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Periodate treatment reduces the tetrodotoxin-sensitivity of voltage-gated Na^+ channels

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(1) Voltage-clamped nerve fibres of the frog *Rana esculenta* were treated with periodate in the extracellular solution. (2) Periodate treatment irreversibly reduced the effect of tetrodotoxin (TTX) on the Na^+ currents. (3) The effect of saxitoxin (STX) was also reduced but less than that of TTX. (4) The presence of STX during the application of periodate to the nerve fibre almost completely prevented the effect of the chemical reagent on the TTX sensitivity of the Na^+ channels. (5) The reduction of the TTX effect is not due to the reaction of small amounts of periodate with the diol group of this toxin, because the effect was seen after prolonged washing with reagent-free Ringer solution with or without high amounts of ribose. (6) Carboxyl groups present in the Na^+ channel seem to be quite important for the binding of TTX and STX. Periodate modifies several amino acid side chains, however, it does not attack carboxyl groups in a peptide chain. Thus, these results suggest that periodate modifies a further group critically involved in the binding of TTX and STX.

Introduction

Tetrodotoxin (TTX) and saxitoxin (STX) are highly specific and potent blockers of voltage-gated sodium channels in nerve and muscle. Both toxins compete for the same receptor site. Tritiated TTX and STX have been used for counting Na^+ channels in various preparations and as tools for the isolation of the channel protein [1–3]. Little is known about the structure of the TTX/STX-binding site [4,5]. Carboxyl groups, however, seem to be quite important. Different chemical reagents known to react covalently with carboxyl groups, reduce the binding of TTX [6,7]. Action potentials and Na^+ currents have been recorded from chemically modified fibres in the presence of relatively high TTX or STX concentrations [8–10]. Sodium currents remain almost unaffected by treatment with the chemical reagent periodate, except that the steady-state inactivation curve, $h_\infty(E)$, is strongly shifted to more negative values of membrane potential [11]. In this study, a marked reduction of the TTX effect on the Na^+ currents by periodate treatment is described. The effect of STX is also reduced, however, this reduction is quantitatively different from that of TTX. In contrast to the other reagents that reduce the effect of TTX [6–10], periodate is not able to attack carboxyl groups in a peptide chain [12].

Abbreviations: STX, saxitoxin; TTX, tetrodotoxin; Mes, morpholineethanesulfonic acid; Mops, morpholinepropanesulfonic acid; TEA, tetraethylammonium chloride.

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Methods

Single myelinated nerve fibres were dissected from the sciatic nerve of the frog *Rana esculenta* [13]. A node of Ranvier was voltage clamped at 12°C by the method of Nonner [14]. The fibre was cut on both sides of the node at a distance of about 0.75 mm. The ends of the fibre were in 113 mM CsCl, 7 mM NaCl. The fibres were held at $E = -100$ mV. All potentials are given as absolute potentials. The command voltage pulses were generated by a 12 bit D/A converter under computer control. Membrane currents were filtered by a 10 kHz lowpass filter and sampled in 10- or 100-μs intervals by a 12 bit A/D converter [15]. Absolute currents were calculated assuming a longitudinal axoplasmic resistance of 10 MΩ, corresponding to a value of 140 MΩ/cm for the unit length of a 14 μm frog nerve fibre [16]. To correct for capacitative and leakage currents, the current produced by a -30 mV pulse was scaled appropriately and subtracted from the currents produced by the depolarizing pulses. Three up to 40 records were averaged.

The preparation was superfused with Ringers solution continuously with or without the chemical reagent. The control Ringers solution contained 110 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 12 mM tetraethylammonium chloride (TEA) and 4 mM morpholinepropanesulfonic acid (Mops). The pH was adjusted to 7.2 with NaOH. After control, the fibre was treated for 8 min with a Ringers solution buffered at pH 5.5 with morpholineethanesulfonic acid (Mes) and containing 20 mM sodium periodate. Then, the preparation was washed with control Ringer for at least 6 min before measurements were continued. After periodate treatment in the presence of STX, the toxin was nearly completely washed out, using an acidic Ringer solution (buffered with 2,6-dimethylpyridine-3-sulfonic acid, pH 4.5) and control Ringer. Low pH accelerates the unbinding of STX (see Ref. 17). To facilitate comparison of the results described here with those of recent chemical modification studies on the same preparation, the experimental set up and the reaction conditions were very similar to those reported previously [11,18–20].

