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ACTIONS OF GALLAMINE AND TETRAETHYLAMMONIUM AT THE FROG NEUROMUSCULAR JUNCTION

BY

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As judged by the dose-response curves derived from the isolated frog rectus preparation to acetylcholine, tetraethylammonium shows both competitive and non-competitive forms of antagonism (Van Rossum & Ariëns, 1959). In addition to a depressant action at the frog neuromuscular junction, Koketsu (1958) has produced evidence suggesting that tetraethylammonium increased the release of acetylcholine following a single nerve stimulus and that, after such a stimulus, this drug produced repetitive firing in the motor nerve by a prejunctional action. The neuromuscular blocking action of gallamine is very similar to that produced by tubocurarine (Riker & Wescoe, 1951 ; Randall, 1951) and a competitive anti-acetylcholine action on the frog rectus muscle has been demonstrated by Van Rossum (1963).

The experiments described here show that, in addition to a competitive action at the frog neuromuscular junction, gallamine has a prejunctional action similar to that of tetraethylammonium.

METHODS

The isolated extensor longus digiti IV muscle (the toe muscle) preparation of the frog was used in the manner described by Fatt (1950) and Nicholls & Quilliam (1956). Preparations showing an injury potential of more than 1 mV were discarded. Recordings were made through non-polarizable silver/silver chloride/agar electrodes which fed through a balanced cathode follower to a DC amplifier, the signals being displayed on an oscilloscope equipped with a camera.

The end-plate potential of the M. extensor longus digiti IV could be recorded either by lowering the fluid-air interface to the region of maximal end-plate density (Castillo & Stark, 1952) or by lowering the upper electrode to the region of maximal end-plate density and lowering the fluid-air interface to the lower end of the muscle. (It was hoped by this latter technique to eliminate any variation in position of the end-plate electrode that might occur when using the fluid-air interface as an electrode and so facilitate comparison of end-plate potentials in the same preparation.) The nerve was stimulated by supramaximal rectangular pulses of 0.1 msec duration.

The effects of the drugs on nerve conduction were assessed separately. An isolated length of frog sciatic nerve with its peroneus lateralis branch was mounted vertically in the same bath. When recording, the fluid-air interface was lowered to the cut distal end and the upper recording electrode placed approximately 5 mm above the meniscus. Platinum stimulating electrodes were placed proximally on the nerve. Owing to the small size of the nerve to the M. extensor longus digiti IV, it was not possible with this technique to record solely from the nerves innervating this muscle.

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With the toe muscle mounted distal end uppermost, a pair of additional stimulating electrodes could be placed directly on the muscle. Placing the upper recording electrode in the region of maximal end-plate density and lowering the meniscus to the lower end of the muscle then permitted recording of the response to either direct or indirect stimulation. For direct stimulation rectangular pulses of 1.0 msec duration and of supramaximal intensity were used.

Intracellular recordings were made on the isolated frog sartorius preparation as described by Fatt & Katz (1951). Drugs could be washed out or added to the bath without disturbing the inserted microelectrode. Glass micro-capillary electrodes of 6 to 12 megohms resistance were used. The well containing the platinum stimulating electrodes was isolated from the bath Ringer solution so that the drugs did not come into contact with the cut end of the nerve.

Antidromic activity in the motor nerve was detected by mounting the M. extensor longus digiti IV preparation as shown in Fig. 1. When recording, the frog Ringer solution surrounding the muscle was drained leaving the muscle and its recording electrodes suspended in air.

All concentrations are final bath concentrations expressed as g/ml. of tetraethylammonium bromide, tubocurarine chloride (B.W.) and gallamine triethiodide (M. and B.).

