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# TRYPSIN-INDUCED MASKING OF TETRODOTOXIN RECEPTOR OF THE SODIUM CHANNELS IN MOLLUSC NEURONES

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## Summary

At the early stage of trypsin treatment of mollusc neurones tetrodotoxin cannot block the $Na^+$ current. In the course of further exposure of neurones to trypsin, tetrodotoxin-sensitivity is restored completely, so its temporal loss results from shielding rather than destruction of the tetrodotoxin-binding site. Pronase and papain do not affect the tetrodotoxin action on the $Na^+$ current.

Tetrodotoxin selectively blocks the voltage-dependent $Na^+$ current while interacting with a specific binding site — the tetrodotoxin receptor of the $Na^+$ channel [1,2]. Tetrodotoxin-sensitivity of the $Na^+$ current differs in various species and even in the same cells depending on their functional state, e.g., it decreases in denervated muscle and increases in the course of differentiation of muscle, cardiac and nerve cells [3]. Modification of the tetrodotoxin receptor structure is currently considered to be the cause of the differences in tetrodotoxin-sensitivity [4,5], although the mechanism responsible for these differences remains unclear. Recently, tetrodotoxin-insensitivity was experimentally provoked in snail neurones by treatment with trypsin, it was accounted for by destruction of the tetrodotoxin receptor [6]. We propose another explanation for natural and enzymatically induced changes in tetrodotoxin-sensitivity: they may result from the influence of channel environment, i.e., they may be due to shielding or conformation of unaltered tetrodotoxin receptor. The present study shows that proteolytic treatment of mollusc neurones can induce only temporal and reversible loss in tetrodotoxin-sensitivity; this fact rules out destruction or modification of tetro-

dotoxin receptor [6] and supports the supposition of environmental influence on the properties of the $Na^+$ channel.

We studied the effects of various types of proteolytic treatment on tetrodotoxin-sensitivity of the $Na^+$ current in neurones of a fresh water mollusc, *Lymnaea stagnalis*. In the experiments with the whole ganglia we used conventional microelectrode techniques. The neurones were penetrated with glass KCl-filled electrodes (resistance 10—30 MΩ) for recording and stimulation. Action potentials were differentiated electronically in order to obtain the maximal rate ($\dot{V}_{max}$) of the action potential upstroke. Membrane potential was held at —60 to —80 mV by means of continuous polarization using a recording electrode. Direct studies of the $Na^+$ currents were carried out in dialyzed neurones under voltage clamp [7] with the use of electrophysiological equipment 'Axon-I' (manufactured by Special Construction Bureau for Biological Instrumentation of the U.S.S.R. Academy of Sciences) [8].

The bathing solution contained: 100 mM NaCl, 1.6 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5) [9]. The solution used for intracellular perfusion was: 120 mM KCl, 10 mM Tris-HCl (pH 7.5). To separate the $Na^+$ current, we blocked the $Ca^{2+}$ and $K^+$ currents of action potential; the $Ca^{2+}$ current was blocked by substituting $F^-$ for $Cl^-$ in the internal solution and, sometimes, by adding $Cd^{2+}$ into the external one [10]; the $K^+$ current was blocked by replacement of $K^+$ with $Cs^+$ in both solutions [10]. In this case only the fast inward current was elicited (Fig. 1).

