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ENHANCEMENT BY OXOTREMORINE OF ACETYLCHOLINE RELEASE FROM THE RAT PHRENIC NERVE

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1 Oxotremorine (10.5 μM) produced a paralytic effect on twitch responses of rat diaphragm *in vitro* to direct and indirect stimulation.

2 The paralytic effect of oxotremorine was absent when the diaphragm was stimulated directly in the presence of hemicholinium-3 (0.42 mM), at a time when twitch responses to indirect stimulation ceased completely.

3 Oxotremorine, at two different pharmacologically active doses, strikingly increased the resting as well as electrically evoked release of acetylcholine into the bathing fluid from the phrenic nerve-diaphragm preparation.

4 This presynaptic effect of oxotremorine may explain its pharmacological effects at the cholinergic synapses studied so far.

Introduction

The tremorogenic agent, oxotremorine (Oxo-T), blocks neuromuscular transmission at the rat phrenic nerve junction (Ganguly & Chaudhuri, 1970); this effect is inhibited by propranolol (Ganguly, 1976). A prejunctional action of Oxo-T seemed likely. The present experiments were designed to test this, by measuring the effect of Oxo-T on the resting as well as electrically evoked release of acetylcholine (ACh) in this tissue. In addition, the effects of Oxo-T on the pharmacologically denervated diaphragm were investigated to determine a cause-effect relationship.

Methods

Isolated phrenic nerve-diaphragm preparations were made from albino rats (150 to 200 g) of either sex, according to the method of Bülbring (1946). Muscle contractions were recorded with a force displacement transducer (Encardiorite). Direct stimulation of the diaphragm was carried out conventionally by attaching two thin platinum electrodes to the muscle.

Pharmacological denervation was achieved by the direct stimulation of the diaphragm in the presence of hemicholinium-3 (0.42 mM), at a time when the responses to indirect stimulation were completely abolished (Vedasiromoni & Ganguly, 1976). Since hemicholinium also impaired responses to direct stimulation under such conditions, presumably reflecting current spread to nerve terminals, a higher voltage was invariably needed to elicit a sizeable direct response in its presence.

Supramaximal square-wave pulses of 0.2 ms duration at a frequency of 0.2 Hz were used to stimulate the phrenic nerve.

Collection and bioassay of released acetylcholine

The diaphragm was suspended in a 4 ml organ bath containing Krebs solution (mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.9, $NaHCO_3$ 25, dextrose 11, KH_2PO_4 1.2 and $MgSO_4$ 1.2) plus physostigmine sulphate 8 μM.

The schedule of collection of samples was exactly according to the procedure of Bowman & Hemsworth (1965) except that only two samples were collected from a single diaphragm and that the samples were assayed immediately after collection on the dorsal muscle of the leech, suspended in a 2 ml bath containing physiological solution of the following composition (mM: NaCl 119.8, KCl 4.4, $CaCl_2$ 1.2, $NaHCO_3$ 1.4 and physostigmine sulphate 8 μM).

The effect of Oxo-T on resting (without nerve stimulation) release of ACh was determined by use of the same schedule of collection of samples. The tissue was kept in Krebs solution alone or in Krebs