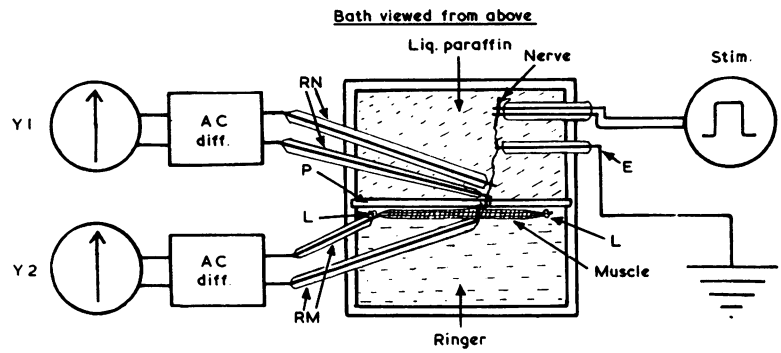

Fig. 1. Diagram of the apparatus for simultaneous recording of nerve and muscle activity in the M. extensor digiti IV. RN=platinum recording electrodes on nerve; RM=platinum recording electrodes on muscle; E=earth electrode; P=perspex partition dividing the bath. The muscle was suspended on the platinum loops L. AC diff, differential amplifier channel feeding to either the upper or lower beams, Y1 and Y2, of a Cossor oscilloscope, model 1049. Stim, electronic stimulator leading to platinum stimulating electrodes.

RESULTS

Effects on carbachol depolarization curves

Curves relating the depolarization of the end-plate region of the toe muscle to the concentration of carbachol added to the bath were constructed both in the presence and in the absence of gallamine or tetraethylammonium, and each was compared in the same preparation with that constructed in the presence of tubocurarine. As a control dose-response curves were also constructed after washing out the test drug or the tubocurarine. As no appreciable deviation of these curves from the original control occurred they are not illustrated. The concentration of blocking drug was gradually increased until the response to nerve stimulation was an end-plate potential uncomplicated by a muscle spike. The development of depolarization to added carbachol was then followed by photographing responses at 5, 15, 30, 60 and 120 sec after its addition to the bath fluid. The maximal response recorded was used in the construction of the dose-response curves.

Just blocking concentrations of gallamine or tubocurarine (namely the lowest concentrations which produced an end-plate potential uncomplicated by muscle action potentials) each produced similar shifts of the dose-response curve to the right (Fig. 2,*a*). No increase in the level of depolarization necessary to initiate action potentials was observed. In only one experiment out of three in which the just blocking concentrations of tetraethylammonium were used was it possible to produce depolarization by added carbachol and then the effect obtained was small as illustrated in Fig. 2,*b*. Sub-blocking concentrations of tetraethylammonium produced some shift of the dose-response curve to the right but this was always accompanied by marked flattening of the curve.

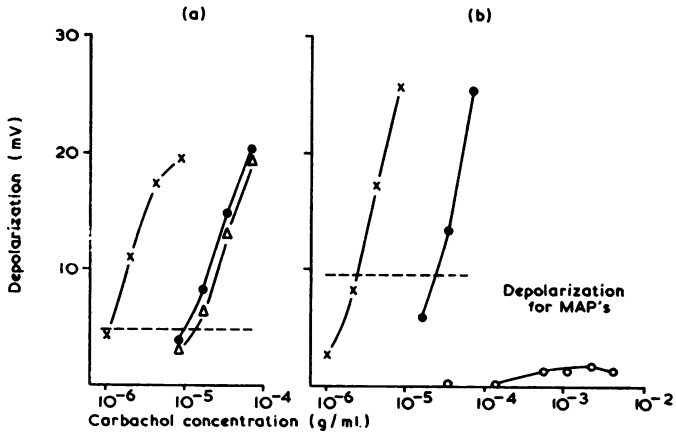

Fig. 2. M. extensor longus digiti IV. Carbachol dose-depolarization curves. Crosses, controls; solid circles, in the presence of tubocurarine 5×10^{-6}; triangles, in the presence of gallamine 1×10^{-4}; open circles, in the presence of tetraethylammonium 3×10^{-3}. The concentrations of tubocurarine, gallamine and tetraethylammonium used were the lowest found to abolish the muscle action potential evoked by nerve stimulation so leaving an uncomplicated end-plate potential. The depolarization required to initiate action potentials is indicated by the dotted line.

