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Actions of acetylcholine and carbachol on the chick biventer cervicis muscle

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Summary

1. The possibility that carbachol may act on the chick biventer cervicis muscle by releasing acetylcholine was investigated.

2. Log dose-response curves for acetylcholine and carbachol were constructed in the presence of triethylcholine, tubocurarine and physostigmine, in both innervated and denervated chick biventer cervicis muscles.

3. The log dose-response curve for acetylcholine was shallower than that for carbachol and was moved to the left by triethylcholine, indicating potentiation. In the presence of physostigmine, the curve was as steep as that for carbachol, and like that for carbachol was moved to the right by triethylcholine, indicating antagonism. In the presence of tubocurarine, the log dose-response curves for both acetylcholine and carbachol were moved to the right.

4. The log dose-response curves for both acetylcholine and carbachol were moved to the left by chronic denervation, indicating increased sensitivity to these drugs. Both curves were moved similar distances to the right by excess magnesium ion concentration. No tachyphylaxis developed to repeated administration of carbachol in the presence of triethylcholine or tubocurarine, either in innervated or denervated muscles.

5. It was concluded that the main action of triethylcholine in the chick biventer cervicis muscle is a tubocurarine-like action, which, in the presence of acetylcholine, is masked by a weak anticholinesterase action. It was also concluded that the diminished response to carbachol in the presence of triethylcholine is due to the postjunctional blocking action of triethylcholine, and that carbachol owes none of its activity to an ability to release acetylcholine from the nerve terminals.

Introduction

Evidence suggests that, in sympathetic ganglia, carbachol may act, at least partly, by releasing acetylcholine from the presynaptic terminals (Volle & Koelle, 1961; McKinstry & Koelle, 1967 a & b). Collier & Katz (1970) have shown that acetylcholine itself may also act to release quantities of surplus endogenous acetylcholine, although they believe that the presynaptic effects of acetylcholine are not of physiological importance.

Recently, Chiou & Long (1969) have similarly suggested that the action of carbachol on the biventer cervicis muscle of the chick involves the release of acetylcholine from the presynaptic terminals. From experiments using triethylcholine,

which they assumed to have a specific prejunctional hemicholinium-like action, they concluded that carbachol and other nicotinic stimulants such as decamethonium, nicotine, choline and tetramethylammonium acted prejunctionally to release acetylcholine, although they believed that the action of acetylcholine itself was confined to the postjunctional membrane. Marshall (1969), on the other hand, from results obtained using the same preparation but using another hemicholinium-like drug, troxypyrrolium, concluded that carbachol acted postjunctionally.

In the experiments described in this paper, the actions of acetylcholine and carbachol were further studied in the presence of triethylcholine on the isolated chick biventer cervicis muscle.

Methods

Biventer cervicis muscle preparations from chicks aged 3–10 days were set up as described by Ginsborg & Warriner (1960). However, the temperature was 32° C and dextrose (2 g/l.) was added to the physiological saline (Krebs & Henseleit, 1932). When desired, the nerve within the muscle tendon was stimulated with pulses of 0·1 ms duration, at a frequency of 0·1 Hz, and of sufficient voltage to produce maximal twitches.

Unilateral denervation of biventer cervicis muscles of 2-day old chicks was carried out under ether anaesthesia using aseptic precautions. The nerve within the muscle tendon was sectioned by cutting the tendon between ligatures. After section the cut ends of the tendon were overlapped and tied together. The nerve was allowed to degenerate for 5 days before the chick was killed and the muscle removed and mounted in Krebs-Henseleit solution. Contractions and contractures of the muscles were recorded isometrically using Statham G10B strain gauges connected to an ink-writing dynograph.

Log dose-response curves for acetylcholine and carbachol were constructed by increasing successive doses of the agonists until the respective maximal responses were obtained. The agonists were added to the tissue bath at 5 min intervals. The acetylcholine was allowed to remain in contact with the tissue for 30 s whereas the slower response to carbachol necessitated a longer contact period of 60 seconds. Both agonists were washed out of the bath by overflow for 30 seconds. Submaximal responses to the agonists both in the presence and absence of blocking or potentiating drugs were expressed as a percentage of the maximal response to the agonists in the absence of such drugs. All the positions on the log dose-response curves represent the mean (±S.E.M.) of five separate experiments.

The drugs used were: acetylcholine chloride, carbachol chloride, physostigmine salicylate (B.D.H.), triethylcholine iodide (Ward Blenkinsop), tubocurarine chloride (Burroughs Wellcome). All drugs were dissolved in 0.9% saline and the concentrations quoted in the text refer to the salts.

