Action of α -dendrotoxin on K^+ currents in nerve terminal regions of axons in rat olfactory cortex

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1 In the rat olfactory cortex, unmyelinated axons give rise to synapses *en passant*. This tissue was used to study the pharmacology of axonal K^+ -currents. Responses were measured from a group of these axons as unclamped field currents, with a polarizable suction electrode.

2 A single stimulus to the axons elicited a tetrodotoxin-sensitive Na^+ -dependent transient. K^+ -currents were revealed by positive polarization of the suction electrode and were manifest as a negative current following the Na^+ -component.

3 In the presence of tetraethylammonium (TEA, 5 mM) and Cd^{2+} (100 μM), the K^+ -component was depressed by 3,4-diaminopyridine (3,4-DAP; 1 to 20 μM ; IC_{50} $2.0 \pm 0.4 \mu M$). α -Dendrotoxin (DTX; 15–1500 nM) also attenuated the aminopyridine-sensitive component (IC_{50} 93 ± 4 nM). At the highest DTX concentration, depression of the K^+ -current was incomplete, the residual K^+ current being reduced by 3,4-DAP (0.1 to 5 μM).

4 These results indicate the presence of two aminopyridine-sensitive K^+ currents in this preparation distinguished by their susceptibility to DTX.

Keywords: Dendrotoxin; aminopyridine; K^+ -current; axons; axonal currents; unmyelinated; rat olfactory cortex

Introduction

α -Dendrotoxin (DTX) is a basic protein with a molecular weight of around 7 kD derived from the venom of the snake, *Dendroaspis angusticeps* (Halliwell *et al.*, 1986). It inhibits the voltage-dependent aminopyridine-sensitive 'A' like K^+ -current in rodent hippocampal neurones (Dolly *et al.*, 1984; Halliwell *et al.*, 1986) but DTX is more effective on the slowly inactivating K^+ current found in ganglia (Penner *et al.*, 1986; Stansfeld *et al.*, 1986), frog motor nerve terminals (Weller *et al.*, 1985; Dreyer & Penner, 1987) and myelinated axons (Brau *et al.*, 1990).

DTX is convulsant (Mehraban *et al.*, 1986) and it increases transmitter release (Harvey & Karlsson, 1982; Tibbs *et al.*, 1989a) as a consequence of the K^+ -channel blockade. This idea is confirmed by the inhibition of ^{86}Rb efflux from synaptosomes (Benishin *et al.*, 1988). In the hippocampus, high densities of DTX binding sites are found in the synaptic regions (Halliwell *et al.*, 1986). Therefore, it would be useful to study electrophysiologically the action of DTX on central nerve terminals. Many synapses in the central nervous system are of the *en passant* type produced from unmyelinated axons and typical are those axons in the olfactory cortex. Direct intracellular recordings of membrane currents in central nerve terminals are not possible because of their very small size (about 0.2 μm in diameter in olfactory cortex; Gracey & Scholfield, 1990). However, field current recordings can give some idea of the various conductances present (Mallart, 1983). In the olfactory cortex, K^+ -currents can be revealed by current clamping using suction electrodes (Scholfield, 1990). Since voltage clamping is not possible, the information obtained is limited to pharmacological rather than kinetic characteristics. Notwithstanding this limitation, DTX has been shown to block partially an aminopyridine-sensitive K^+ -current; a preliminary report of this work has been published (McGivern & Scholfield, 1991).

Methods

Female Wistar rats of 200–250 g were decapitated and their brains removed. The brain was trimmed to produce a 3–5 mm thick slab incorporating the olfactory cortex. Pial surface slices of olfactory cortex were cut with a bow cutter and guide (Scholfield, 1980) to give a thickness of 150–200 μm . The slices were maintained in a reservoir of Krebs solution agitated by bubbling with 95% O_2 /5% CO_2 before experimentation. They were divided along the axonal radiations (Scholfield, 1987) by a clean cut made with a razor blade. Immediately before recording, another vertical cut was made at right angles to the axons. The slices were placed in a 0.3 ml recording bath through which Krebs solution flowed at 5 ml min^{-1} ; this solution was recirculated from a 20 ml reservoir. The recording pipette was filled with Krebs solution and connected to 10 cm of water vacuum. The tip diameter of the electrode was 30 μm and this was offered up to the freshly cut face of the slice so drawing in the severed ends of the axons. The electrode was connected to a current amplifier having a 10 k Ω input resistance. Voltage commands (400 ms) were applied to the non-inverting input of the amplifier so the electrode could be simultaneously polarized by ± 200 mV. These commands began 300 ms before the stimulus. Current waveforms were captured by analogue-to-digital conversion, displayed and stored on computer. Stimuli (20 V, 1 ms) were applied to the axons 2 mm away from the recording electrode. The action potential invaded the axons within the recording electrode to elicit the recorded currents.

