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INTERACTIONS BETWEEN TETRAETHYLAMMONIUM AND METHOHEXITONE AT THE CHICK NEUROMUSCULAR JUNCTION

EXPERIMENTS WITH THE MOVING FLUID ELECTRODE TECHNIQUE

R. C. ELLIOTT and F. A. WALI*
Department of Applied Biology, Brunel University, Uxbridge, Middlesex UB8 3PH, U.K.

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Abstract—The effect of methohexitone on the depolarization and contracture responses produced by tetraethylammonium (TEA), acetylcholine (ACh) and repetitive indirect stimulation were investigated, using the moving fluid electrode technique, in the chick biventer cervicis (BVC) nerve muscle preparation. TEA (4.8×10^{-4}–4.8×10^{-2} M) produced contracture and depolarization responses which were concn-dependent. These responses were potentiated by methohexitone (8.8×10^{-5} M). The mean ED_{50}s for the contracture responses in the control Krebs solution and with methohexitone were (mean ± S.E.M.) $6.5 \pm 0.03 \times 10^{-3}$ M and $1.3 \pm 0.04 \times 10^{-3}$ M (N = 6) respectively. The mean ED_{50}s for the depolarizations were (mean ± S.E.M.) $5.9 \pm 0.1 \times 10^{-3}$ and $1.5 \pm 0.06 \times 10^{-3}$ M (N = 6) respectively. ACh (5.5×10^{-6}–1.1×10^{-2} M) produced contracture and depolarization responses which were concn-dependent. These responses were reduced by methohexitone (8.8×10^{-5} M). The mean ED_{50}s for the contracture responses in the control Krebs solution and with methohexitone were (mean ± S.E.M.) $2.4 \pm 0.21 \times 10^{-4}$ and $2.3 \pm 0.1 \times 10^{-3}$ M (N = 6) respectively. The mean (±S.E.M.) ED_{50}s for the depolarizations were $8.4 \pm 0.33 \times 10^{-4}$ and $3.7 \pm 0.14 \times 10^{-3}$ M (N = 6), respectively. Repetitive indirect stimulation, at 1–20 Hz, produced contraction and depolarization responses which were frequency-dependent. These responses were slightly potentiated by methohexitone (8.8×10^{-5} M). The mean (±S.E.M.) $frequency_{50}$s for the contractions produced in the control Krebs solution and with methohexitone were 9.2 ± 0.1 and 8.5 ± 0.2 Hz (N = 6) respectively. The mean $frequency_{50}$s for the depolarizations were (mean ± S.E.M.) 7.2 ± 0.1 and 5.8 ± 0.19 Hz (N = 6) respectively. It is concluded that TEA may have a direct post-synaptic action, in addition to releasing ACh from the presynaptic nerve terminals. TEA produces more contracture tension than does ACh for a given level of membrane depolarization. Methohexitone, non-competitively, reduces the responses produced by applied ACh whereas it potentiates those produced by TEA and repetitive nerve stimulation.

The chick biventer cervicis (BVC) muscle contains both fast and slow muscle fibres [1]. The fast fibres respond to electrical stimulation and depolarizing drugs with twitch contraction, while the slow fibres respond only to depolarizing drugs with a tonic contracture.

In the present experiments, the responses from both fast and slow muscle fibres were investigated.

Previous experiments [2, 3] have shown that the barbiturate agent, methohexitone, markedly potentiates the contractures produced by tetraethylammonium (TEA) in the chick BVC muscle. The experiments suggested that TEA might act by increasing the presynaptic release of acetylcholine (ACh), in addition to a direct agonist action. The occurrence of presynaptic facilitation is supported by the work of Collier and Exley [4] and Koketsu [5] who showed that TEA increased ACh release at the vertebrate neuromuscular junction.

The prolongation of the depolarization phase of the nerve terminal action potential by TEA, which blocks K^+ conductance [6], might lead to an increased Ca^{2+} influx and therefore increase ACh output [7, 8]. Beaulieu and Frank [9] suggested that TEA might exert a direct action on Ca^{2+} channels. High concns of TEA exert a curariform action [2, 10].

If TEA acts indirectly, by releasing ACh, it should be possible to measure the depolarization produced by this released ACh. The object of the present experiments was to measure concurrently this depolarization and accompanying contracture responses, using the moving fluid electrode technique [11], and to compare these responses with those produced by applied ACh and repetitive indirect stimulation. Methohexitone was used as an experimental tool to differentiate between the pre- and post-synaptic actions of TEA.