Results

Dose-response curves for acetylcholine and carbachol

As previously reported (Marshall, 1969; Chiou & Long, 1969), the log dose-response curve for acetylcholine was much shallower than that for carbachol (Figs. 1 and 3). However, when redetermined in the presence of physostigmine (1 μg/ml; 3×10^{-6}M) the curve for acetylcholine was moved to the left, indicating potentiation.

and became steeper (Fig. 2) and was then similar to that for carbachol, which was not altered by physostigmine (Fig. 3).

In the absence of physostigmine, triethylcholine (3 mg/ml ; $1{\cdot}1 \times 10^{-2}$M) shifted the log dose-response curve for acetylcholine slightly to the left (Fig. 1) whereas that

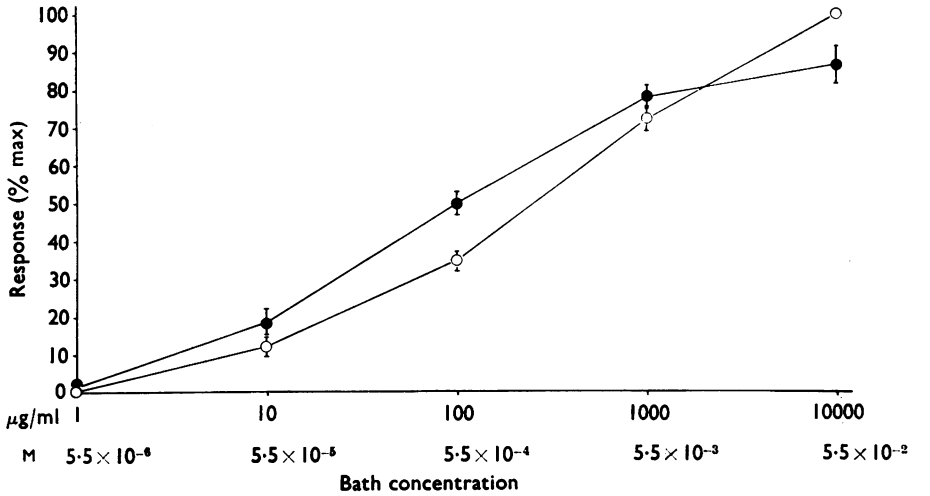

FIG. 1. Log dose-response curves for acetylcholine on the chick biventer cervicis muscle preparation. The response is expressed as a percentage of the maximal response produced by acetylcholine (—○—) in the tissue. The log dose-response curve for acetylcholine in the presence of triethylcholine (3 mg/ml) (—●—) is moved to the left with the lower concentrations of acetylcholine (indicating potentiation). Each point represents the mean (±S.E.M.) of five separate determinations.

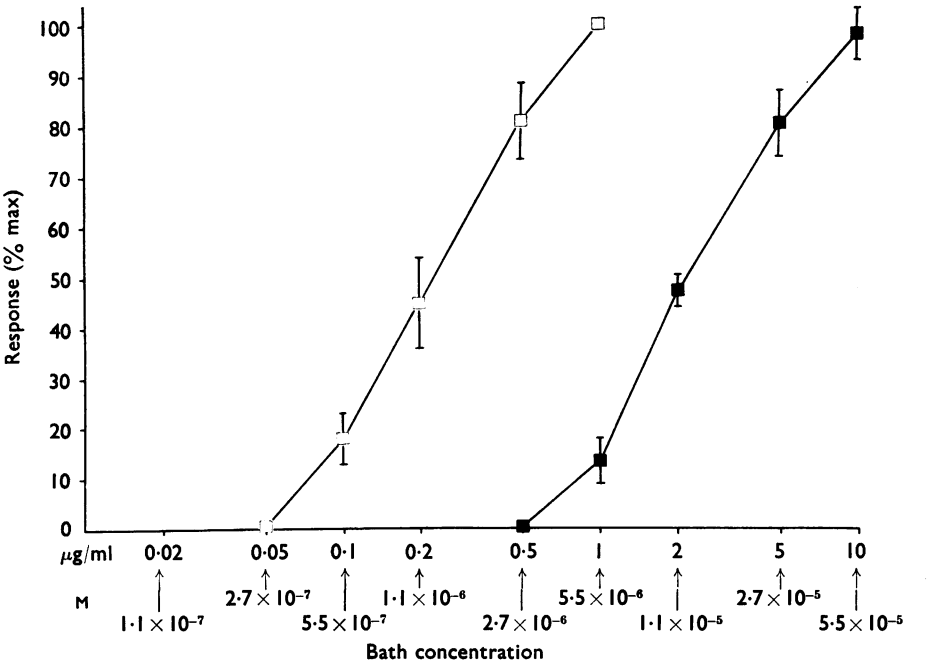

FIG. 2. Log dose-response curves for acetylcholine as in Fig. 1, but in the presence of physostigmine (1 μg/ml). The log dose-response curve for acetylcholine (—□—) is steeper than in Fig. 1, and in the presence of triethylcholine (3 mg/ml) (—■—) is moved approximately 1 log unit to the right (indicating antagonism).

for carbachol was shifted to the right, indicating antagonism (Fig. 3). Chiou & Long (1969) also recorded a shift to the right of the carbachol curve in the presence of the same concentration of triethylcholine. However, in contrast to the results of Chiou & Long (1969), the maximal response to carbachol in the present experiments was only slightly reduced by triethylcholine (Fig. 3). Tubocurarine (1 μg/ml ; 1.3×10^{-6} M) caused a shift to the right of the log dose-response curves for both acetylcholine and carbachol, without reducing the maximal responses to either agonist.

