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INFLUENCE OF CHRONIC NEOSTIGMINE TREATMENT ON THE NUMBER OF ACETYLCHOLINE RECEPTORS AND THE RELEASE OF ACETYLCHOLINE FROM THE RAT DIAPHRAGM

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SUMMARY

1. Rats were treated twice daily for 7 days with neostigmine and the diaphragm was isolated for study of its acetylcholine content, release upon nerve stimulation and the number of receptors in the end-plate.

2. While the content of total acetylcholine was unchanged, the release of acetylcholine on stimulation with trains of 500 pulses at 100 Hz every 20 sec was reduced by about 50 %. Five days after the end of neostigmine treatment the release of acetylcholine recovered to normal.

3. The total number of acetylcholine receptors in the end-plate as measured from the binding of *N*, *O*-di[^{3}H]acetyl α-bungarotoxin was reduced from $2{\cdot}1 \times 10^7$ to $1{\cdot}2 \times 10^7$ per end-plate.

4. The above-mentioned changes are not due to acute pharmacological effects of neostigmine, nor to an immediate effect of cholinesterase inhibition but presumably due to chronic accumulation of acetylcholine at the neuromuscular junction.

INTRODUCTION

Motor nerve is known to exert trophic effects on skeletal muscle (Guth, 1968). One of the most important effects is to regulate the sensitivity of the end-plate as well as the other part of the muscle membrane to acetylcholine (Axelsson & Thesleff, 1959; Miledi, 1960). Recently, it has become possible to determine the number of acetylcholine receptors in the end-plate (Miledi & Potter, 1971; Barnard, Wieckowski & Chiu, 1971; Fambrough & Hartzell, 1972; Chang, Chen & Chuang, 1973) by using isotopically labelled derivatives of α-bungarotoxin isolated from the venom of *Bungarus multicinctus* (Chang & Lee, 1963). Denervation of the rat diaphragm is thus shown to increase the number of receptors by about

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thirty-fold along the whole muscle fibre (Miledi & Potter, 1971; Chang *et al.* 1973). In order to get further insight into the trophic effect of nerve on muscle, it was of interest to see what effect the prolonged inhibition of cholinesterase might produce, since this treatment may affect neuromuscular transmission in a way opposite to that of denervation.

It has been shown by Roberts & Thesleff (1969) that chronic treatment with neostigmine in rats caused muscle weakness and reduction of the quantal content of end-plate potentials. The release and content of acetylcholine were therefore also studied.

METHODS

Neostigmine treatment

Long Evans rats of either sex weighing about 200 g were used. Neostigmine methylsulphate (0·1 mg) was administered subcutaneously twice daily for 7 days as previously described by Roberts & Thesleff (1969). Atropine sulphate (1 mg) was also given 30 min before injection of neostigmine in the first 2 days in order to minimize the muscarinic effect. There was about 10 % decrease of the body weight after this chronic treatment. The rats were sacrificed at 16 hr after the last injection of neostigmine and the diaphragm isolated for the following investigations.

Assay of acetylcholine receptor

Both hemidiaphragms with the phrenic nerves attached were immersed in 30 ml. Tyrode solution (composition in mM: NaCl, 137; KCl, 2·7; $CaCl_2$, 1·8; $MgCl_2$, 1·1; $NaHCO_3$, 11·9; NaH_2PO_4, 0·33, and glucose, 11·2) at 37° C and oxygenated with 95 % O_2 + 5 % CO_2. Several changes of Tyrode solution were made in 2 hr in order to remove any neostigmine retained by the tissue. The number of acetylcholine receptors in the motor end-plate was estimated from the irreversible binding of *N*, *O*-di[^{3}H]acetyl α-bungarotoxin 400 mci/m-mole (Chang *et al.* 1973). Diaphragms were incubated with the toxin at $1{\cdot}25 \times 10^{-7}$ M for 2 hr, a condition known to saturate the receptor, and then washed for 5 hr with twelve changes of Tyrode solution to remove the non-specific binding. The muscle was cut parallel to the end-plate zone into ten segments of 1·5 mm width and their radioactivity counted by liquid scintillation spectrography after digestion with 0·2 ml. 0·5 N-KOH. From the counts of the central two to three segments containing the end-plates, the number of acetylcholine receptors per end-plate was calculated by assuming that one hemidiaphragm contained 10,000 muscle fibres (Krnjević & Miledi, 1958) and one end-plate on each fibre.