METHODS

Preparation. Chicks (1–3 days old) were used, as they gave better depolarization responses than older chicks. The chicks were killed by chloroform and the BVC nerve muscle preparation [12] was dissected and removed from the muscles of the neck. The preparation was set up in the moving fluid electrode bath (modified from Fatt [11] containing Krebs–Henseleit solution maintained at 38° and bubbled with 95% O_2 and 5% CO_2.

*Present address (and all correspondence to): Anaesthetics Unit, London Hospital Medical College, Whitechapel, London E1 1BB, U.K.

Recording. The moving fluid electrode technique was used to record simultaneously the depolarization and contracture responses in the chick BVC muscle induced by drug action. The preparation was set up vertically in the bath (200 ml) to allow isometric recording and repetitive indirect stimulation at 0.2–20 Hz with 5 V and 0.2 ms pulse duration. A pair of Ag/AgCl/agar electrodes fitted with balsa wicks were used for the recording of depolarizations. The lower recording electrode was earthed while the upper electrode was placed on the tendon just below the stimulating electrodes. In this preparation, the tendon encloses the motor nerve. The signal was amplified by the Y1 amplifier of a Tektronix RM 502 A oscilloscope. The amplified signal was then fed into an X, Y recorder, plotter type PL 100 giving an overall gain of ×1000. The contracture responses were recorded with a force transducer (isometric Washington transducer type D1 50 g) and a Washington pen recorder, model 400 MD 2C. The depolarization responses were recorded by moving the meniscus level of the Krebs solution in the bath upwards and downwards along the entire length of the preparation, producing two lines in one record. In the figures, upward movement indicates membrane depolarization. Control records were taken and then either ACh or TEA was added. The first depolarization record for ACh was obtained at 15 s and for TEA at 60 s. Successive recordings of ACh- and TEA-induced depolarizations were made at 15 and 60 s respectively. Maximum depolarizations of ACh and TEA occurred at 30 and 120 s respectively. No desensitization occurred in this period (15 and 60 s). The interval selected depended on the rate of the onset of drug action. The procedure was then repeated after equilibration of the preparation with Krebs solution containing methohexitone for 30 min. A typical set of recording is shown in Fig. 1. The depolarization (mV) and the contracture (g tension) were measured at the peak of the response. The maximum response in each experiment was expressed as 100% and all other responses were expressed as a percentage of the maximum response.

Solutions. All solutions were made up in deionized water. The Krebs–Henseleit solution used had the following composition (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 6H_2O$ 2.5, and glucose 11.1.

Drugs. The drugs used were: ACh chloride (Sigma), TEA bromide (Sigma), sodium methohexital (Breital sodium, Lilly), tubocurarine chloride (Sigma), and eserine sulphate (Sigma). The pH of the Krebs solution and that of the Krebs solution containing methohexitone remained around 7.2.

RESULTS

Actions of ACh and methohexitone

ACh (5.5×10^{-6}–1.1×10^{-3} M) produced concn-dependent contractures and depolarizations in the chick BVC muscle. These responses were reduced by methohexitone (8.8×10^{-5} M). The time course of the contracture response varied between 2 and 4 min, and the response reached its maximum in about 20 s. Maximum depolarization occurred in about 30–40 s relative to the contracture response. Fig. 1 shows the depolarizations produced by ACh in the control Krebs solution (A) and with methohexitone (B). Note that methohexitone greatly reduced these depolarizations (compare records 2A and 2B). The mean (± S.E.M.) ED_{50}s for the con-

