ (b%) were measured. Mean ($n = 3$ – 4) with s.e. (shown by vertical lines).

Figure 4 Effects of tetrodotoxin and lignocaine on the inward current of sea anemone toxin (ATX II)-treated guinea-pig single ventricular cells isolated with collagenase. Whole-cell voltage-clamp recordings at 33°C . Membrane was held at -80 mV (resting potential) and test pulses to 0 mV for 1 s were delivered every 10 s . Perfusate contained 2 mM CoCl_2 . (a) The cell was treated with ATX II for 10 min . (b) Sodium inward currents before and after application of 300 nM tetrodotoxin in the presence of ATX II are superimposed. (c) Sodium inward currents before and after application of $3\mu\text{M}$ lignocaine in the presence of ATX II are superimposed. Concentration of ATX II at which ATX II doubled action potential duration (20 – 30 nM) was used. The holding current at -80 mV was zero.

Figure 5 Effects of tetrodotoxin and lignocaine on sea anemone toxin (ATX II)-modified sodium current at different points of time during 1 s test pulses in ATX II-treated single ventricular cells. Inset shows how the percentage amplitude of inward sodium current (c%) was calculated. The current amplitude was measured from the recordings of the experiments in Figure 4. Mean ($n = 3-4$) with s.e. shown by vertical lines. (a) Tetrodotoxin: (●) 300 nM; (○) 1 μ M. (b) Lignocaine: (●) 3 μ M; (○) 10 μ M.

The amplitudes of the slowly decaying current before and after application of tetrodotoxin or lignocaine were compared as shown in the inset of Figure 5 and the ratios (c% in Figure 5) were plotted against the time after onset of clamp pulse. Tetrodotoxin suppressed ATX II-modified inward current to the same extent at all the time points measured during the depolarizing test pulse, whereas the inhibition by lignocaine progressively increased during the depolarization.

Discussion

ATX II (20–30 nM) prolonged the action potential duration of guinea-pig papillary muscle without affecting the $\dot{V}_{\max}$ and resting potential. Under whole-cell voltage-clamp conditions with single guinea-pig ventricular cells, ATX II at such concentrations produced a slowly decaying inward current, which was absent in cells not treated with ATX II. These results are consistent with the previous reports that ATX II prolonged action potentials by inhibiting the sodium channel inactivation in crayfish giant axon (Romey *et al.*, 1976; Low *et al.*, 1979), myelinated nerve fibres (Bergman *et al.*, 1976; Romey *et al.*, 1976) and guinea-pig ventricular cells (Honerjäger, 1982; Isenberg & Ravens, 1984).

The prolonged action potential was shortened by the addition of either tetrodotoxin or lignocaine in concentrations at which they failed to affect action potential duration and $\dot{V}_{\max}$ in muscles not treated with ATX II. These phenomena were consistent with the results obtained from voltage-clamp experiments, in which slowly decaying inward current devel-

oped during treatment with ATX II was inhibited by extremely low concentrations of tetrodotoxin (300 nM) or lignocaine (3 μ M) i.e. at such low concentrations, tetrodotoxin or lignocaine selectively inhibited the prolonged part of the action potential and the slowly decaying inward sodium current. It has been recognized that there are two sodium channel subtypes (Barchi, 1988; Trimmer & Agnew, 1989): one population of channels is blocked by nanomolar concentrations of tetrodotoxin (e.g. neural sodium channels), whereas the other is resistant or not very sensitive to tetrodotoxin (e.g. mammalian cardiac sodium channels). Recently, single channel measurements in cultured muscle have separated two populations of sodium channels having distinct biophysical properties that might correspond to these tetrodotoxin-sensitive and tetrodotoxin-resistant channel subtypes (Weiss & Horn, 1986).

The sensitivity of sodium channels to tetrodotoxin can also be altered according to the state of innervation. In skeletal muscle, sodium channels in neonatal muscle are resistant to tetrodotoxin, but high affinity binding develops after innervation (Pappone, 1980; Frelin *et al.*, 1983). Denervation of mature skeletal muscle induces the appearance of tetrodotoxin-resistant sodium channels (Pappone, 1980; Rogart, 1985). Sodium channels expressed in *Xenopus* oocytes from mRNA isolated from cardiac cells show only high sensitivity (Sutton *et al.*, 1988).

Taking these findings and our present results together, two possible explanations can be considered for changes in sensitivity of cardiac sodium channels to tetrodotoxin and lignocaine after treatment with ATX II: (1) ATX II induces a new class of sodium channels with high affinity for tetrodotoxin and lignocaine; (2) ATX II modifies the existing sodium channels resulting in a change of sensitivity to tetrodotoxin and lignocaine. Some marine toxins have been reported to induce or create new classes of channels (Dubois *et al.*, 1983; Kobayashi *et al.*, 1987; Muramatsu *et al.*, 1988; Ikeda *et al.*, 1988). Selective inhibition of the prolonged part of the action potential and of the slowly decaying inward current by tetrodotoxin and lignocaine may favour the former possibility. However, $\dot{V}_{\max}$ and the resting potential were not affected by ATX II. Voltage-dependency of the slowly decaying inward current is much the same as that of the normal sodium inward current (Isenberg & Ravens, 1984). Therefore, it seems that ATX II does not induce new channels with activation kinetics different from that of the existing sodium channels. Rather, it is more likely that ATX II inhibits the inactivation process of existing sodium channels and that only the modified sodium channels become more sensitive to tetrodotoxin and lignocaine. Biphasic curves of concentration-inhibition relationships for tetrodotoxin and lignocaine may reflect the complexity of the modification of sodium channels by ATX II.

Recently, Muramatsu *et al.* (1990) reported that tetrodotoxin-sensitive sodium channels of crayfish giant axon become more sensitive to tetrodotoxin and lignocaine after treatment with anthopleurin A, another site 3 toxin. This evidence also supports the second possibility that susceptibility of sodium channels to tetrodotoxin or lignocaine is enhanced after modification with site 3 toxins such as ATX II and anthopleurin A, regardless of whether they are neuronal (tetrodotoxin-sensitive) or cardiac (tetrodotoxin-low sensitive) sodium channels. Site 3 toxins have been reported to enhance the binding and actions of lipid-soluble toxins acting at the receptor site 2 in neuroblastoma cells (Catterall, 1980) and cardiac myocytes (Sheldon *et al.*, 1986).

Lignocaine and other local anaesthetics are known to block sodium channels differently according to the state of the channels; i.e. resting, open and inactivated states (Hille, 1977; Hondeghem & Katzung, 1977; Matsubara *et al.*, 1987). Open channel block by lignocaine may be more likely to occur under conditions where the inactivation of sodium channels is inhibited by ATX II. As shown in Figures 4 and 5, a progressive increase in the lignocaine-induced inhibition of slowly decaying inward current during the depolarizing pulse may

represent such an additional block (open channel block) in contrast to the case of tetrodotoxin. However, detailed analysis is required in further experiments using single channel recordings.

Neurotoxins and local anaesthetics have been demonstrated to bind to several different sites in the sodium channel and to produce different electrophysiological effects. The present

study further suggests the existence of close and complex interactions between the binding sites.

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