The mean concentrations found necessary to just block neuromuscular transmission of the muscle extensor longus digiti IV were $1.2\pm0.6\times10^{-4}$ g/ml. of gallamine ($n=13$) and $3.8\pm0.3\times10^{-3}$ g/ml. of tetraethylammonium ($n=9$).

End-plate potentials

Extracellular recording of the end-plate potentials after gallamine and tetraethylammonium showed small variations in the amplitudes and time courses when compared with those seen after tubocurarine in the same preparation (Fig. 3). The end-plate potentials were measured to the nearest 0.1 mV, the times from onset to peak of rise and onset to half decay were measured to the nearest msec and the difference between these parameters and those seen after tubocurarine in the same preparation was analysed for significance. There was no statistically significant difference in the amplitudes of gallamine or tetraethylammonium end-plate potentials when compared with tubocurarine.

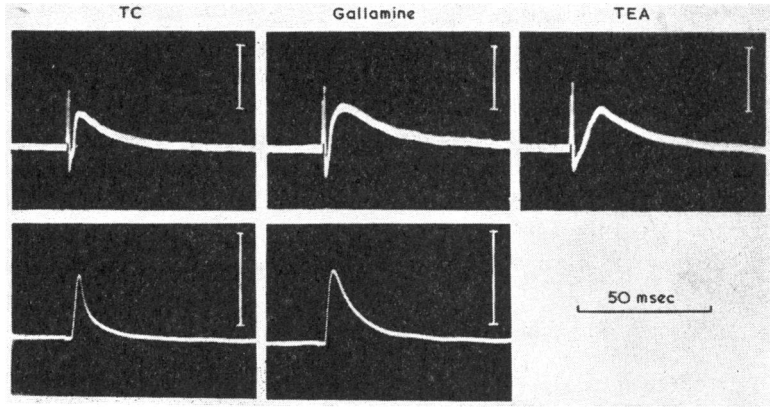

Fig. 3. Upper row: M. extensor digiti IV. Extracellular recording of end-plate potentials, preceded by nerve action potentials in the presence of tubocurarine 3.5×10^{-6}, gallamine 1×10^{-4} and tetraethylammonium 4×10^{-3}. Voltage calibration 2 mV. Lower row: M. sartorius. End-plate potentials recorded intracellularly after tubocurarine 4×10^{-6} and gallamine 1×10^{-4}. Voltage calibration 10 mV.

However, the time courses of the end-plate potentials after both gallamine and tetraethylammonium, as measured by the differences in the rise and half decay times, were significantly longer (gallamine, $P<0.001$, $n=13$; tetraethylammonium, $P<0.01$, $n=9$). Mean amplitudes, rise and half decay times with their standard errors are given in Table I. The more prolonged end-plate potential in the presence of gallamine was also recorded intracellularly in M. sartorius (Fig. 3, lower record).

TABLE 1

END-PLATE POTENTIAL AMPLITUDES, RISE TIMES AND TIMES TO HALF DECAY

Drug	Amplitude mV	Rise time msec	Time to half decay msec	n
Tubocurarine	1·5±0·1	2·3±0·1	8·7±0·6	59
Gallamine	1·0±0·1	5·4±1·1	21·0±2·4	13
Tetraethylammonium	1·0±0·2	9·4±1·4	29·8±3·7	9

Repetitive firing

In addition to the effects described above all the experiments with tetraethylammonium and eleven out of twenty-one with gallamine showed evidence of repetitive firing after a single nerve stimulus. This repetitive firing in the toe muscle could be recorded after treating with both blocking and sub-blocking concentrations of the two drugs. The repetitive nature of the response was usually more marked at sub-blocking concentrations when it appeared as multiple muscle action potentials, but repetitive end-plate potentials could be distinguished (Figs. 4 and 7). Control records taken on the same preparations showed no repetitive firing in the presence of tubocurarine. Similar responses were recorded in sartorius muscle fibres under conditions of complete and partial block (Fig. 5).