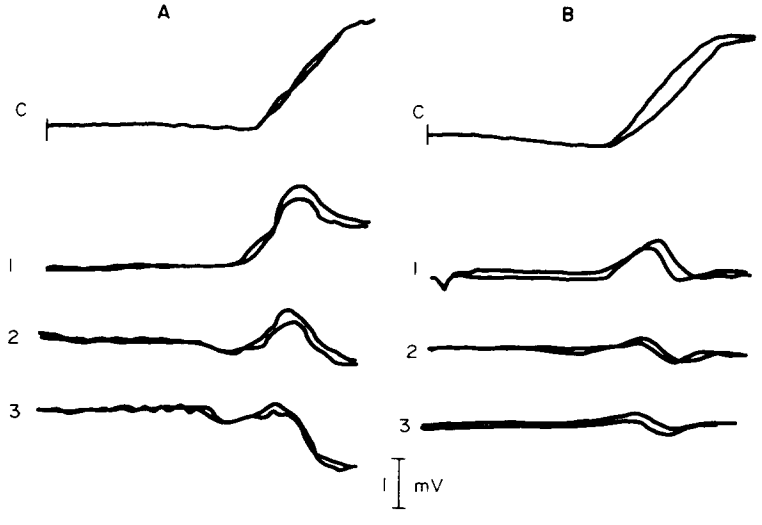

Fig. 1. The effect of methohexitone on ACh-induced depolarizations recorded by the moving fluid electrode technique. C, control records with no drug added, showing the potential profile moving from the tendon end of the preparation on the left of the record to the cut end of the muscle on the right. 1–3, records of the depolarization induced by ACh (2.8×10^{-3} M) taken: (1) at 15 s, (2) at 30 s, and (3) at 45 s after C. A, records taken in Krebs solution; B, records from the same preparation after equilibration with Krebs solution containing methohexitone (8.8×10^{-5} M) which reduced the ACh-induced depolarization. Calibration: 1 mV.

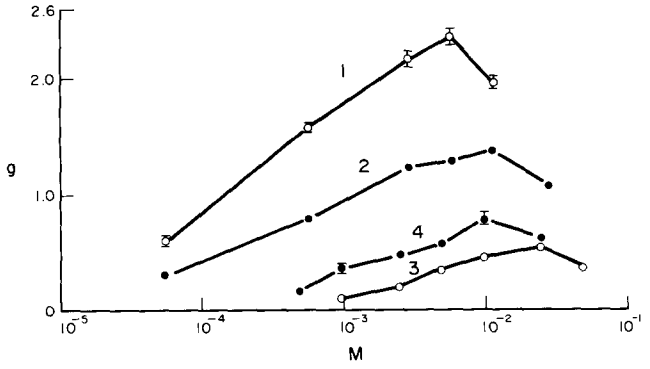

Fig. 2. The effect of methohexitone on the contracture tension produced by ACh and TEA: log concn–response curves for: (1) ACh, (2) ACh in muscle equilibrated for 30 min with methohexitone (8.8×10^{-5} M), (3) TEA, (4) TEA in muscle equilibrated with methohexitone (8.8×10^{-5} M). Bars are ± S.E.s; where they are absent in this figure and Figs 3 and 5, the S.E. lies within the diameter of the symbol at the point. Empty circles, controls; filled circles, in the presence of methohexitone.

tractures produced by ACh in the control Krebs solution and with methohexitone were $2.4 \pm 0.21 \times 10^{-4}$ and $2.3 \pm 0.1 \times 10^{-3}$ M (N = 6) respectively. A mean (± S.E.M.) maximum contracture of 2.4 ± 0.7 g tension (N = 6) was obtained by 5.5×10^{-3} M ACh in the control Krebs solution. The mean (± S.E.M.) ED_{50}s for the depolarizations were $8.4 \pm 0.33 \times 10^{-4}$ and $3.7 \pm 0.14 \times 10^{-3}$ M (N = 6) respectively. A mean (± S.E.M.) maximum depolarization of 1.7 ± 0.06 mV (N = 6) was obtained by 5.5×10^{-3} M ACh in the control Krebs solution. Fig. 2 shows the concn–contracture curves (CCCs) for ACh responses in the control Krebs solution (1) and with methohexitone (2). Note that methohexitone shifted the ACh CCC non-competitively to the right (curve 1 to curve 2). Methohexitone also shifted the concn–depolarization curves (CDCs) produced by ACh in the control Krebs solution to the right (Fig. 3, curve 1 to curve 2). In addition, methohexitone did not seem to alter the relation between the depolarizations (mV) and contractions (g tension) (Fig. 4A).