Assay of acetylcholine release

Isolated diaphragms were first treated with Mipafox, 1 mg/ml., at 37° C for 150 min in 50 ml. modified Krebs solution (composition in mM: NaCl, 130; KCl, 4·7; $CaCl_2$, 2·5; KH_2PO_4, 1·2; $NaHCO_3$, 13·1, and glucose, 11·2) to inactivate cholinesterase and then placed in a flat small organ bath containing 4 ml. modified Krebs solution. The phrenic nerve was stimulated with 500 supramaximal pulses at 100 Hz every 20 sec at 37° C. After 20 min of stimulation, the bath fluid was removed for assay of acetylcholine. The assay was performed on the guinea-pig isolated ileum pre-treated with 10 μg/ml. Mipafox as previously described by Johnson (1963). Pyribenzamine (5 ng/ml.) and morphine sulphate (50 ng/ml.) were

added to antagonize the possible histamine interaction and to depress the spontaneous movement of the ileum.

Acetylcholine content

Immediately after isolation of the diaphragm, acetylcholine contained in the muscle was extracted by homogenizing the tissue with 2 ml. cold 95 % ethyl alcohol containing 0·2 % acetic acid. The homogenate was kept in the cold for 30 min to complete extraction. The supernatant was then dried in the vacuum and the residue diluted with modified Krebs solution for assay of acetylcholine as described above.

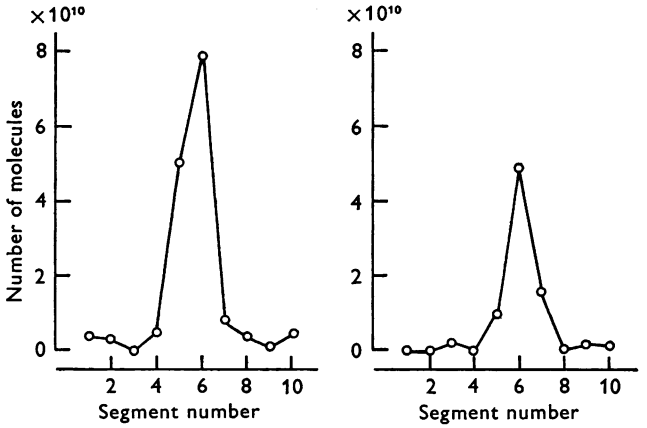

Fig. 1. Distribution of *N*, *O*-di[^{3}H]acetyl α-bungarotoxin in the normal (left) and chronic neostigmine treated- (right) rats. All segments represent 1·5 mm width of diaphragm and numbered from the central tendon in abscissa. The ordinate represents the number of toxin molecules in each segment.

RESULTS

Number of acetylcholine receptors

The number of acetylcholine receptors in one end-plate of diaphragms of rats weighing about 250 g has been determined to be $1{\cdot}9\text{–}2{\cdot}2 \times 10^7$ (Chang *et al.* 1973) and $2{\cdot}9 \times 10^7$ (Fambrough & Hartzell, 1972) by using various purified derivatives of α-bungarotoxin. In the present experiment we obtained $2{\cdot}11 \pm 0{\cdot}12 \times 10^7$ (mean ± S.D., $n = 9$) receptive sites per end-plate with *N*, *O*-di[^{3}H]acetyl α-bungarotoxin. After 7 days of chronic treatment with neostigmine, the number was found to be reduced by 42 % to $1{\cdot}22 \pm 0{\cdot}21 \times 10^7$ ($n = 6$). As in the normal diaphragm, the *N*, *O*-diacetyl α-bungarotoxin binding site was found only in the end-plate zone (Fig. 1). On stimulation of the phrenic nerve with repetitive pulses at 100 Hz, no Wedensky inhibition, a typical phenomenon of cholinesterase inhibition, was observed, indicating that the action of neostigmine had disappeared. With similar treatment, Roberts & Thesleff (1969) also found that the enzyme activity was within normal.

Release of acetylcholine

As shown in Table 1, the output of acetylcholine during 20 min stimulation at 100 Hz for 5 sec every 20 sec from normal hemidiaphragms was 27 ng ± 5·2 (S.D.) whereas hemidiaphragms isolated from rats treated with neostigmine for 7 days released only 15 ± 3·1 ng. The output of acetylcholine did not recover on further stimulation after several washings (Table 1). This result indicates that the decrease of output was not due to an acute pharmacological effect of neostigmine. When rats were killed 5 days after the end of the chronic neostigmine treatment, the acetylcholine output had returned to normal (Table 1).

