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CONTRACTURES ELICITED BY TETRAETHYLAMMONIUM IN AVIAN MUSCLE TREATED WITH METHOHEXITONE

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1 The chick biventer cervicis muscle immersed in methohexitone (8.8×10^{-5} M) responded to tetraethylammonium with contractures which were dose-related. The ED_{50} for tetraethylammonium was 2.1×10^{-3} M.

2 In the absence of methohexitone, tetraethylammonium produced contractures only at much higher concentrations; these contractures were accompanied by fasciculations and neuromuscular block of the twitch fibres.

3 The contractures produced by tetraethylammonium in the presence of methohexitone were not reduced by exposure to botulinum toxin which eliminated all response of the muscle to indirect stimulation.

4 Tubocurarine (1.2×10^{-6} M) displaced the dose-response curve for tetraethylammonium-methohexitone-induced contractures to the right. The dose-ratio was 15.63 ± 1.98.

5 Physostigmine (1.8×10^{-6} M) potentiated the activity of tetraethylammonium-methohexitone 3.26 or 3.84 fold, depending on the method of calculation used.

6 Physostigmine potentiated contractures elicited by indirect repetitive stimulation 4.8 to 6.0 fold more than it potentiated contractures due to tetraethylammonium-methohexitone.

7 It is concluded that in the presence of methohexitone, tetraethylammonium produces contractures of the chick muscle by releasing acetylcholine but also by a direct agonist action on the cholinoceptor.

Introduction

Tetraethylammonium (TEA) has an anti-curare action at the neuromuscular junction in frog and mammalian preparations. This action may be due to facilitation by TEA of the release of acetylcholine from the presynaptic nerve terminals (Ing, 1936; Kensler, 1950; Stovner 1957; Koketsu, 1958; Bowman, Hemsworth & Rand, 1962; Riker & Okamoto, 1968). TEA may produce repetitive firing in the motor nerve terminals (Payton & Shand, 1966); fibrillatory twitching has been noted in voluntary muscle with TEA (Ing, 1936).

Methohexitone (Metho) produced complete neuromuscular block at a concentration of 1 mM in the phrenic nerve-diaphragm preparation; this was accompanied by a reduction in miniature endplate potential (m.e.p.p.) amplitude but there was also a marked increase in m.e.p.p. frequency (Westmoreland, Ward & John, 1971). Similar findings were reported for phenobarbitone (Thompson & Turkanis, 1973; Weakley & Proctor, 1977). In contrast, Galindo (1971) observed a marked depressant action by pentobarbitone on the presynaptic terminal. The neuromuscular block which barbiturates can produce may be partly due to a shortening in the duration of the acetylcholine (ACh)-induced increase in conductance (Adams, 1974; Torda & Gage, 1977). The present paper is concerned with the interaction of TEA and Metho in the chick biventer cervicis muscle preparation (Ginsborg & Warriner, 1960). The muscle contains fast fibres which are focally innervated and respond to an indirect stimulus with a single twitch contraction. There are also slow multiply innervated fibres which respond to low frequency indirect stimulation (Ginsborg, 1960) with slowly developing contractions. The slow fibres also produce contractures in response to 'depolarizing' drugs (Ginsborg, 1960). Elliott (1978) found that the chick muscle immersed in low concentration of Metho responded with contractures when TEA was applied. The same doses of TEA were devoid of contractile activity in the absence of Metho. The present paper describes these experiments in greater detail and examines the nature of TEA-induced contractures in Metho-treated muscles.

Methods

Chicks (3 to 10 days old) were killed with chloroform and the two biventer cervicis muscles set up as de-