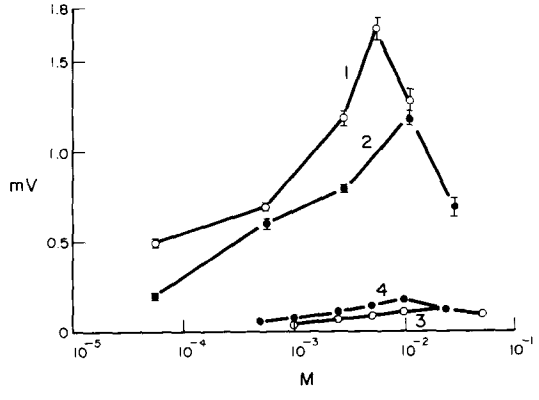

Fig. 3. The effect of methohexitone on the depolarization produced by ACh and TEA: log concn–response curves for: (1) ACh, (2) ACh in muscle equilibrated for 30 min with methohexitone (8.8×10^{-5} M), (3) TEA, (4) TEA in muscle equilibrated with methohexitone (8.8×10^{-5} M). Empty circles, controls; filled circles, in the presence of methohexitone.

Methohexitone reduced these responses [see control (empty circles) and test (filled circles)]. The straight-line regressions were fitted by method of least squares. The slopes of the lines in the control Krebs solution (empty circles) and with methohexitone (filled circles) were 1.52 and 1.09 g/mV respectively. The intercepts on the *Y*-axis were 0.11 and 0.12 g respectively, and the corresponding correlation coefficients were 0.93 and 0.91.

Actions of TEA and methohexitone

TEA (4.8×10^{-4}–4.8×10^{-2} M) produced concn-dependent depolarization and contracture responses in the chick BVC muscle. These responses were potentiated by methohexitone (8.8×10^{-5} M). The time course for the contracture response varied between 6 and 8 min, and the response reached its maximum in about 2–4 min. Maximum depolarization occurred in about 2.5–4.5 min. The mean (± S.E.M.) ED_{50}s for the contractures produced by TEA in the control Krebs solution and with methohexitone were $6.5 \pm 0.03 \times 10^{-3}$ and $1.3 \pm 0.04 \times 10^{-3}$ M (N = 6) respectively. A mean (± S.E.M.) maximum contracture of 0.56 ± 0.02 g (N = 6) was obtained with 2.4×10^{-2} M TEA in the control Krebs solution. The mean (± S.E.M.) ED_{50}s for the depolarizations were $5.9 \pm 0.1 \times 10^{-3}$ and $1.5 \pm 0.06 \times 10^{-3}$ M (N = 6) respectively. A mean (± S.E.M.) maximum depolarization of 0.18 ± 0.01 mV (N = 6) was obtained with TEA (9.5×10^{-3} M) in the presence of methohexitone. Fig. 2 shows the CCCs for TEA-induced responses in the control Krebs solution (3) and with methohexitone (4). Note that methohexitone shifted the TEA CCC to the left (curve 3 to curve 4), indicating potentiation of responses. Similarly, methohexitone also shifted the TEA CDCs to the left (Fig. 4, curve 3 to curve 4). Fig. 4B shows the depolarization (mV) produced by TEA plotted against the contractures (g tension), in the control Krebs solution (empty circles) and with methohexitone (filled circles). Methohexitone did