In the presence of physostigmine (1 μg/ml), triethylcholine no longer shifted the acetylcholine curve to the left. Instead, a shift to the right similar to that for carbachol occurred (Fig. 2). Tubocurarine (1 μg/ml) also shifted both curves to the right as it did in the absence of physostigmine.

In another series of experiments, the nerve within the tendon was stimulated at a frequency of 0·1 Hz. At intervals of not less than 5 min electrical stimulation was temporarily stopped and acetylcholine or carbachol, in concentrations sufficient to produce a contracture approximately equal in height to a maximal twitch, was added to the bath. When neuromuscular transmission was partially impaired by tubocurarine (2–4 μg/ml), the responses to carbachol were reduced more than the

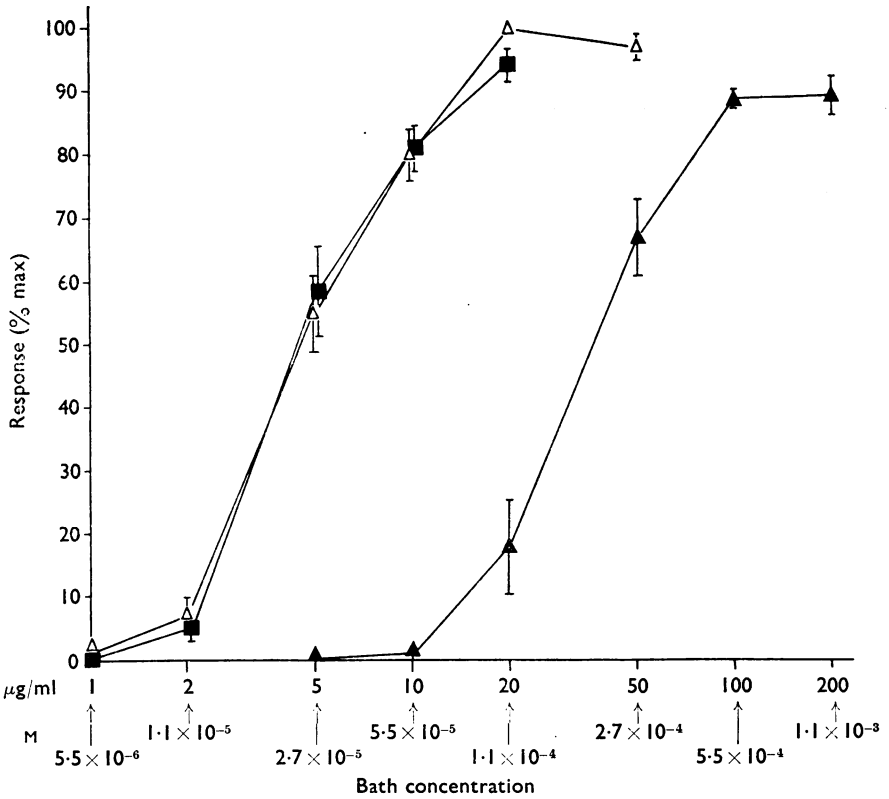

FIG. 3. Log dose-response curves for carbachol on the chick biventer cervicis muscle preparation. The response is expressed as a percentage of the maximal response produced by carbachol in the tissue. The log dose-response curve for carbachol (—△—) is steeper than that for acetylcholine (Fig. 1), but is of similar slope to that for acetylcholine in the presence of physostigmine (Fig. 2). In the presence of physostigmine (1 μg/ml) the log dose-response curve for carbachol is unchanged (—■—). In the presence of triethylcholine (3 mg/ml) the log dose-response curve for carbachol (—▲—) is moved approximately 1 log unit to the right. Each point represents the mean (±S.E.M.) of five separate determinations.