Materials

4-Morpholinepropanesulfonic acid (Mops) and 4-morpholineethanesulfonic acid (Mes) were from Serva, Heidelberg, F.R.G. Tetrodotoxin (TTX) was from Sankyo, Tokyo, Japan, saxitoxin (STX) from Calbiochem, Frankfurt, F.R.G. All other chemicals were analytical grade or in the purest form available and were purchased from Merck, Darmstadt, F.R.G.

Results and Discussion

Fig. 1 shows the basic finding. An 8 min treatment of the nerve fibre with a Ringers solution containing 20 mM periodate irreversibly reduced the Na^+ current to 83% of its original size. The current was then further reduced by increasing amounts of TTX. For a K_d value of TTX of 3.4 nM [21], a reduction of the Na^+ currents to about 53, 10.2 and 1.1% can be calculated for 3, 30 and 300 nM TTX, respectively (see Eqn. 1). However, on the periodate treated fibre, 3 nM TTX reduced the current to 55.8%, 30 nM TTX to 17.7% and 300 nM to 3.7% (Fig. 1). Na^+ currents were clearly

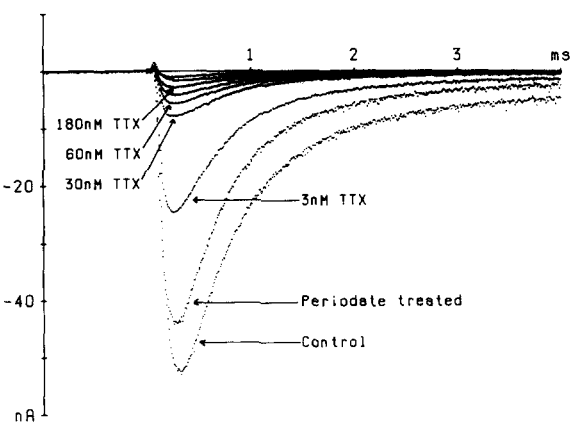

Fig. 1. Effect of TTX on the Na^+ current after periodate treatment. Na^+ currents associated with 4 ms depolarizing pulses from the holding potential (-100 mV) to a potential of 0 mV. Na^+ currents before and after periodate treatment and their further reduction by increasing amounts of TTX are shown. TTX concentrations (nM): 3, 30, 60, 90, 180, 300 and 600. Despite the presence of high amounts of TTX, Na^+ currents are clearly visible. K^+ currents blocked by internal Cs^+ and external TEA. Three up to 40 records averaged.

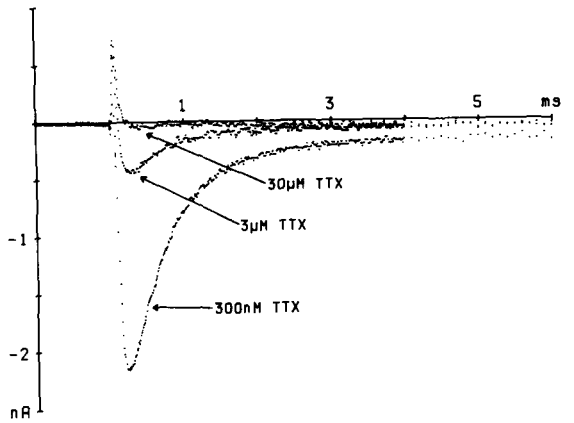

Fig. 2. Na^+ current of a periodate treated fibre in the presence of 300 nM, 3 μM and 30 μM TTX. Same treatment as in Fig. 1, but different fibre and higher TTX concentrations. The early transient outward current is the gating current. 30 records averaged.