Drug solutions were applied directly to the bathing solution superfusing the slice. To economize on the DTX, it was necessary to reduce the circulating volume of Krebs solution to 5 ml. With large volumes of solution, gassing the reservoir with 95% O_2 /5% CO_2 was adequate while for smaller reservoir volumes it was also necessary to pass the gas mixture over the surface of the recording bath. The Krebs solution had the following composition (mM): Na^+ 144, K^+ 5.0, Ca^{2+} 2.5, Mg^{2+} 1.3, Cl^- 128, HCO_3^- 25, D-glucose 11 and it was equilibrated with 95% O_2 /5% CO_2 . The DTX was purified from crude snake venom by a combination of conventional chromatography and high performance liquid chromatography (h.p.l.c.) cation-exchange (Black *et al.*, 1986).

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Results

A single stimulus delivered to the unmyelinated axons produced a positive transient (Figure 1) which was tetrodotoxin-sensitive (10–100 nM, McGivern & Scholfield, 1990). When the recording electrode was positively polarized, a negative current was also observed (Figure 1). Only the negative current showed a polarization dependency and it was blocked by aminopyridines (Scholfield, 1990). Since the electrode resistance and electrode potential remained constant, the recordings were of the current clamp type and as such they gave little information about the kinetics of the respective currents; thus their characterization was limited to the pharmacological properties. To avoid any confusion with voltage clamp records, these positive-negative currents are termed the Na⁺- and K⁺-components, respectively.

The negative current was depressed by the application of the 3,4-diaminopyridine (3,4-DAP), a more potent analogue of 4-aminopyridine (Kirsch & Narahashi, 1978; Berger *et al.*, 1989). The depression was dose-dependent in the concentration range of 1 to 20 μ M: the half-maximal effect (IC_{50}) was at $2.0 \pm 0.4 \mu$ M in 12 slices (Figure 3b). The above experiments were performed in the presence of tetraethylammonium (TEA) (5 mM) and Cd²⁺ (100 μ M) to block the K-currents which were resistant to aminopyridine. Unless otherwise noted all experiments below were done with TEA and Cd²⁺.

DTX attenuated the K⁺-component (Figures 1 and 2) in a manner similar to 3,4-DAP. The action of DTX was concentration-dependent over the range 15–1500 nM, with a IC_{50} of 93 ± 4 nM (Figure 3a). At lower concentrations, the depression was similar to that produced by 3,4-DAP but at the highest concentration, the effect was incomplete. Another series of experiments was conducted in the absence of TEA and Cd²⁺. DTX also produced a dose-dependent depression although the potency was less (IC_{50} 550 ± 60 nM) and the depression was also incomplete at the highest concentration.

With DTX, the amplitude of the Na⁺-component was slightly decreased in some preparations, as in Figure 1, whereas in other cases, the Na⁺-component was increased somewhat (Figure 3). These small effects on the Na⁺-component were also seen with 3,4-DAP (Figure 3).

3,4-DAP was tested in the presence of 300 nM DTX (TEA and Cd²⁺ were also included); under these conditions, 3,4-DAP (0.1–5.0 μ M) depressed the residual K-component in a concentration-dependent manner (Figure 3b). The shape of the dose-effect curve was similar to that seen without DTX but 3,4-DAP was slightly more potent (IC_{50} = 0.75–0.10 μ M in DTX compared to 2.0 ± 0.4 , $P = 0.006$ by unpaired Student's *t* test comparing effect of 3,4-DAP alone and in TEA/Cd²⁺). Neither the effect of DTX nor 3,4-DAP could be readily reversed by washing out (over a period of 60 min).

Figure 2 Time course for the action of α -dendrotoxin (DTX, 300 nM, added at the first arrow) followed by 3,4-diaminopyridine (3,4-DAP, 0.2, 0.5 and 1.0 μ M added at the points indicated). Throughout the experiment, the axons were electrically stimulated at 0.05 Hz. At each stimulus cycle, the electrode was polarized by +100 mV and the maximum amplitudes of the positive transient (labelled Na component) and negative transient measured (labelled K-component). Each point was joined to the next to produce a continuous time course. The dashed line indicates the recording base-line.