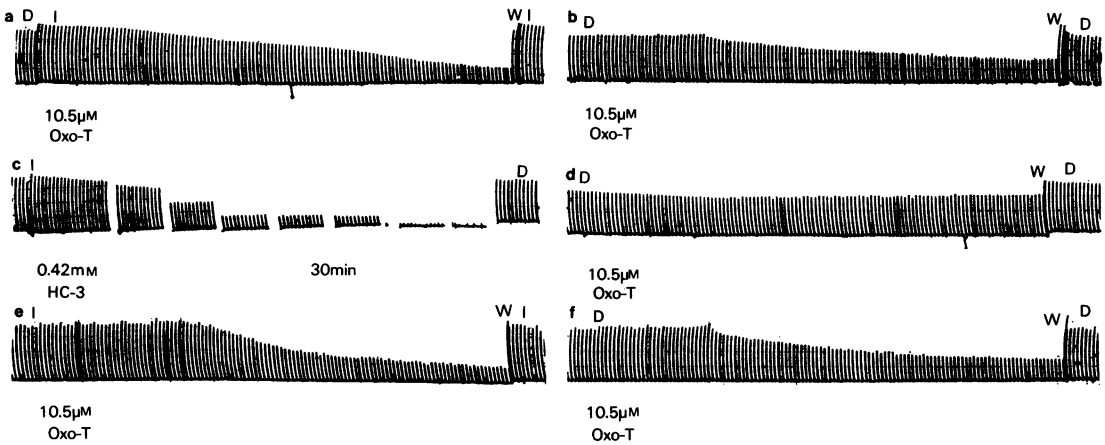

Figure 1 Twitch responses of a rat isolated diaphragm muscle to indirect (I, left-hand traces) and direct (D, right-hand traces) stimulation. Initially, oxotremorine reduced responses to both indirect and direct stimulation (a and b). After addition of hemicholinium (HC-3), which blocked indirect stimulation (c) oxotremorine had no effect on direct stimulation (d). Traces (e) and (f) show effects of oxotremorine obtained 90 min after washing out the hemicholinium.

solution with added Oxo-T (2.63 or 10.5 μM) for 15 min followed by collection of the bathing fluid and bioassay on dorsal muscle of leech.

A bracketing-cum-matching assay was employed for estimation of the released ACh. A cross-over design was employed for both collection and bioassay of the control and treated samples in all experiments. Those leech muscles which were not sensitive to standard ACh in the range of 0.03 to 0.125 ng/ml were discarded. Prior to assay all samples were diluted at least 10 times in leech solution to minimize any change in sensitivity due to difference in composition of the two physiological fluids.

The following drugs were used: oxotremorine sesquifumarate (Aldrich), physostigmine (eserine) sulphate (E. Merck), hemicholinium-3 (Aldrich) and acetylcholine chloride (E.Merck). Concentrations of drugs are expressed in terms of molarity of the salts.

Results

Pharmacologically denervated rat diaphragm

Oxo-T (10.5 μM) impaired twitch responses of the diaphragm to both 'direct' and 'indirect' stimulation. Effects on 'direct' stimulation were prevented by pretreating the preparation with hemicholinium, indicating that they arise from an action on the nerve terminals that had been excited by current spread (Figure 1). It is concluded that Oxo-T inhibits transmission without having any direct action on the muscle.

Resting and electrically evoked acetylcholine release

In the absence of Oxo-T (control) the total amount of ACh released in the bathing fluid due to supramaximal stimulation of the phrenic nerve at a frequency of 1 Hz (0.2 ms duration) for 15 min was found to be 4.05 ± 0.73 ng and 3.4 ± 0.48 ng (mean ± s.e) in two separate sets of experiments. Addition of a fasciculatory (2.63 μM) or a paralytic dose (10.5 μM) of Oxo-T to the bath for 15 min was followed by a sharp increase in ACh release from the phrenic nerve. The quantities of ACh found were 135.14 ± 20.16 ng and 236.5 ± 33.12 ng respectively. In experiments with Oxo-T, the standard ACh solutions used for bioassay purpose invariably contained an equivalent amount of Oxo-T, to nullify its direct influence on leech muscle, if any. This precaution was taken in spite of the fact that Oxo-T failed to influence the responses of the leech muscle to exogenous ACh in separate experiments. The control release from the same diaphragm was invariably estimated either before or after (cross-over) treatment with Oxo-T. A summary of all experiments is shown in Figure 2. The results are expressed in terms of the weight of ACh chloride.

The amount of spontaneously released ACh, in the absence of Oxo-T could not be measured since the responses of these samples were below the linear sensitivity range of the bioassay preparation. As with nerve stimulation, Oxo-T (2.63 or 10,5 μM) produced a large increase in the amount of ACh released during the resting period. The amounts found were 12.0 ± 1.46 ng and 26.33 ± 7.01 ng (6 experiments each) in the presence of 2.63 and 10.5 μM Oxo-T respectively (control was not measurable).