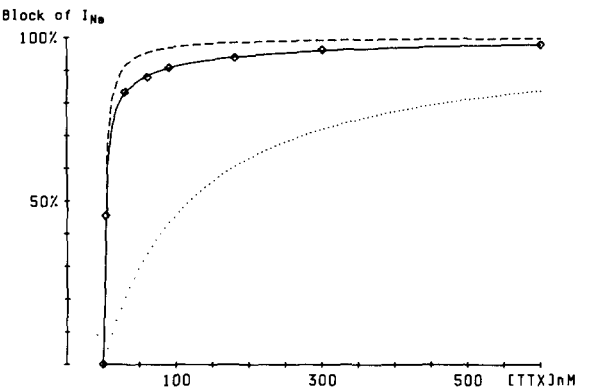

Fig. 3. Block of Na^+ current by various TTX concentrations. The fibre was treated with 20 mM periodate for 8 min. Same experiment as in Fig. 1. Points were fitted by Eqn. 2 with $A = 0.884$, $K_{d2} = 118$ nM, $K_{d1} = 2.9$ nM and $B_{max} = 100\%$ (uninterrupted curve). Simulations of the blocking effect of TTX with $A = 0$, $K_{d2} = 118$ nM (100% modification) and $A = 1$, $K_{d1} = 2.9$ nM (no modification) are illustrated by the dotted line and the interrupted curve, respectively.

visible in 3 μM TTX; however, they were almost completely abolished in the presence of 30 μM TTX (Fig. 2). The very small Na^+ current remaining after periodate treatment in 30 μM TTX is similar in size to the Na^+ currents seen in 30 or 35 μM TTX after treatment with the carboxyl group reactive reagents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) [22] or *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) [23]. There is, however, an important difference between the results obtained with periodate and those obtained with an oxonium salt. The carboxyl group reactive oxonium salt, but not periodate, converts a certain number of Na^+ channels (15%) into a form that is completely insensitive towards TTX (up to 90 μM) [9].

K_d values in nM for different TTX concentrations can be calculated from the equation

$$K_d = (B_{max} \cdot [T]/B) - [T] \quad (1)$$

where B_{max} is the maximum of the blocking effect, [T] the concentration of the toxin in nM and B is the percentage of block caused by [T]. For example, for 30 nM and 300 nM TTX (Fig. 1), K_d values of 6.5 and 11.5 nM are obtained. Obviously, on periodate-treated nerve fibres, the block of Na^+ channels by TTX cannot be described by a single K_d value.

The simplest explanation is, that only a fraction $(1-A)$ of Na^+ channels is modified during exposure of the nerve fibre to periodate, while the rest (A) is unaffected. In Fig. 3, the block of I_{Na} by various TTX concentrations was fitted by the equation

$$B = A \cdot (B_{max} \cdot [T]/[T] + K_{d1}) + (1 - A) \cdot (B_{max} \cdot [T]/[T] + K_{d2}) \quad (2)$$

where K_{d1} and K_{d2} are the K_d values for unmodified and modified Na^+ channels, respectively. The fit revealed that 11.6% of the Na^+ channels are modified ($A = 0.884$) and have a K_{d2} value of 118 nM. Furthermore, a K_{d1} value of 2.9 nM for TTX on unmodified Na^+ channels was obtained. In three experiments the values were: $A = 0.846 \pm 0.079$, $K_{d2} = 139 \pm 44$ nM and $K_{d1} = 3.5 \pm 0.6$ nM (mean $\pm$ S.D.). The latter value is in good agreement with that obtained on untreated nerve fibres [21]. The number of Na^+ channels with reduced TTX sensitivity might increase when the exposure time to periodate is prolonged. Unfortunately, it was not feasible to test this, because a substantial prolongation of the chemical modification procedure results in a sudden drastic increase of the leakage current.