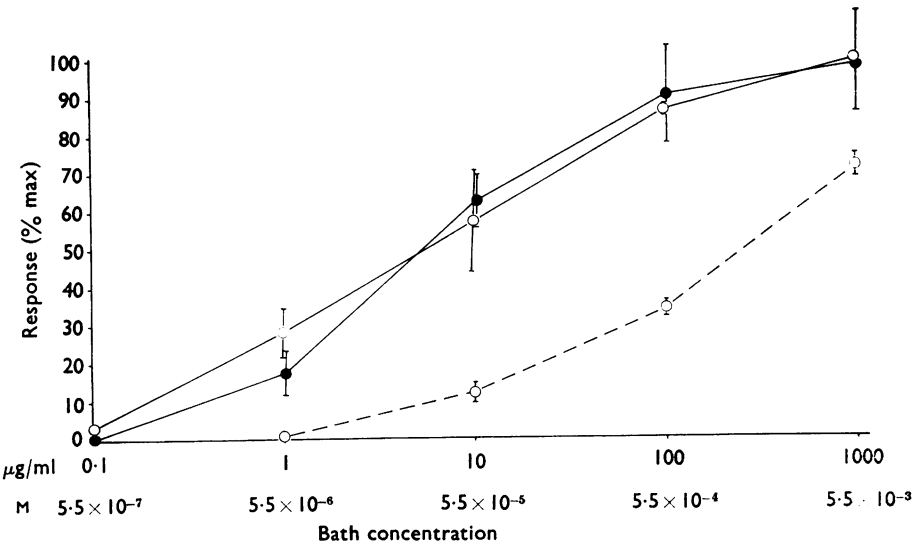

FIG. 4. Log dose-response curves for acetylcholine in the chronically denervated chick biventer cervicis muscle, expressed as in Fig. 1. The log dose-response curve for acetylcholine (—○—) is moved to the left of that obtained in innervated muscles (broken line) but is of similar steepness. The log dose-response curve in the presence of triethylcholine (3 mg/ml) (—●—) is not significantly different from that in its absence.

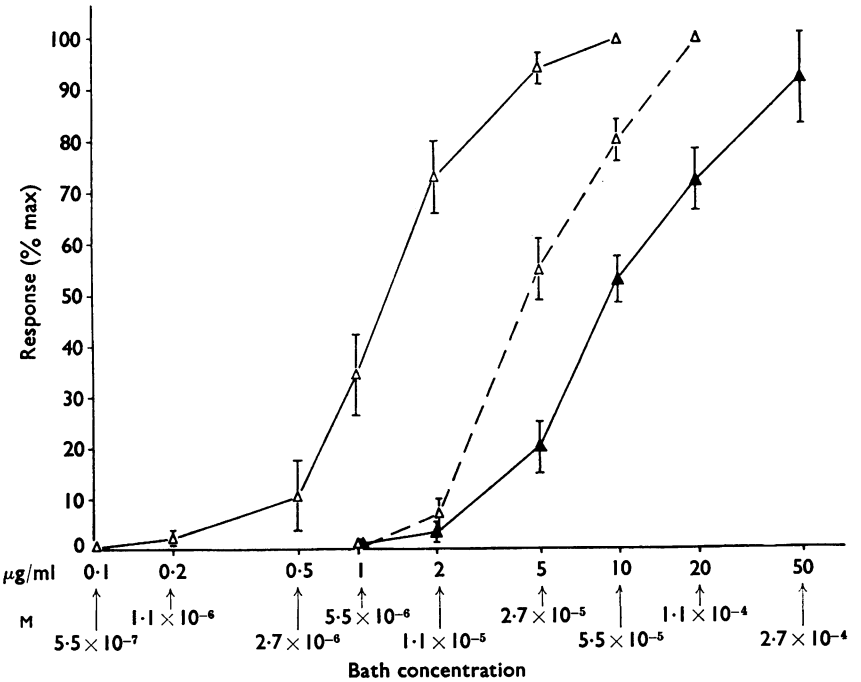

FIG. 5. Log dose-response curves for carbachol in the chronically denervated chick biventer cervicis muscle expressed as in Fig. 3. The log dose-response curve for carbachol (—△—) is moved to the left of that obtained in innervated muscles (broken line). As in innervated preparations (Fig. 3), in the presence of triethylcholine (3 mg/ml) the log dose-response curve for carbachol (—▲—) is moved approximately 1 log unit to the right.

responses to acetylcholine, presumably because of the difference in slopes of the log dose-response curves for the two agonists. Physostigmine (1 μg/ml) returned the response to nerve stimulation to the control level, while the response to acetylcholine was augmented above its control level. However, the reduced response to carbachol was not increased by the anticholinesterase.

Chronically denervated muscles

In chronically denervated muscles the concentrations of acetylcholine and carbachol required to produce contractures equal to 50% of the maximal responses were respectively 50 and 4 times less than those required in innervated preparations, and the slopes of the log dose-response curves were similar to those obtained from innervated preparations (Figs. 4 & 5). This degree of increased sensitivity to carbachol is in accordance with that recorded by Blaber & Bowman (1962) using other cholinesterase stable nicotinic stimulants in the *in situ* denervated fowl gastrocnemius muscle, whereas the degree of increased sensitivity to acetylcholine is rather more than that recorded by other workers using adult fowls (Brown & Harvey, 1938; Blaber & Bowman, 1962).