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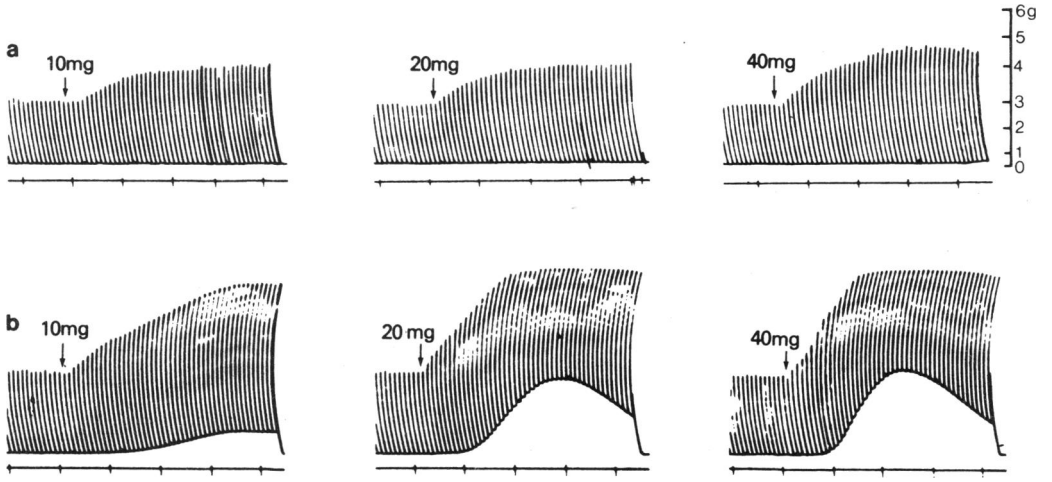

Figure 1 The effect of methohexitone (Metho) on the response of the chick biventer cervicis muscle to tetraethylammonium (TEA). (a) TEA (10, 20 and 40 mg added to the bath at arrows) facilitated indirectly elicited twitch contractions but did not produce contractures. (b) After immersion in Metho 25 μg/ml for 30 min the muscle produced contractures in response to the same doses of TEA.

scribed by Ginsborg & Warriner (1960) in 20 ml baths containing Krebs-Hensleit solution at 37°C bubbled with 95% O_2 and 5% CO_2. The muscles were indirectly stimulated with square wave pulses usually of 5 V and 0.1 ms duration at a repetitive rate of 0.2 Hz, unless otherwise stated. Semi-isometric recording was used. When Metho was used it was added to the Krebs solution in the reservoir (Metho-Krebs). TEA was added directly to the bath in which the muscle lay. The Metho concentration used was 8.8×10^{-5} M. The drugs used were methohexitone sodium, tetraethylammonium bromide, acetylcholine chloride, and physostigmine. Botulinum toxin was supplied by the Wellcome Institute as a dry powder, the glycerinated stock solution was prepared as described by Ambache (1949) at a concentration of 4×10^5 median lethal doses (MLD)/ml.

Results

Action of methohexitone

The twitch contractions elicited by indirect stimulation were not reduced by immersion of the preparation in Metho 8.8×10^{-5} M for 6 h. A small facilitation was noted but may not be significant since twitch contractions tended to increase in untreated preparations over a long period. No effect of Metho on slow fibres was noted.

Action of tetraethylammonium

TEA in a final concentration of 1.2×10^{-3} M to 9.5×10^{-3} M produced an increase in twitch contractions in response to indirect electrical stimulation. There was no significant contracture when TEA was added. With very high concentrations of TEA, 3.8×10^{-2} M to 1.5×10^{-1} M, fasciculations of the muscle occurred and these sometimes fused to give a contracture which was accompanied by block of the twitch responses.

Action of tetraethylammonium in the presence of methohexitone

When the muscle was immersed for 30 min in Metho-Krebs and then tested in the continued presence of Metho-Krebs it was found that TEA produced substantial contractures (Figure 1). The amplitude of the contractures varied with the concentration of TEA (Figure 2). The ED_{50} derived from the graph for the collected results of over 20 experiments was 2.1×10^{-3} M. The amplitude of the response diminishes with repeated application of the same dose of TEA; this was avoided by giving the TEA at not less than 30 min intervals. No fasciculations occurred with TEA in Metho-Krebs.

Action of botulinum toxin on tetraethylammonium-methohexitone contractures

Control contracture responses to TEA in the presence of Metho-Krebs were obtained. The Metho was then washed out and the preparation immersed in Krebs solution containing botulinum toxin 1 to 2×10^{-4} MLD per ml for 3 h. During 2 h of this period the muscle was stimulated repetitively at 20 Hz. During this time all response to indirect stimulation ceased.