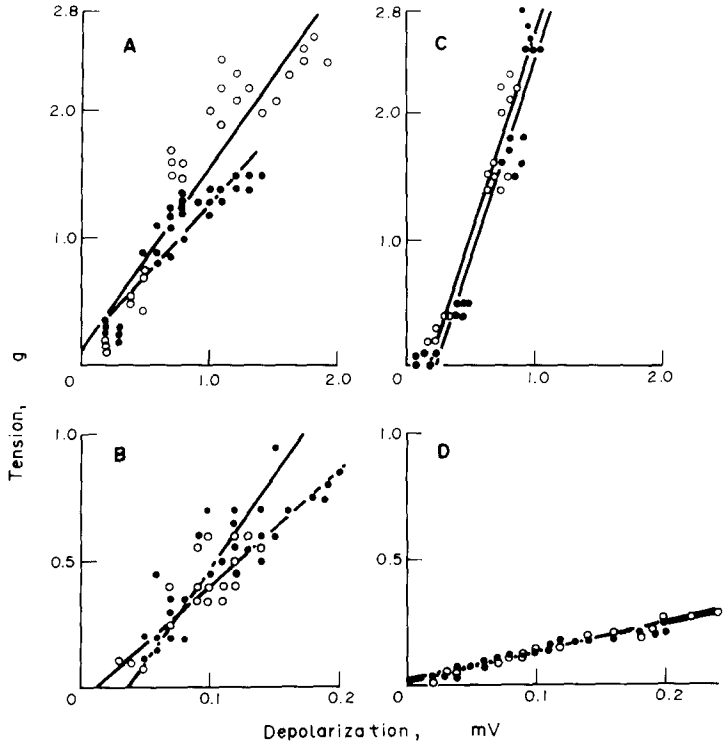

Fig. 4. The relation between the depolarization and contracture tension in the chick BVC muscle: each point represents the depolarization (mV) and tension (g), recorded in drug-induced contracture or contraction. A, contractures elicited by ACh (10^{-6}–10^{-2} M); B, contractures elicited by TEA (10^{-4}–10^{-2} M); C, contractions elicited by repetitive indirect stimulation; D, contractures elicited by low concns of ACh (5.5×10^{-7}–1.1×10^{-5} M). In each graph, empty circles are controls, filled circles are after equilibration with methohexitone (8.8×10^{-5} M). Half circles, coincidence of control and methohexitone points. The straight-line regressions were calculated by the method of least squares. Note the different scales for A and C in contrast to B and D.

not seem to alter the relation between the depolarization and contracture responses, but increased these responses (see empty and filled circles). The straight-line regressions and the slopes of the responses in the control Krebs solution (empty circles) and with methohexitone (filled circles) were 4.69 and 4.31 g/mV respectively. The intercepts on the *Y*-axis were −0.07 and −0.02 g respectively, and the corresponding correlation coefficients were 0.89 and 0.85.

If Fig. 4B is compared with Fig. 4A, it can be seen that the depolarization produced by TEA covers only a small range of the depolarization produced by ACh (note the difference between the two scales). A separate set of experiments was, therefore, undertaken with very low concns of ACh (Fig. 4D), for direct comparison with TEA. The calculated slopes of the regression lines in the control Krebs solution and with methohexitone were 1.26 and 1.11 g/mV respectively. The intercepts on the *Y*-axis were 0.01

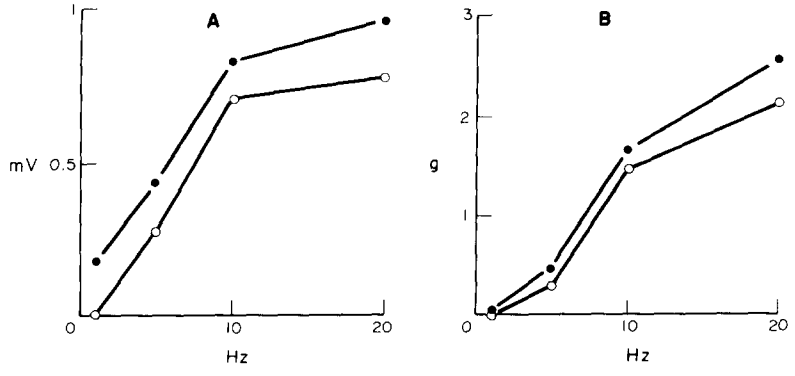

Fig. 5. The effect of methohexitone on the responses produced by repetitive indirect stimulation: A, depolarization vs frequency of stimulation, B, contracture tension vs frequency of stimulation. Empty circles are controls, and filled circles are after equilibration with methohexitone (8.8×10^{-5} M).

and 0.02 g respectively, and the corresponding correlation coefficients were 0.99 and 0.99.

Repetitive indirect stimulation and action of methohexitone

Repetitive indirect stimulation at 1–20 Hz produced frequency-dependent contractions and depolarizations in the chick muscle. These responses were slightly increased by methohexitone (8.8×10^{-5} M). The mean (± S.E.M.) $frequency_{50}$s for the contractions produced in the control Krebs solution and with methohexitone were 9.2 ± 0.1 and 8.5 ± 0.2 Hz (N = 6) respectively. A mean (± S.E.M.) maximum contraction of 2.17 ± 0.04 g tension (N = 6) was obtained by repetitive nerve stimulation at 20 Hz in the control Krebs solution. This was potentiated by methohexitone by 17 ± 1.63% ($P < 0.05$). The mean (± S.E.M.) $frequency_{50}$s for the depolarizations were 7.2 ± 0.1 and 5.8 ± 0.19 Hz (N = 6) respectively. A mean (± S.E.M.) maximum depolarization of 0.78 ± 0.02 mV (N = 6) was produced by 20 Hz in the control Krebs solution and this was increased by methohexitone by 19 ± 1.38% ($P < 0.01$).