Neurones were treated with trypsin (Reanal), pronase (Merck) or papain

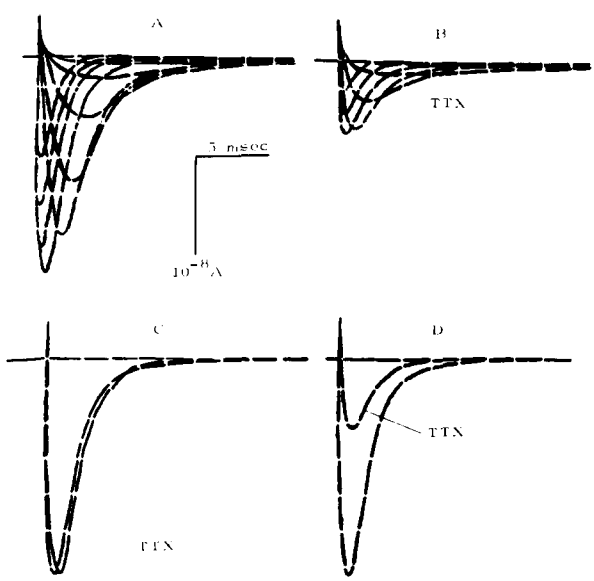


**Fig. 1. Dependence of tetrodotoxin (TTX)-sensitivity of the $Na^+$ current on the type of proteolytic treatment for isolated dialyzed neurones. The $Na^+$ current was elicited by depolarizing steps of 5 or 10 mV from the holding membrane potential level, —60 mV. The $Ca^{2+}$ and $K^+$ currents were blocked, the leakage current was subtracted automatically. (A) The set of the $Na^+$ current curves for the neurone isolated after intensive pronase treatment (0.35%, 40 min, 20°C). (B) The set of the $Na^+$ current curves for the same neurone (as in A) in the presence of $2 \cdot 10^{-5}$ M tetrodotoxin. (C) Maximal $Na^+$ current insensitive to tetrodotoxin ($8 \cdot 10^{-5}$ M) for another neurone, isolated after mild trypsin treatment (1%, 8 h, 0—2°C). (D) Restoration of tetrodotoxin-sensitivity after additional exposure of the same neurone (as in C) to pronase (0.35%, 15 min, 20°C).**

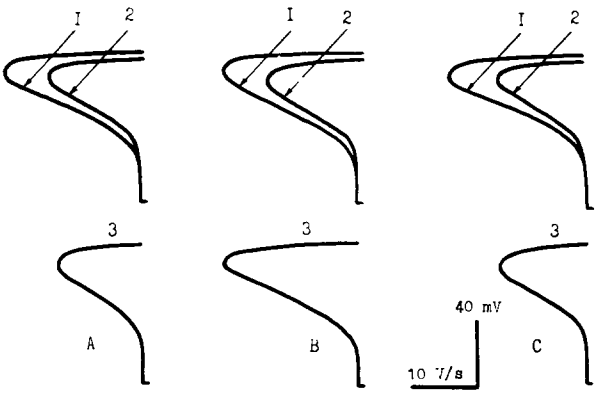


Fig. 2. Effects of tetrodotoxin and replacement of $Na^+$ in the bathing solution on the rate of rise of action potential for neurones in ganglia. Phase-plane display of the rate of rise of action potential: rate as a function of instantaneous level of generated potential. Abscissa: the rate of rise of action potential, $\dot{V}$; ordinate: the level of membrane potential in the course of generation of action potential. The highest point of each curve corresponds to the action potential peak, the lowest point to the undershoot level. Each curve is the mean of 10 recordings of action potentials induced by a sequence of 10 depolarizing voltage pulses. The rising phase of action potential only is represented. Estimations were performed according to the levels of $\dot{V}_{max}$. Initial resting potential of a given neurone was −62 mV. Throughout the experiment the neurone was hyperpolarized up to 70 mV by continuous current polarization. The amplitude of action potential varied little and was about 85 mV. (A) An intact neurone. (B) The same neurone (as in A) after mild exposure to trypsin (0.35%, 15 min, 18°C). (C) The same neurone (as in B) after additional treatment of ganglia with pronase (0.35%, 15 min, 18°C). 1, normal bath solution with 100 mM $Na^+$; 2, 50 mM $Na^+$ plus 50 mM $Tris^+$; 3, 100 mM $Na^+$ plus $1.3 \cdot 10^{-5}$ M tetrodotoxin.

(Merck) either in the course of preparation of isolated cells [11] or during the experiment; two modes of enzyme treatment were used: intensive and mild. In the case of intensive treatment, ganglia were incubated with 0.35% trypsin or 1% papain for 1 h at 37°C or with 0.35% pronase for 45 min at room temperature. These were the routine conditions for isolation of the cells excepting the case when the neurones were isolated with mild enzymatic treatment. The conditions for mild trypsin treatment (1% trypsin, 8 h, 0—2°C) were found empirically. When ganglia were treated in the course of an experiment with 0.35% trypsin at room temperature (18—20°C) it was also mild treatment. In both cases mild treatment led to the loss in tetrodotoxin-sensitivity of the neurones as opposed to intensive treatment.

Measurements carried out on isolated neurones showed that substitution of half of the $Na^+$ by $Tris^+$ leads to a 2-fold decrease of the $Na^+$ current. Complete substitution of $Na^+$ by $Tris^+$ caused full disappearance of the $Na^+$ current. This indicates that enzyme treatment has no effect on selectivity of the $Na^+$ channel for other ions in the bathing solution. $\dot{V}_{max}$ of action potentials of neurones in ganglia determined as the sum of $Na^+$ and $Ca^{2+}$ currents was only 20—30 V/s. $\dot{V}_{max}$ fell linearly with the decrease in the bulk concentration of $Na^+$ from 100 to 5 mM. When the external $Na^+$ concentration was halved by replacement with $Tris^+$ or sucrose, $\dot{V}_{max}$ decreased by 15—40% (Fig. 2); thus, in the medium containing 100 mM NaCl and 4 mM $CaCl_2$, 30—80% of the inward current is transferred by $Na^+$.