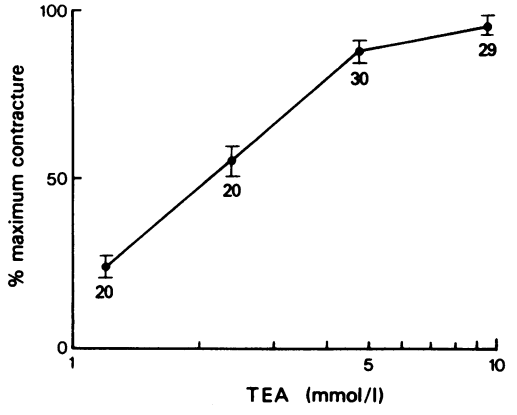

Figure 2 Graph of pooled results for tetraethylammonium (TEA)-induced contracture in the presence of methohexitone (Metho) 8.8×10^{-5} M. The vertical bars show ± s.e. mean; number of contractures measured is given beside each point.

The toxin was washed out and Metho-Krebs was applied for 30 min, after which the muscle responded to TEA with contractures which were larger than those in the control period. In control preparations which were stimulated repetitively at 20 Hz for the same period without application of the toxin, the TEA-induced contractures were also larger at the end of the experiment than at the start. Comparison of toxin-treated muscle ($n = 8$) with controls ($n = 3$) showed that the increase in contracture amplitude at the end of the period of repetitive stimulation was on average 20% less in the toxin-treated muscles than in the controls. However, the important finding was that TEA could still elicit contractures in the presence of Metho-Krebs after pretreatment with toxin.

Does the contracture produced by tetraethylammonium-methohexitone involve a cholinoceptor?

Contractures were evoked by TEA in the presence of Metho-Krebs after which the preparation was immersed in Metho-Krebs containing 1.2×10^{-6} M tubocurarine and after 30 min tested again with TEA. The dose-response curve for TEA-Metho-induced contractures was displaced to the right. The ratio of the ED_{50}s before and in the presence of tubocurarine was 15.63 ± 1.98 (s.e.) $n = 8$. It should be noted that the upper part of the dose-response curve for TEA in the presence of tubocurarine overlaps the region of TEA concentrations (1.2×10^{-3} M upwards) at which TEA has an action on its own (i.e. in the absence of Metho), producing fasciculations and sometimes contracture. If this 'direct' component of TEA's contractile activity is present then it would tend to lower the dose-ratio obtained. The antagonistic activity of tubocurarine suggests that TEA/Metho contractures involve a cholinoceptor.

The effect of physostigmine on tetraethylammonium/methohexitone contractures

In preliminary experiments it was found that physostigmine (1.8×10^{-6} M) produced some potentiation of TEA/Metho contractures but the potentiation was very small in comparison with the potentiation of ACh-induced contractures. For example, in one experiment the dose-ratio was 1.2 for TEA but 274 for ACh. Large doses of ACh tended to desensitize the preparation. It appeared desirable to compare the activity of TEA with that of ACh released by nerve impulses rather than with exogenous ACh. Slow muscle fibres respond with contractures which are graded with respect to the frequency of indirect repetitive stimulation. The frequency range required to elicit these contractures is far lower than that necessary to produce tetanic contraction of the fast fibres (Ginsborg, 1960). The preparation in Metho-Krebs was repetitively stimulated for 1 min, frequencies in the range of 5 to 15 Hz elicited contractures. From these results a frequency-response curve was obtained and used to estimate the frequency giving a 50% maximum response ($Freq_{50}$). TEA/Metho contractures were also elicited and an ED_{50} calculated. The preparation was then exposed to Metho-Krebs, containing physostigmine (1.8×10^{-6} M) and the frequency-response and TEA-Metho response determinations repeated. Physostigmine potentiated TEA-induced contractures, the ratio of ED_{50}s after physostigmine to the previous control was 1:3.84 ± 0.65 s.e., $n = 9$. The corresponding ratio of the $Freq_{50}$ was 1:13.91 ± 2.63, $n = 9$. The ratio, $Freq_{50}$ ratio/ED_{50} ratio was calculated for each experiment, the mean of these ratios was 4.81 ± 1.49 $n = 9$. Thus physostigmine appears to potentiate the contractures due to ACh release by nerve impulses some five times more than it potentiates TEA/Metho-induced contractures.