The effects of triethylcholine and tubocurarine in denervated muscles were similar to those on innervated preparations. Triethylcholine had no significant effect on acetylcholine, but shifted the log dose-response curve for carbachol to the right (Figs. 4 & 5), whereas tubocurarine shifted both curves to the right.

Physostigmine potentiated acetylcholine in denervated muscles as it did in innervated muscles, but the effect was less pronounced. In denervated muscles the dose of acetylcholine required to produce 50% of the maximal response was reduced 100-fold, compared with a 1,000-fold reduction in innervated muscles. As observed in innervated muscles, the log dose-response line was steeper in the presence of physostigmine than in its absence.

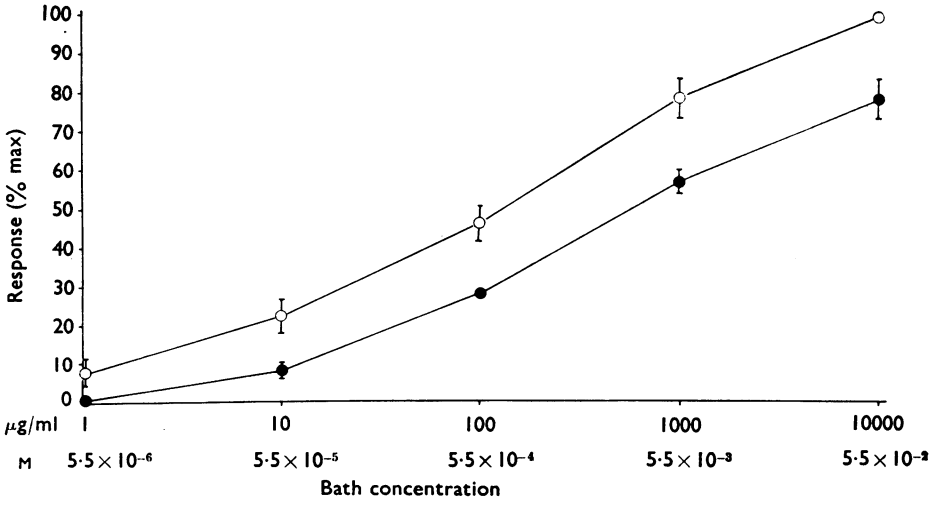

FIG. 6. Log dose-response curves for acetylcholine as in Fig. 1. The log dose-response curve for acetylcholine (—○—) is moved to the right when determined in the presence of 5 times the normal concentration of magnesium ions (—●—), sufficient to reduce acetylcholine release. Compare with Fig. 7.

Increased magnesium concentration

An excess concentration of magnesium ions impairs neuromuscular transmission in two ways: it decreases transmitter release from motor nerve endings (del Castillo & Engbaeck, 1954; Dodge & Rahamimoff, 1967) and it stabilizes the postjunctional membrane, thereby depressing its excitability (Engbaeck, 1947; del Castillo & Engbaeck, 1954). Magnesium ions also prevent the depolarization of motor nerve terminals produced by acetylcholine (Hubbard, Schmidt & Yokota, 1965).

The effects of excess magnesium were determined both on responses to nerve stimulation and on responses to acetylcholine. When the magnesium concentration was increased 5-fold, responses to nerve stimulation gradually diminished until the muscle no longer responded to single shock stimulation. At 75% block of twitch height responses to acetylcholine were partially reduced but to a much lesser extent than in the presence of a concentration of tubocurarine with an equivalent effect on twitch amplitude. These results indicated that in this tissue the main inhibitory action of excess magnesium ions is exerted on the nerve endings to reduce the release of transmitter.

When the magnesium concentration was increased 5-fold, log dose-response curves for acetylcholine and carbachol were moved by a similar degree to the right (Figs.