Tetrodotoxin (Sankyo) reduced the $Na^+$ current in all neurones isolated after intensive treatment with any enzyme ($n$ = 50) (Fig. 1). A 2-fold decrease of the $Na^+$ current occurred at $1 \cdot 10^{-5}$ M tetrodotoxin. The tetrodotoxin

effect developed for 1—2 min and was completely reversible. Tetrodotoxin up to $1 \cdot 10^{-4}$ M had no effect on the time course, voltage-dependence of the $Na^+$ current and its dependence on external $Na^+$ concentration, and decreased only the maximal $Na^+$ conductance. Neither changes in the $K^+$ and $Ca^{2+}$ currents nor marked increase in the leakage current were detected, so the tetrodotoxin effect could be considered to be specific as opposed to that described in Ref. 12. This discrepancy is, possibly, due to the fact that the authors [12] estimated the efficiency of the tetrodotoxin effect according to its influence on the total transmembrane current without separating it into its constituents —$Na^+$, $Ca^{2+}$ and $K^+$ currents. Tetrodotoxin-sensitivity of intact neurones did not differ from that of isolated ones treated with enzymes. Fig. 2 shows that identical reduction of $\dot{V}_{max}$ occurs at $1 \cdot 10^{-5}$ M tetrodotoxin and at half substitution of external $Na^+$. Thus, intensive treatment with any of three proteolytic enzymes does not affect the tetrodotoxin-sensitivity of the $Na^+$ current in mollusc neurones. So, the possibility of destruction of the tetrodotoxin-binding site can be excluded.

However, in the case of mild trypsin treatment it was possible to obtain the cells with tetrodotoxin-resistant $Na^+$ current (Figs. 1 and 2). The magnitude, kinetics, potential dependence and dependence on external $Na^+$ concentration were similar to those of the tetrodotoxin-sensitive $Na^+$ currents. The state of tetrodotoxin-insensitivity could be stable if trypsin treatment were stopped by washing the neurones with fresh solution without enzyme. But, if after mild trypsin treatment the neurones were additionally treated with pronase or papain, their tetrodotoxin-sensitivity restored completely (Figs. 1 and 2). Lee et al. [6] did not use trypsin for longer than 4 min, therefore, only the tetrodotoxin-resistant state was observed.

It is not necessary to change the enzyme used in order to restore tetrodotoxin-sensitivity. A transient characteristic of the loss of the tetrodotoxin-sensitivity was clearly demonstrated in the experiments recording the tetrodotoxin-dependence of the $Na^+$ current in the course of incubation of ganglia with trypsin at room temperature. During first 15—30 min tetrodotoxin reduced $\dot{V}_{max}$, over the next 15—30 min $\dot{V}_{max}$ was tetrodotoxin-resistant, then tetrodotoxin-sensitivity was restored gradually up to the initial level. Mild trypsin treatment itself was not responsible for tetrodotoxin-resistance: additional exposure of sensitive isolated neurones with trypsin (10—15 min) did not affect the tetrodotoxin-sensitivity of the $Na^+$ currents. We did not observe the state of the tetrodotoxin-insensitivity in the neurones treated with pronase or papain.

The fact that the loss of tetrodotoxin-sensitivity due to mild trypsin treatment is completely reversible, indicates that this cannot be the result of tetrodotoxin receptor destruction. This coincides with the supposition on the environmental influence on the $Na^+$ channel function. In fact, no parameter of the $Na^+$ current changes under the action of extracellular proteolytic enzymes. Consequently, the $Na^+$ channel, as an integral membrane protein, has no functional part exposed onto the surface of the neurone membrane and, thus, accessible to bath-applied enzymes. For other objects, it is known that on the inner surface of the axonal membrane there is an inactivation particle of the $Na^+$ channel accessible to pronase digestion [13] but tetrodotoxin re-

ceptor, as an intrinsic membrane protein, can be destroyed by enzymes only when the channel complex is solubilized [14] even at lower enzyme concentrations than we used. However, external membrane proteins can be subjected to degradation with extracellular proteases. Cleavage of some external proteins (their function is unknown) situated in the vicinity of the $Na^+$ channel due to mild trypsin treatment can result in shielding of the tetrodotoxin receptor. More intensive treatment leads to removal of interfering fragments. Pronase and papain induce cleavage of proteins at other points (see, for example, Refs. 15—17) so they either do not provoke shielding or abolish it faster than trypsin does.

It seems very likely that external proteins hinder interaction of tetrodotoxin with its receptor not only under experimental conditions but in natural states as well, e.g., in the course of denervational changes in muscles. If this is the case, solubilization of the channel complex or some proteolytic treatment would increase tetrodotoxin binding to the receptor.

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