Discussion

TEA-Metho contractures were blocked by tubocurarine and they are therefore not due to an action of TEA on the excitation-contraction coupling mechanism but presumably involve a cholinoceptor. TEA-Metho contractures differ from TEA contractures in that the former are elicited with lower concentrations of TEA, and do not involve fasciculations or block of the twitch fibres. The work of previous authors, described in the Introduction, suggests that TEA contractures may be due to the release of ACh from presynaptic nerve terminals by TEA. The following

results suggest that TEA-Metho contractures are not entirely due to the release of ACh: (1) TEA-Metho still produces contractures when botulinum toxin has completely eliminated transmission as judged by the absence of any response to indirect repetitive stimulation. The toxin interferes with the release of ACh (Brooks, 1956); it could be that TEA still releases ACh from a store not susceptible to the action of the toxin. (2) If TEA acts by releasing ACh, physostigmine might be expected to potentiate TEA-Metho-induced contractures to the same extent as it potentiates ACh-Metho contractures. However, the results of preliminary experiments indicated that the potentiation of TEA is insignificant in comparison with the potentiation of ACh. As ACh postulated to be released by TEA from nerve endings is in close proximity to its receptors whereas exogenous ACh is not, it could be argued that an anticholinesterase would be more effective in potentiating exogenous ACh. (3) In an attempt to meet this point the action of physostigmine on TEA-Metho-induced contractures was compared with its action on sub-maximal contractures induced by low frequency repetitive stimulation. Ginsborg (1960) showed that although slow fibres can conduct action potentials they can also probably be activated by the depolarization produced by the summation of e.p.ps following repetitive indirect stimulation. The assumption has been made that the release of ACh per nerve impulse is fairly constant in the range of frequency employed and that consequently frequency is directly related to the total ACh released during the 1 min stimulation period. The results indicate that TEA-Metho-induced contractures are potentiated by physostigmine some five times less than are contractures due to ACh released by nerves.

Larger contractures were usually obtained with repetitive stimulation than with TEA-Metho; consequently the $Freq_{50}$ contracture was greater than the ED_{50} contracture produced by TEA-Metho. A comparison of the potentiating effect of physostigmine on the basis of its effect on unequal contractures may be thought unsatisfactory. An alternative method was to calculate the TEA-Metho contractures as a percentage of the maximum response to repetitive stimulation. Since on this basis the TEA-Metho contractures did not surpass 50% maximum in all experiments, ED_{30} values were used for comparison. When the results were recalculated on this basis the ratio of $Freq_{30}$s after, to that before physostigmine, was $1:15.7 \pm 2.95$, $n = 8$ and for ED_{30}s $1:3.26 \pm 0.64$, $n = 8$. The mean of the $Freq_{30}$ ratio/ED_{30} ratio was 6.01 ± 1.84, $n = 8$. In one experiment it was not possible to calculate an ED_{30}. Thus in whichever way the results are calculated they indicate a marked difference between the effect of physostigmine on TEA-Metho contractures and on nerve-mediated contractures.

A difference between the nerve impulse mediated release of ACh and TEA-Metho mediated release might be that the nerve impulse would release ACh more synchronously than TEA-Metho. However, one would expect slowly released ACh to be more likely to be hydrolysed by cholinesterase before it reaches the receptors than synchronously released ACh. Consequently an anticholinesterase should be more effective in potentiating ACh released asynchronously. Since physostigmine potentiated TEA-Metho contractures between 3 and 4 fold, it seems reasonable to conclude that there is normally some ACh release, however there must also be a considerable element of direct agonist action of TEA on the cholinoceptor which is not potentiated by physostigmine.

Why pretreatment with Metho should cause the chick muscle to respond to TEA with contractures is not certain. In so far as ACh release is involved, the increase in m.e.p.p. frequency which Westmoreland *et al.* (1971) noted in the phrenic nerve-diaphragm preparation might suggest that Metho could facilitate TEA-induced ACh release, but the experiments with botulinum toxin suggest that vesicular release of ACh is not involved in TEA-Metho contractures. Lowering the potassium concentration by 70% in the Krebs-Hensleit solution bathing the muscle, causes TEA to produce contractures with no Metho present, and ACh contractures are potentiated (Elliott, unpublished observations). This suggests the possibility that Metho might act by producing some hyperpolarization in this preparation. Further studies with microelectrode techniques are in hand to elucidate these points.

It will be interesting to see if this action of Metho is also apparent at other peripheral and central synapses.

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