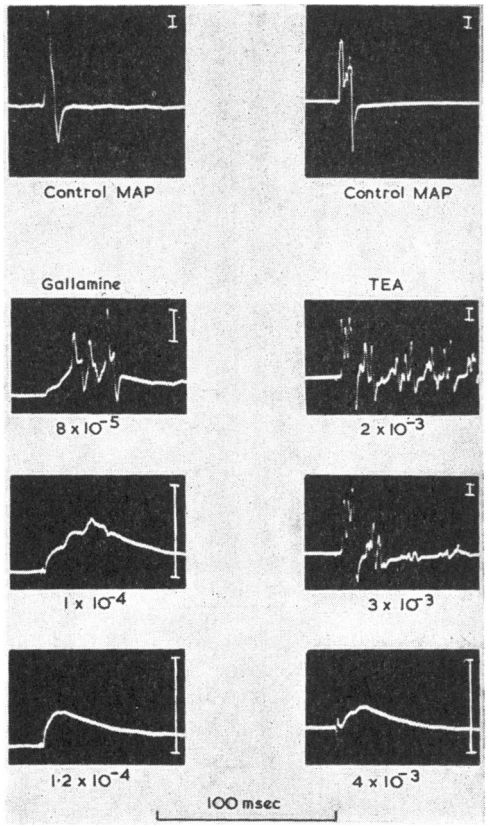

Fig. 4. M. extensor digiti IV. Extracellular recordings of end-plate potentials and of muscle action potentials at the end-plate region in response to maximal single stimuli to the motor nerve before and after increasing concentrations of gallamine (left-hand column) and tetraethylammonium (right-hand column) in two different preparations. Voltage calibration 4 mV.

Effect of neostigmine on block

The ability of neostigmine to reverse any block was assessed by adding 3×10^{-6} g/ml. of neostigmine to the bath containing the blocking drug and allowing the neostigmine to act for half an hour before stimulating. A reversal of the gallamine block was shown by the production of action potentials instead of an end-plate potential. The block by tetraethylammonium was unaffected by neostigmine. In gallamine-treated preparations, those showing repetitive responses to gallamine alone responded by showing a marked potentiation of this effect when neostigmine was added to the bath. Preparations which had not responded repetitively to gallamine alone, all showed repetitive firing after the neostigmine (Figs. 5 and 6). In two experiments in which eserine (10^{-5} g/ml.) was used in place of neostigmine, a similar potentiation of the repetitive firing after gallamine was observed. Increasing the gallamine concentration restored the block and an increase in the time course of the end-plate potential was noted. Half decay times were increased from 27.7 to 58.7 msec (mean of five experiments). Control experiments in the same

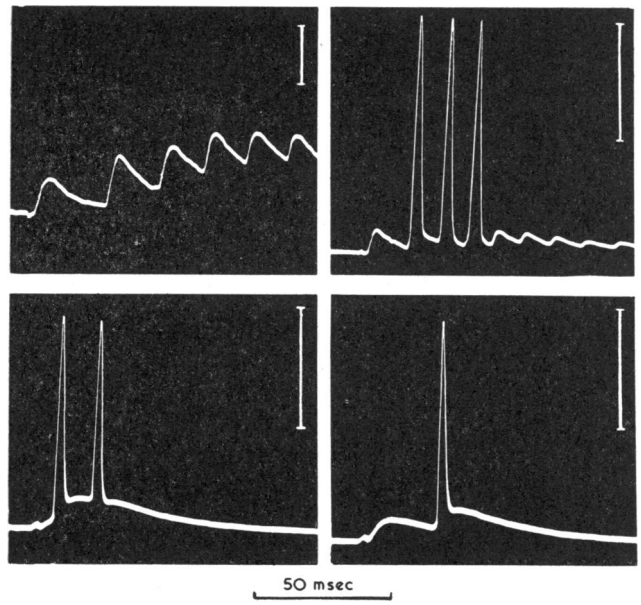

Fig. 5. M. sartorius. Repetitive end-plate and muscle action potentials recorded intracellularly in response to a single maximal stimulus to the motor nerve after gallamine and gallamine+ neostigmine. Top left: gallamine 8×10^{-5}. Voltage calibration 10 mV. Top right: partial block in another fibre of the same muscle. Voltage calibration 50 mV. Bottom: varying response of the same fibre to different stimuli, gallamine 1.6×10^{-4} and neostigmine 3×10^{-6}. Voltage calibration 50 mV.