TABLE 1. Effect on the release of acetylcholine from phrenic nerve stimulated at 100 Hz for 5 sec every 20 sec. Acetylcholine was collected for the period of 20 min stimulation. Twenty min of rest intervened between the 1st and 2nd period of stimulation

		Acetylcholine output/hemidiaphragm	
Treatment	*n*	1st period (ng ± S.D.)	2nd period (as % of 1st period)
Control	6	27 ± 5·2	102 ± 4
0·2 mg neostigmine per day for 7 days	6	15 ± 3·1	104 ± 6
Same treatment as above, 5 days after the last injection	6	29 ± 3·5	104 ± 5

Acetylcholine store

The total content of acetylcholine in a hemidiaphragm from normal rats was 40 ± 4·0 ng (S.D., $n = 8$) while that from the chronic neostigmine treated rats was 38 ± 4·0 ($n = 8$). Evidently, no appreciable change occurred in the total store of acetylcholine.

DISCUSSION

The present experiments demonstrate that upon chronic treatment with neostigmine both the number of post-synaptic acetylcholine receptors and the amount of transmitter released by nerve impulses are remarkably decreased. It is apparent that these effects are not due to a direct immediate action of neostigmine since the measurements were performed in *in vitro* experiments where neostigmine, if any, was washed out. Besides, no sign of cholinesterase inhibition was observed in the isolated muscle. Furthermore, acute treatment of the diaphragm preparation *in vitro* with an irreversible organophosphorus anticholinesterase, with subsequent

wash-out, did not appreciably affect the number of acetylcholine receptors (Barnard *et al.* 1971; C. C. Chang & S. T. Chuang, unpublished).

Our results confirmed the conclusion by Roberts & Thesleff (1969) that the release of transmitter was reduced by chronic treatment with neostigmine. The extent of inhibition of acetylcholine output in our experiment is similar to the inhibition of quantal contents of end-plate potentials in Roberts & Thesleff's experiments. These two data together may indicate that the quantum size was not reduced. Since the total store of acetylcholine was not reduced, a change might have occurred in the small releasable pool of acetylcholine as pointed out by Roberts & Thesleff (1969) or in the release mechanism *per se*. It is interesting that chronic treatment with neostigmine also decreased the number of acetylcholine receptors. This may account for the decreased amplitude of miniature end-plate potentials found by Roberts & Thesleff (1969) in similarly treated rat muscles. It is apparent that the decrease of acetylcholine receptors may contribute to the muscle weakness in rats and the cholinergic crisis in myasthaenia gravis after chronic neostigmine treatment. Chronic treatment with anticholinesterase has been shown also to reduce the sensitivity of rat cardiovascular system, ileum and salivary gland (McPhillips & Dar, 1967; McPhillips, 1969; Buckley & Heading, 1970) and cat iris sphincter (Bito, Hyslop & Hyndman, 1967; Bito & Dawson, 1970) to choline esters. Since the pA_2 value of atropine was unchanged, Bito & Dawson (1970) inferred that the resistance to choline esters might be due to a decrease of the number of cholinergic receptors. The present experiment thus provides analogous evidence for such a possibility. It is conceivable that the facilitatory effect induced by anticholinesterases on neuroeffector transmission could be thus offset by the reduction of transmitter release and number of acetylcholine receptors.

The mechanism for the decrease of release of the neurotransmitter and of the number of acetylcholine receptors after chronic treatment with anticholinesterase has not yet been clarified. Although the release of acetylcholine was reduced, the actual concentration at the receptor as well as the nerve endings must be still higher than normal because of the inhibition of cholinesterase. Therefore, acetylcholine could be a 'trophic' factor for these pre- and post-synaptic changes. Neostigmine, on the other hand, is known to induce repetitive nerve impulses (Masland & Wigton, 1940). This effect of neostigmine might cause excess release of a trophic substance other than acetylcholine and result in the phenomena observed. In a preliminary experiment, we found that chronic treatment of rats with hemicholinium-3 for 7 days caused a 26 % increase of the number of acetylcholine receptors in the diaphragm. If one assumes that the effect of hemicholinium-3 is only to interfere with the synthesis of acetylcholine

by inhibiting the incorporation of choline, then acetylcholine could be the regulatory factor. It may be proposed that nerve has a 'trophic' influence on both pre- and post-synaptic sites via the concentration of the transmitter for regulation of neuromuscular transmission.

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