Fig. 4C shows the relation between the depolarization (mV) and contraction (g tension) produced by repetitive nerve stimulation in the control Krebs solution (empty circles) and with methohexitone (filled circles). Methohexitone did not seem to alter the relationship but slightly increased these responses (see empty and filled circles). The straight-line regressions and the slopes of the responses produced in the control Krebs solution and with methohexitone were 3.2 and 3.0 g/mV respectively. The intercepts on the *Y*-axis were −0.56 and −0.59 g respectively, and the corresponding correlation coefficients were 0.96 and 0.96. Fig. 5 shows the effect of methohexitone on the depolarization (A) and contraction (B) responses produced by repetitive nerve stimulation at 1–20 Hz. Methohexitone (filled circles) slightly increased these responses.

Comparison between the responses produced by ACh, TEA and repetitive indirect stimulation

In a single experiment, the responses produced by concns of ACh, TEA, and a rate of repetitive stimulation which produced near maximum response, were recorded in the same preparation. ACh (5.5×10^{-4} M) produced 1.9 g tension and 0.8 mV depolarization. TEA (4.8×10^{-3} M) produced 0.33 g tension and 0.07 mV depolarization. Repetitive indirect stimulation at 10 Hz produced 1.47 g tension and 0.52 mV depolarization. Note that TEA produced the smallest contracture and depolarization responses in the chick BVC muscle.

Action of tubocurarine on ACh- and TEA-induced responses

Tubocurarine (1.27×10^{-5} M) reduced the contracture and contraction responses produced by applied and neurally-released ACh [by 62 ± 1.55% (N = 16) ($P < 0.001$)]. Tubocurarine also reduced, but to a lesser extent, the TEA-induced contractures [by 48 ± 0.87% (N = 16) ($P < 0.001$)].

Action of eserine on ACh- and TEA-induced responses

Eserine (1.8 ± 10^{-7} M) increased the contraction, contracture and depolarization responses produced by ACh and TEA [by 173 ± 6.63% (N = 16) ($P < 0.001$) and by 11 ± 0.48% (N = 16) ($P < 0.01$) respectively].

The pharmacological agents, eserine and tubocurarine, were used to ensure that the observed responses were really due to ACh, TEA and repetitive nerve stimulation, and not just due to a change in the cholinesterase activity.

DISCUSSION

Effect of methohexitone on ACh-induced responses

Methohexitone significantly reduced the ACh-induced responses and shifted the log concn–response curves to the right in a non-competitive manner. However, recovery upon washing out the methohexitone was achieved after about 1 hr. Desensitization readily occurred with high concns of ACh. Similar results were obtained by Elliott [2, 13] for the contractures produced by ACh in the chick BVC muscle.

The actions of barbiturates on ACh-induced responses have been studied by many workers. Quilliam [14] showed that barbiturates reduced the ACh-induced contractures in the frog ileofibularis muscle without affecting the response to indirect stimulation. Depolarization responses produced by ACh in the frog toe muscle [15] were also reduced by barbiturates without affecting the muscle action potentials. Adams *et al.* [16], using intracellular recording in the frog sartorius muscle, found that amylobarbitone and thiopentone (2.8×10^{-5} M) abolished the responses to iontophoretically applied ACh without affecting the endplate potential or the miniature endplate potential. They dismissed the possibility that barbiturates may facilitate the release of ACh in view of the known presynaptic depressant actions of barbiturates [17, 18]. Adams [19, 20], using an intracellular recording technique in the frog sartorius muscle, suggested that the duration of the endplate channel open time produced by ACh might be reduced by barbiturates. Prior receptor activation and channel opening by ACh was required for the barbiturate agent to enter the channel and reversibly bind. Adams [21] suggested that the binding site was located within the channel itself. Lee-Son *et al.* [22], working on the guinea-pig lumbrical muscle, using the moving fluid electrode technique, showed that barbiturates reduce the depolarization produced by carbachol. They suggested that, although barbiturates may affect the lipid solubility and protein binding in the muscle membrane, a specific receptor for the action of barbiturates could be involved.