preparations produced, as expected, a reversal of a tubocurarine block by neostigmine but no repetitive discharge of a similar nature was ever seen. Addition of further tubocurarine to restore block also unmasked an increase in the time course of the end-plate potential half decay time, 9 to 16 msec (mean of thirty experiments). Thus the end-plate potentials produced by gallamine were longer than those seen in the presence of neostigmine plus tubocurarine. Although there was no reversal of a block produced by tetraethylammonium in the presence of neostigmine, the time course of the end-plate potentials was also prolonged from 19 to 25 msec (mean of three experiments).

Nerve conduction

Nerve action potentials recorded from three isolated frog sciatic nerve preparations showed no evidence of repetitive firing after the addition of 4 or 8×10^{-3} tetraethylammonium. Similarly no repetitive firing was seen in three other preparations treated with 1 and 2×10^{-4} gallamine.

Muscle action potentials following direct stimulation

Four experiments were made to assess the responses to direct muscle stimulation after tetraethylammonium. None of these showed repetitive firing after direct stimulation

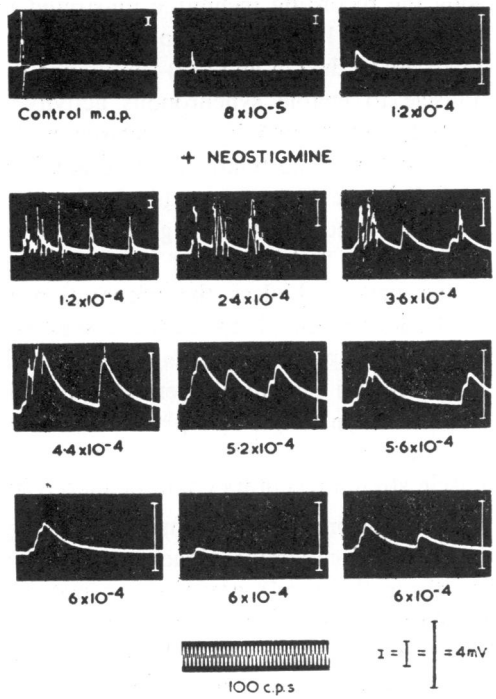

Fig. 6. M. extensor digiti minimi IV. The effect of neostigmine on block by gallamine. The upper row of records shows the response before, during partial block, and at complete block by gallamine. The lower three rows show the responses after addition of neostigmine 3×10^{-6} and at increasing gallamine concentrations. Voltage calibration 4 mV.

although all responded to indirect stimulation by firing repetitively. Another four preparations failed to show repetitive responses to direct muscle stimulation after gallamine but none of these responded repetitively to nerve stimulation either.

Effect on miniature end-plate potentials

Both gallamine and tetraethylammonium completely abolished the miniature end-plate potentials of sartorius muscle fibres at concentrations found necessary to produce neuromuscular block. When using between one-hundredth and one-tenth of the concentrations necessary to produce neuromuscular block, the degree of depression in the amplitude of the miniature end-plate potentials occurring made it difficult to assess change in frequency. However, concentrations which reduced the amplitude without abolishing the miniature end-plate potentials completely did not appear to cause any gross change in frequency.