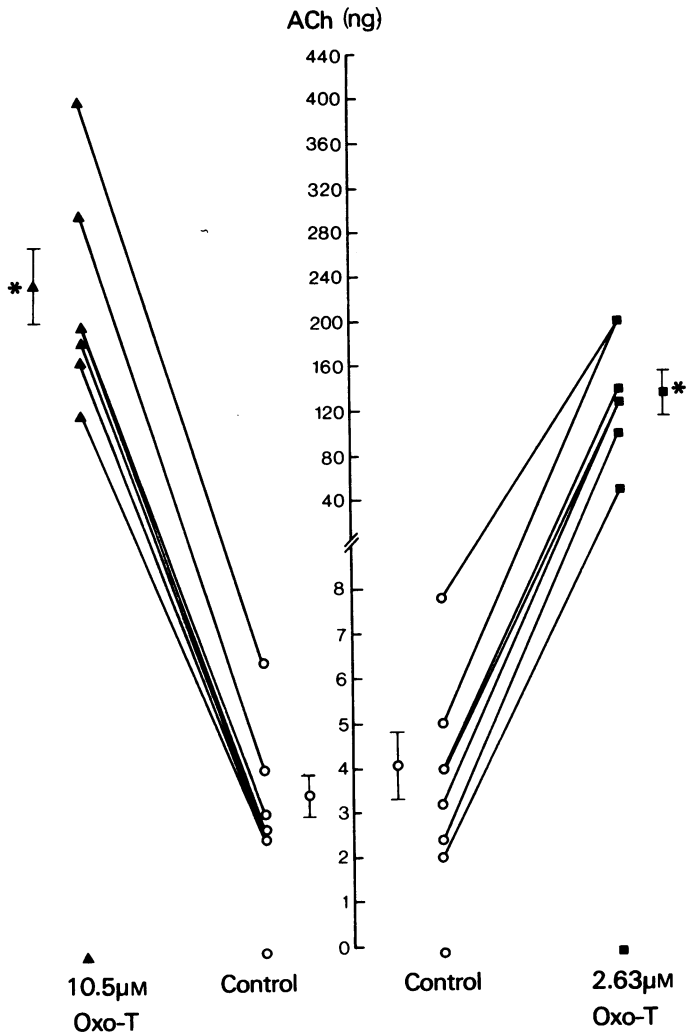

Figure 2 Summary of effects of oxotremorine (2.63 and 10.5 μM) on the electrically evoked release of acetylcholine into the bathing fluid of rat diaphragm. The values indicate the total amount of acetylcholine released in a 4 ml bath in terms of acetylcholine chloride. Each experiment had its individual control. Vertical bars indicate s.e. * $P < 0.001$.

Discussion

The present experiments suggest that both the initial muscle fasciculations and the subsequent neuromuscular blockade produced by Oxo-T may arise from a massive increase in the amount of ACh released by the motor nerve impulse. A similar effect may occur in the central nervous system as suggested by Holmstedt & Lundgren (1966). Oxo-T raises ACh levels in the central nervous system (Pepeu, 1963; Holmstedt, Lundgren & Sundwall, 1963; Ganguly & Saha, 1969). Szerb & Somogyi (1973) observed a reduced release of ACh from isolated cerebral cortex slices of the rat, but Guggenheimer & Levinger (1975) and Nistri (1976) have reported an increased spontaneous release of ACh from cat and frog spinal cord respectively. One of us has previously reported that Oxo-T increases the spike discharges of Renshaw cells evoked by antidromic motor nerve stimulation and also produces spontaneous Renshaw cell discharges (Ganguly, Ross, Haase & Cleveland, 1976). In the present study, Oxo-T caused increase in resting as well as evoked release of ACh in the bathing fluid. More recently Fackler, Ross, Cleveland & Haase

(1977) have observed an increase in the muscle spindle afferent discharge and stretch reflex after injection of Oxo-T in cats. The phenomenon of excess release of ACh reported here, may successfully explain the action of Oxo-T at all of the aforementioned sites.

It is worth mentioning that not only propranolol (Ganguly, 1976) and the cholinolytic anti-Parkinson drugs (Das & Ganguly, 1977) antagonize the skeletal myoneural (extrafusal) effect of Oxo-T, but also TK 174, a quarternary compound, inhibits tremor even though it does not permeate the central nervous system (Leszkovszky & Tardos, 1971). In view of these reports it is tempting to conclude that the effects at the motor nerve junction, the muscle spindle, and the cholinergic synapse from motor axon collaterals to Renshaw cells could contribute to the tremorogenic action of Oxo-T.

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