Effect of methohexitone on responses produced by repetitive stimulation

Methohexitone potentiated the amplitudes of the depolarization and contraction responses produced by repetitive indirect stimulation [17 and 19% ($P < 0.05$)], perhaps by enhancing the rate of release of ACh (Westmoreland *et al.* [23]). The present results showed that methohexitone acted differen-

tially on the responses produced by applied and neurally-released ACh, i.e. it greatly reduced the former while it slightly potentiated the latter. Similar results for the potentiation by methohexitone of the contraction responses produced by neurally-released ACh in the chick BVC muscle were obtained by Elliott [2]. Thesleff [24], working on frog sartorius muscle, suggested that pentobarbitone had a greater effect in reducing the responses produced by applied ACh than those produced by neurally-released ACh. Since Adams [21] has suggested that barbiturates block open but not closed ACh channels, it may be that a substantially greater proportion of ACh-controlled channels are opened for a longer period by applied ACh than by ACh released from the nerve terminals. The difference between the slopes of the depolarization and contraction regressions for the applied and neurally-released ACh presumably reflects the greater efficiency of ACh released from the nerve terminal near its receptors when compared with applied ACh. This would imply that depolarization at endplates is more effective than at extrajunctional sites in promoting a rise in intracellular Ca^{2+} concn. The slow muscle of the chick BVC does not normally conduct action potentials when stimulated indirectly so that any advantage of junctional over extrajunctional depolarization in initiating contraction does not relate to action potential production. An alternative explanation is that for a given contracture tension both applied and neurally-released ACh produce the same amount of depolarization, but, whereas applied ACh acts mainly on surface fibres, neurally-released ACh acts on fibres inside the muscle as well as at the surface. The recording technique used may have picked up changes in the surface fibres preferentially. Although this possibility cannot be excluded, it should be noted that the BVC muscle in 1–3-day-old chicks only weighs about 10 mg and is about 1.5 mm thick.

Effect of methohexitone on the responses produced by TEA

There are at least two possible explanations for the action of TEA at the chick BVC neuromuscular junction: (1) TEA may act by facilitating the release of ACh from the nerve terminals; and (2) TEA might be a weak nicotinic agonist, i.e. it has a direct post-synaptic action. The slight potentiation with eserine and reduction by hemicholinium of TEA responses in two experiments, and in the experiments reported by Bell and Wali [25], suggested that TEA, in part, may act by releasing ACh from the nerve terminals. However, after depleting the presynaptic ACh stores by prolonged repetitive nerve stimulation for several hours and in the presence of hemicholinium, TEA still produced small depolarization and contracture responses, indicating that it had a definite post-synaptic action. If TEA acted by releasing ACh, then TEA and ACh should produce the same contractures for equal depolarizations. However, our experiments indicated that TEA produced contractures with 2–3 times less accompanying depolarization than did ACh. This, therefore, suggested that TEA may have other actions apart from producing an increase in presynaptic ACh release and membrane depolarization. Elliott [3] showed that TEA produced contractures in the chick BVC muscle in the presence of a concn of lignocaine which blocked both nerve and muscle action potentials, and ACh-induced contractures. It is possible that TEA could produce contractures by increasing Ca^{2+} influx into slow muscle fibres. If this Ca^{2+} were to trigger further Ca^{2+} release from the sarcoplasmic reticulum, then the actual Ca^{2+} influx might involve relatively little movement of charge across the membrane resulting in only a small membrane depolarization. Methohexitone is known to facilitate caffeine-induced contractures [3], suggesting that methohexitone reduces Ca^{2+} uptake by the sarcoplasmic reticulum. Thus if TEA directly increases Ca^{2+} influx, methohexitone might potentiate TEA-induced contractures by reducing the rate at which this Ca^{2+} is sequestered. In so far as TEA has an indirect action, by releasing ACh from nerve terminals, the increase in miniature endplate potential frequency produced by methohexitone [23] suggests that methohexitone also might facilitate the action of TEA on the presynaptic nerve terminals. This would account for the slight facilitation by methohexitone of the depolarization produced by TEA. A more extensive discussion of the evidence for a direct and indirect actions of TEA at the neuromuscular junction has recently appeared [26].

In conclusion, by simultaneously recording the contracture and depolarization it has been shown that TEA produces contractures which are accompanied by relatively little membrane depolarization. This is in contrast to the contractures produced by ACh and repetitive indirect stimulation. Methohexitone potentiates the contractures produced by TEA and repetitive indirect stimulation, while it reduces those produced by applied ACh.

It is hoped that the present extracellular recording results would be confirmed by using a direct intracellular recording technique at the level of membrane conductance and intracellular calcium concn.

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