Antidromic nerve action potentials after gallamine

Although the preceding results suggest that the repetitive firing seen after gallamine or tetraethylammonium is due to an action in the region of the nerve terminals, the responses in the M. extensor longus digiti IV could be due to an action at the cut end

of the motor nerve. Using the recording technique illustrated in Fig. 1, this possibility may be eliminated in two ways: (1) by immersing the motor nerve in liquid paraffin so that the drugs have no access to the cut end ; (2) simultaneous recording of the activity in the nerve and the muscle to enable synchronous activity to be detected and the direction of any such activity determined by comparison with the orthodromic nerve spike set up by the nerve stimulus. The relatively large amplitude of the muscle action potential led to " cross talk " between the two sets of recording electrodes. When the muscle action potential was not blocked by a drug, the shape of the orthodromic nerve action potential was distorted by " pick-up " of the muscle action potential by the recording electrodes on the nerve. Unless the preparation were almost completely blocked, it was impossible to discriminate between the true nerve activity and this muscle artifact. However, when only end-plate potentials or very small action potentials were being initiated in the muscle, it was easy to differentiate slow distortion of the base line on the nerve recording channel due to the end-plate potentials from nerve spikes. In these records the gain of the nerve recording channel was such that the nerve action potentials initiated by muscle stretch receptors could be clearly seen.

The responses from a preparation of M. extensor longus digiti IV which did show repetitive firing in the presence of gallamine alone are shown in Fig. 7. At amplifications adequate to display the orthodromic nerve action potential following stimulation no additional activity synchronous with the repetitive response in the muscle could be detected (Fig. 7,*a*). Greater amplification was necessary to display antidromic activity in a few nerve fibres supplying the muscle and, at such gains, nerve action potentials arising from stretch receptors in the absence of stimulation are seen in Fig. 7,*b*. At this

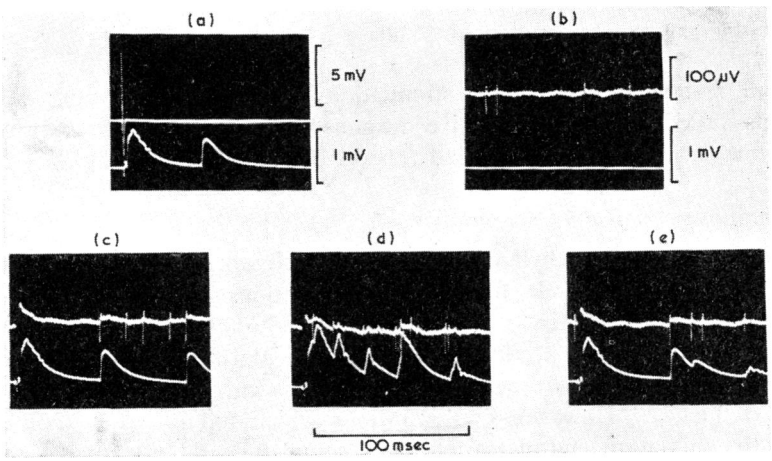

Fig. 7. M. extensor digiti IV. Antidromic nerve action potentials during gallamine (1.2×10^{-4}) block. Upper trace, nerve response ; lower trace, muscle response. *a*, At low gain only a single orthodromic nerve action potential is visible ; *b*, at high gain and in the absence of nerve stimulation ascending action potentials from stretch receptors are seen ; (*c*, *d* and *e*, after similar high gain and following nerve stimulation, additional antidromic nerve action potentials, synchronous with the post-synaptic activity, can be seen. Voltage calibrations for *c*, *d* and *e* as for *b*.

amplification, after a single stimulus to the motor nerve, additional action potentials of similar amplitude and direction are seen (in the upper trace of Fig. 7,*c*, *d* and *e*) synchronous with post-junctional activity visible in the lower trace. In the preparation illustrated in Fig. 7, 88 records showing repetitive activity were taken, and in these 158 repetitive post-junctional responses were counted, each of which was associated with a synchronous action potential passing antidromically up the nerve. In addition, 140 other nerve action potentials, due presumably to stretch receptor activity, were seen.

In one experiment it was possible to cut one of the final terminal branches of the motor nerve to the muscle and thus to abolish the spontaneous antidromic action potentials without destroying the muscle response to nerve stimulation. Treatment with tetraethylammonium 2×10^{-3} g/ml. then produced antidromic firing after complete abolition of the muscle response to nerve stimulation.