TTX might be able to protect its binding site,

when present during the modification procedure. TTX, however, contains an α-diol, and this part of the molecule is easily cleaved by periodate. Therefore, a protection of the TTX-binding site by this toxin is not feasible. The presence of 2 μM STX during periodate treatment (see Methods) almost completely prevented the reduction of the TTX sensitivity of the sodium channels. This is strong indication that periodate directly modifies a part of the TTX/STX-receptor.

The reduction in TTX sensitivity resulting from periodate treatment is not removed by prolonged washing with Ringers solution with or without 20 mM of an α-diol containing substance like ribose. Thus, the reduction in TTX sensitivity is not caused by the reaction of small amounts of periodate with the α-diol part of the TTX molecule.

TTX and STX compete for the same receptor site [1]. Therefore, periodate treatment could be expected to reduce the effect of both toxins to the same extent. However, the nerves of e.g. the Puffer fish are highly resistant to TTX, but not to STX [24] and denervated mammalian skeletal muscle is more resistant to TTX than to STX [25]. The same is observed after treatment of nerve fibres with periodate. Fig. 4 compares Na^+ currents in the presence of 300, 600 and 3000 nM TTX or 140 and 280 nM STX. With K_d values of 3.4 nM for TTX and of 1.4 nM for STX on *Rana esculenta* [17,21] a block of the Na^+ current by 98.9% for 300 nM TTX and by 99.0% for 140 nM STX can be calculated; i.e. 300 nM TTX and 140 nM STX should be almost equally effective. On periodate-treated fibres, however, the Na^+ current remaining in 300 nM TTX was 5.4-fold larger than to be expected for this TTX concentration, but the Na^+ current remaining in 140 nM STX was only 2.9-fold larger. The Na^+ current remaining in 600 nM TTX was 33% larger than that in 140 nM STX (see Fig. 4). In four further experiments on different fibres, using various high concentrations (> 100 nM) of TTX and STX, this differential action of both toxins on periodate treated fibres was also observed. Thus, the chemical treatment reduced the effect of STX much less than that of TTX.

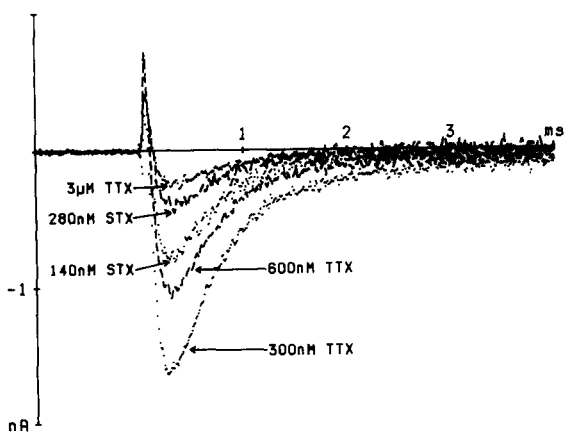

Fig. 4. Periodate treatment of a nerve fibre reduces the effect of TTX and STX differently. Na^+ current of a periodate-treated fibre in the presence of 300, 600 and 3000 nM TTX or 140 and 280 nM STX. Same treatment as in Fig. 1, but different fibre. 140 nM STX blocked 97.1% of the Na^+ current, while a TTX concentration as high as 600 nM TTX blocked only 96.1% of the Na^+ current. In toxin free Ringers solution, the peak Na^+ current was 27.8 nA. On two different untreated fibres, 1.4 nM STX (diluted from the same stock solution) blocked 59 and 51% of the Na^+ current, respectively. 30 records averaged.

The chemical nature of the part of the TTX/STX-binding site modified by periodate has not been elucidated. It is clear, however, that periodate does not react with carboxyl groups in a peptide chain [12]. Periodate covalently reacts with a number of amino acid side chains and other groups present in a biological membrane. Thiols, disulfides and the side chains of methionine, tyrosine, histidine, tryptophan as well as serine and threonine as N-terminal residues are possible targets [12]. Moreover, diols, present in the heavily glycosylated Na^+ channel protein [26], are easily cleaved by periodate. Further experiments must show which of these groups is essentially, but differently, involved in the binding of TTX and STX.

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