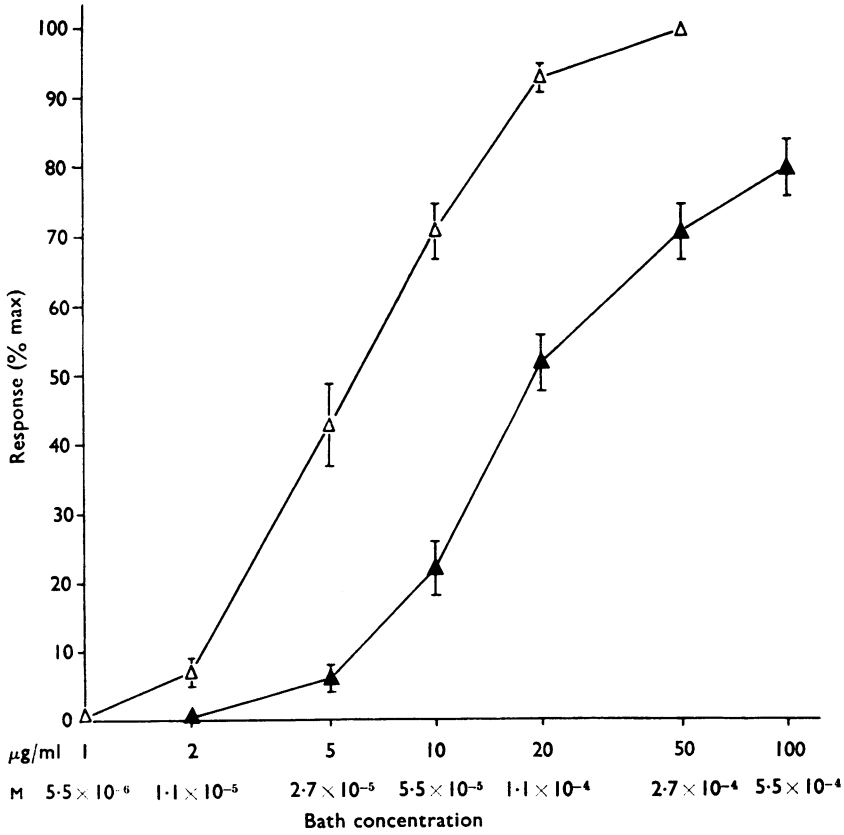

FIG. 7. Log dose-response curves for carbachol as in Fig. 3. The log dose-response curve for carbachol (—△—) is moved to the right in the presence of 5 times the normal concentration of magnesium ions (—▲—). The shift to the right is similar, but somewhat less than that observed with acetylcholine in Fig. 6.

6 & 7). Thus, a reduction in acetylcholine release had no greater effect on responses to carbachol than on responses to acetylcholine.

Tachyphylaxis

Triethylcholine impairs acetylcholine synthesis by inhibiting the uptake of choline into nerve endings (Bull & Hemsworth, 1965 ; Hemsworth, Darmer & Bosmann, 1971). Failure of neuromuscular transmission develops gradually in rapidly stimulated nerve-muscle preparations as a result of depletion of the available stores of acetylcholine (Bowman & Rand, 1961 ; Bowman, Hemsworth & Rand, 1962 ; Bowman & Hemsworth, 1965). Thus any agonist which acts by releasing endogenous acetylcholine should, in the presence of triethylcholine, deplete the nerve terminals of stored acetylcholine and, since resynthesis is prevented, tachyphylaxis to the agonist should develop rapidly.

The responses to repeated doses of carbachol (50 μg/ml, approx. $2{\cdot}5 \times 10^{-4}$M), which produced a slightly submaximal response in the presence of triethylcholine (3 mg/ml) and of tubocurarine (2 μg/ml), were studied. The degree of reduction of the responses to carbachol in the continued presence of triethylcholine was indistinguishable from that in the presence of tubocurarine. As is commonly noted with antagonists, the degree of block was time dependent, the responses diminishing slightly over the first 10–15 min after addition of the antagonist. After this initial period the responses to carbachol (added every 5 min) remained constant, with no evidence of tachyphylaxis. Additionally, no tachyphylaxis to carbachol was observed in denervated muscles, or in denervated muscles treated with triethylcholine.

Discussion

Chiou & Long (1969) have divided nicotinic agonists into three groups on the basis of their susceptibility to blockade by triethylcholine, which was used for its prejunctional hemicholinium-like action: (i) drugs which act only on the postjunctional membrane by occupying acetylcholine receptors, although from their evidence they suggested that, of the drugs tested, only acetylcholine itself acted in this way ; (ii) drugs which are taken up by the nerve terminals and subsequently displace acetylcholine which then acts on the postjunctional receptors, for example, carbachol, decamethonium, choline, nicotine and tetramethylammonium ; and (iii) drugs which share both actions (i) and (ii), for example dimethylphenylpiperazinium and 6-trimethylamino-*n*-hexanol. Two obvious differences exist between the drugs in groups (i) and (ii). First the slope of the log dose-response curve for acetylcholine is shallower than those for the drugs in group (ii), and second acetylcholine is susceptible to hydrolysis by acetylcholinesterase, whereas the drugs in group (ii) are stable to the enzyme. My results confirm previous findings (Marshall, 1969) that the difference is probably related simply to the fact that acetylcholine is hydrolysed by cholinesterase, because in the presence of an anticholinesterase drug the log dose-response curves for acetylcholine and carbachol were similar in slope.