Although the potency of DTX was around 20 fold greater than that of 3,4-DAP, its IC_{50} in this preparation was lower than in some tissues and its onset of action was slow (Figure 2). Thus, the possibility exists that degradation by proteolytic enzymes in interstitial spaces, uptake, absorption or restricted penetration may reduce the effectiveness as it does with some drugs in this preparation (Brown & Scholfield, 1984). Therefore, a series of experiments was performed to investigate the possibility of binding and enzymatic breakdown. This was done by comparing the effect of 70 nM DTX in normal solution and in the presence of various test agents. Bovine serum albumin (0.02 mg ml⁻¹) was added to offset any non-specific binding. Brystatin (50 μ M), captopril (1 mM) or N-carboxymethyl-phe-leu (0.12 mM) for protease inhibition were applied 20 min before DTX and during toxin application. To reduce the hindrance of extracellular matrix to DTX diffusion, collagenase (type 1, 1 mg ml⁻¹), trypsin (type 3, 0.8 units ml⁻¹) and hyaluronidase (1 mg ml⁻¹) were applied for 30 min and then washed out for 30 min before the application of DTX. The enzyme concentrations were the highest that could be used without attenuation of the current responses. None of these treatments had any influence on the

Figure 1 Single responses recorded from the unmyelinated axons of the rat olfactory cortex. Throughout the experiment, the electrode was polarized by +100 mV to reveal the negative current component. In the first response, the slice was bathed in normal solution followed by 700 nM α -dendrotoxin (DTX) for 30 min and by DTX and 5 μ M 3,4-diaminopyridine (3,4-DAP) for 30 min. The arrows indicate the point of stimulus application.

Figure 3 Concentration-depression curves for 3,4-diaminopyridine (3,4-DAP) and α -dendrotoxin (DTX). For each plot, the Na component is the open symbols ($\square$, $\circ$) and K-component during +100 mV electrode polarization the solid symbols ($\blacksquare$, $\bullet$). (a) Slices were treated with increasing concentrations of DTX (15–1500 nM). (b) This shows two series of experiments where slices were either pretreated with 700 nM DTX for 30 min and with DTX still present, 0.1 to 5 μ M 3,4-DAP tested (circles) or tested with 3,4-DAP in the absence of DTX (squares). All experiments were conducted in the presence of tetraethylammonium (TEA, 5 mM) and Cd (100 μ M). The ordinate scale is the peak amplitude of the respective ionic current component expressed as a percentage of that in normal solution. Each point represents the mean (with s.e.mean) of 7–12 slices.

time-course or magnitude of DTX action. Although this list of substances cannot be considered exhaustive, it does suggest that the effect of DTX was not attenuated by the above influences.

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Discussion

The present results clearly show that DTX depresses the aminopyridine-sensitive K^+ -current in unmyelinated axons of the rat olfactory cortex. In some other preparations, DTX has been shown to affect more than one channel or binding sites. For *Xenopus* myelinated axons, it has an IC_{50} of 70 nM and 1.8 μ M (Braun *et al.*, 1990) whereas in nodose ganglion A cells it is much more potent (IC_{50} of 2 nM for slowly inactivating current, Penner *et al.*, 1986; Dolly *et al.*, 1987). For ^{86}Rb fluxes in synaptosomes, the IC_{50} for DTX is 1.7 to 20 nM (Benishin *et al.*, 1988; Tibbs *et al.*, 1989a,b) whilst inhibition of voltage-dependent K^+ currents in hippocampal neurones (Halliwell *et al.*, 1986) and axons (Schauf, 1987; Braun *et al.*, 1990) lies between 50 and <800 nM. In contrast, the K_D for binding is around 0.3 nM (Halliwell *et al.*, 1986; Bidard *et al.*, 1987). In cloned K^+ channels, DTX also has high potency (Stuhmer *et al.*, 1989; Kirsch *et al.*, 1991) although these K^+ channels are also sensitive to TEA (Schroter *et al.*, 1990; Kirsch *et al.*, 1991) in contrast to the present study. For the olfactory cortex, the IC_{50} was 93 nM which suggests that they are of the fast inactivating type seen in the hippocampus and in myelinated axons. There was no evidence for a second DTX-sensitive component in the olfactory cortex although very high concentrations were not tested.

We also considered that the potency might have been attenuated by restricted access of the extracellular space. Our experiments suggest that this was unlikely. Furthermore, 3,4-DAP appeared to be very potent in our experiments having an IC_{50} at 2 μ M. In other tissues somewhat higher 3,4-DAP concentrations of 4 μ M upwards are required: the neuromuscular junction (Hong & Chang, 1990), squid giant axon nerve terminal (Augustine, 1990), pituitary cells (Racke *et al.*, 1988; Rogawski, 1988), smooth muscle (Berger *et al.*, 1989). Thus, the observed IC_{50} for DTX appears to be a true reflection of K^+ channel potency.

The blockade of the K^+ -current by DTX was incomplete, the residual being blocked by 3,4-DAP. This suggests the presence of two aminopyridine-sensitive currents, as seen in some other tissues (see Introduction). The residual negative current after DTX was significantly more sensitive to 3,4-DAP than the DTX sensitive component which is opposite to the results with other preparations (see Dolly *et al.*, 1987). This suggests that the two 3,4-DAP binding sites are not identical. Thus DTX was selective for part of the aminopyridine-sensitive component and had a negligible effect on the Na component.

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