The difference in the action of triethylcholine on the responses to acetylcholine and to the drugs in group (ii) also may be explained in terms of the differing sensitivities of acetylcholine and the latter drugs to enzymatic hydrolysis. In the presence of physostigmine, that is when the differing susceptibilities of acetylcholine and carbachol (used in this study as a representative of group (ii) of Chiou & Long) to

enzymatic destruction were reduced and the slopes of the log dose-response curves were similar, triethylcholine had a similar antagonistic action against both agonists. This action of triethylcholine is probably a reflection of the postjunctional blocking action of the drug which, although weak in focally innervated mammalian muscle (Bowman & Rand, 1961 ; Bowman *et al.*, 1962), is more appreciable in multiply innervated amphibian or avian muscle (Roberts, 1962 ; Marshall, 1969) especially at the concentrations used. The lack of blocking activity of triethylcholine against acetylcholine in the absence of physostigmine is probably a reflection of the weak anticholinesterase action of triethylcholine in chicken muscle (Marshall, 1969) which at a concentration of 3 mg/ml (approximately 10^{-2}M) is probably sufficient to counteract the postjunctional blocking action.

Triethylcholine has a complex action in multiply innervated preparations, showing (1) a hemicholinium-like action reversible by choline (Marshall, 1969) ; (2) a tetraethylammonium-like action in increasing acetylcholine release in response to nerve stimulation (Roberts, 1962 ; Marshall, 1970) ; (3) a tubocurarine-like action (Roberts, 1962 ; Marshall, 1969) ; and (4) an anticholinesterase action (Marshall, 1969). My results, and those of Chiou & Long (1969), emphasize the need for careful interpretation of results obtained using such a drug with multiple actions at high concentrations. The inhibitory actions of triethylcholine against carbachol in denervated muscle and against acetylcholine in the presence of physostigmine, indicate that the main action of triethylcholine in the chicken biventer cervicis muscle is its tubocurarine-like action rather than its hemicholinium-like action. This is not unexpected as the concentration of triethylcholine used is approximately 10 times that required for a specific hemicholinium-like action (Bowman *et al.*, 1962 ; Marshall, 1969).

The lack of tachyphylaxis in the presence of sufficient triethylcholine to inhibit acetylcholine synthesis, the only slightly diminished response in the presence of sufficient magnesium ions to inhibit acetylcholine release, and the increased sensitivity in the absence of functional nervous tissue in chronically denervated muscle confirm that carbachol is acting independently of the nervous element in transmission, probably by an action on the postjunctional membrane. The increased sensitivity to carbachol after chronic denervation is in accordance with previous findings in chicken muscle using cholinesterase stable agonists (Blaber & Bowman, 1962). This denervation supersensitivity, and the depolarizing action of nicotinic stimulants in cultured chicken muscle, which is devoid of nervous tissue (Dryden, 1970 ; Dryden, Erulkar & De La Haba, personal communication), would be difficult to explain in terms of the agonists acting by releasing acetylcholine.

In focally innervated mammalian muscle the increased sensitivity to acetylcholine after chronic denervation is thought to be due to a spread of receptors over the surface of the muscle out with the endplate region, an area normally devoid of acetylcholinesterase (Axelsson & Thesleff, 1959 ; Koelle, 1963). In multiply innervated muscles the receptors and acetylcholinesterase are already widespread, and therefore the increased sensitivity to acetylcholine after denervation is usually less pronounced than in focally innervated muscle (Brown, 1937 ; Brown & Harvey, 1938 ; Blaber & Bowman, 1962). In this series of experiments the greater increase in sensitivity to acetylcholine than to carbachol suggests that in the biventer cervicis muscle, which is partially focally innervated (Ginsborg & Mackay, 1961), a spread of receptors to areas devoid of acetylcholinesterase is taking place. However, the fact that the

slopes of the log dose-response lines for acetylcholine, and the actions of physostigmine, were similar in both innervated and denervated preparations suggests that a large proportion of the added acetylcholine is still broken down by acetylcholinesterase in the denervated muscle.

Chiou & Long (1969) base their arguments on the idea that carbachol and similar substances are taken up into nerve endings by the choline transport mechanism, and they suggest that triethylcholine inhibits their action by blocking this uptake. However, there is no published evidence to support the possibility that carbachol and similar drugs are taken up into nerve endings, and indeed, evidence obtained from isolated red blood cells, which have a choline transport mechanism similar to that of nerve endings (Martin, 1968), indicates that uptake of radioactive carbachol and decamethonium is independent of the choline transport mechanism and is unaffected by hemicholinium (Martin, 1969).

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REFERENCES

AXELSSON, J. & THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol., Lond.*, **147**, 178–193.

BLABER, L. C. & BOWMAN, W. C. (1962). A comparison between the responses of avian and mammalian muscles to substances which facilitate neuromuscular transmission. *Arch. int. Pharmacodyn. Ther.*, **138**, 185–208.

BOWMAN, W. C. & HEMSWORTH, B. A. (1965). Effects of triethylcholine on the output of acetylcholine from the isolated diaphragm of the rat. *Br. J. Pharmac. Chemother.*, **24**, 110–118.

BOWMAN, W. C., HEMSWORTH, B. A. & RAND, M. J. (1962). Triethylcholine compared with other substances affecting neuromuscular transmission. *Br. J. Pharmac. Chemother.*, **19**, 198–218.

BOWMAN, W. C. & RAND, M. J. (1961). Actions of triethylcholine on neuromuscular transmission. *Br. J. Pharmac. Chemother.*, **17**, 176–195.

BROWN, G. L. (1937). The actions of acetylcholine on denervated mammalian and frog's muscle. *J. Physiol., Lond.*, **89**, 438–461.

BROWN, G. L. & HARVEY, A. M. (1938). Reactions of avian muscle to acetylcholine and eserine. *J. Physiol., Lond.*, **94**, 101–117.

BULL, G. & HEMSWORTH, B. A. (1965). The action of triethylcholine on the biological synthesis of acetylcholine. *Br. J. Pharmac. Chemother.*, **25**, 228–233.

CHIOU, C. Y. & LONG, J. P. (1969). Acetylcholine-releasing effects of some nicotinic agents on chick biventer cervicis nerve muscle preparation. *Proc. Soc. exp. Biol. Med.*, **132**, 732–737.

COLLIER, B. & KATZ, M. S. (1970). The release of acetylcholine by acetylcholine in the cat's superior cervical ganglion. *Br. J. Pharmac.*, **39**, 428–438.

DEL CASTILLO, J. & ENGBAEK, L. (1954). The nature of the neuromuscular block produced by magnesium. *J. Physiol., Lond.*, **124**, 370–384.

DODGE, F. A. JUN. & RAHAMIMOFF, R. (1967). Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *J. Physiol., Lond.*, **193**, 419–432.

DRYDEN, W. F. (1970). Development of acetylcholine sensitivity in cultured skeletal muscle. *Experientia*, **26**, 984–986.

ENGBAEK, L. (1947). Investigations on the course and localisation of magnesium anaesthesia. A comparison with ether anaesthesia. *Acta pharmac. tox.*, **4**, Suppl. 1, pp. 189.

GINSBORG, B. L. & MACKAY, B. (1961). A histochemical demonstration of two types of motor innervation in avian skeletal muscle. *Bibl. Anat.*, **2**, 174–181.

GINSBORG, B. L. & WARRINER, J. (1960). The isolated chick biventer cervicis nerve-muscle preparation. *Br. J. Pharmac Chemother.*, **15**, 410–411.

HEMSWORTH, B. A., DARMER, K. I. jun. & BOSMANN, H. B. (1971). The incorporation of choline into isolated synaptosomal and synaptic vesicle fractions in the presence of quaternary ammonium compounds. *Neuropharmacology*, **10**, 109–120.

HUBBARD, J. I., SCHMIDT, R. F. & YOKOTA, T. (1965). The effect of acetylcholine upon mammalian motor nerve terminals. *J. Physiol., Lond.*, **181**, 810–829.

KOELLE, G. B. (1963). Cytological distributions and physiological functions of cholinesterases. In: *Cholinesterases and Anticholinesterase Agents*, ed. Koelle, G. B. Berlin: Springer-Verlag.

KREBS, H. A. & HENSELEIT, K. (1932). Untersuchungen über die Hamstoffbildung im Tierkörper. *Hoppe-Seyler's Z. physiol. Chem.*, **210**, 33–66.

MCKINSTRY, D. N. & KOELLE, G. B. (1967a). Acetylcholine release from the cat superior cervical ganglion by carbachol. *J. Pharmac. exp. Ther.*, **157**, 319–327.

MCKINSTRY, D. N. & KOELLE, G. B. (1967b). Effects of drugs on acetylcholine release from the cat superior cervical ganglion by carbachol and by preganglionic stimulation. *J. Pharmac. exp. Ther.*, **157**, 328–336.

MARSHALL, I. G. (1969). The effects of some hemicholinium-like substances on the chick biventer cervicis muscle preparation. *Eur. J. Pharmac.*, **8**, 204–214.

MARSHALL, I. G. (1970). Actions of tetraphenylboron on neuromuscular transmission in the biventer cervicis muscle of the chick. *Eur. J. Pharmac.*, **13**, 51–54.

MARTIN, K. (1968). Concentrative accumulation of choline by human erythrocytes. *J. gen. Physiol.*, **51**, 497–516.

MARTIN, K. (1969). Effects of quaternary ammonium compounds on choline transport in red cells. *Br. J. Pharmac.*, **36**, 458–469.

ROBERTS, D. V. (1962). Neuromuscular activity of the triethyl analogue of choline in the frog. *J. Physiol., Lond.*, **160**, 94–105.

VOLLE, R. L. & KOELLE, G. B. (1961). The physiological role of acetylcholinesterase in sympathetic ganglia. *J. Pharmac. exp. Ther.*, **133**, 223–240.