DISCUSSION

The action of gallamine at the neuromuscular junction has generally been held to consist simply of a post-junctional competitive anti-acetylcholine action similar to that of tubocurarine (Riker & Wescoe, 1951; Randall, 1951) whereas, in frog muscle, tetraethylammonium is known to have both pre- and post-junctional actions (Koketsu, 1958). An increase in twitch tension after low doses of gallamine was initially reported by Riker & Wescoe (1951) in experiments on cats, and more recently Jones & Laity (1965) have reported an increase in twitch tension of the rat diaphragm preparation after both gallamine and tubocurarine; this effect was related to repetitive post-synaptic activity, but no pre-synaptic activity was detected. The results of the latter workers were found only when stimulating the nerve with pulses whose durations were much longer than those usually necessary to initiate a single nerve action potential.

The similar shifts of the dose-depolarization response curves after tubocurarine and gallamine described in this paper confirm a competitive antagonist action for gallamine. Some evidence for an anti-acetylcholine action of tetraethylammonium at the neuromuscular junction is also presented, but an additional post-synaptic depressant action appears to be involved when using the extremely high concentrations necessary to produce a complete block of transmission. The repetitive post-synaptic responses after treatment with either gallamine or tetraethylammonium were similar in many respects. Both appeared to be due to a pre-synaptic event as shown by the repetitive end-plate potentials. Koketsu (1958) has reported repetitive antidromic pre-synaptic activity in frog muscle after tetraethylammonium following orthodromic nerve stimulation. No repetitive post-synaptic activity associated with this effect was reported but his preparations were also heavily curarized and this could have masked any post-synaptic response. Hagiwara and Watanabe (1955) have shown that tetraethylammonium gives rise to repetitive firing of directly stimulated toad muscle fibres and the frequency of the repetitive action potentials decreased with high concentrations of tetraethylammonium. Repetitive firing of isolated frog sciatic nerve preparations was originally described by Cowan & Walter (1937), but such an action was not detected in our experiments and, if a type of firing similar to that described by them had occurred in our experiments, it is doubtful if it could have accounted for the type of repetitive post-synaptic responses

which we observed with tetraethylammonium, for the responses often continued for as long as 0.5 sec after the single initial stimulus. The repetitive responses observed in muscle (Hagiwara & Watanabe, 1955) and nerve (Koketsu, 1958) were associated with a prolongation of the falling phase of the action potentials. Repetitive post-synaptic responses associated with synchronous antidromic nerve action potentials which also showed a prolongation of the falling phase have been described by Burke, Katz & Machne (1953) in crustacean nerve muscle preparations treated with tetraethylammonium. An effect on the form of the action potentials in the pre-synaptic terminals was postulated by Koketsu (1958) as a possible mechanism to account for an increase in acetylcholine release after tetraethylammonium. Such an effect might also prolong the duration of release and alter the time course of the end-plate potential. It is unlikely that the longer durations of the end-plate potentials observed in the presence of gallamine and tetraethylammonium are due to an anticholinesterase action, as the time courses with each drug were longer than those observed after tubocurarine and neostigmine. It is possible, however, that the concentration of neostigmine used did not completely abolish cholinesterase activity, but it was not possible to use higher concentrations of the anti-cholinesterase as these depress the depolarization response to acetylcholine.

The potentiation by neostigmine and physostigmine of the repetitive firing seen after gallamine suggests that the released acetylcholine may also play a role in initiating the repetitive response. Douglas & Ritchie (1960) have shown that acetylcholine can excite both myelinated and non-myelinated nerve fibres and this effect is more marked in non-myelinated C fibres. It is therefore possible that the increased quantity of acetyl-choline persisting after cholinesterase inhibition could initiate impulses by exciting the non-myelinated nerve terminals.

There is a good deal of evidence that anticholinesterase drugs alone can cause repetitive back responses in mammalian motor nerve-muscle preparations (Masland & Wigton. 1940; Feng & Li, 1941; Lloyd, 1942; Eccles, Katz & Kuffler, 1942). More recent studies by Riker, Roberts, Standaert & Fujimori (1957), Werner (1960a & b, 1961) and Riker, Werner, Roberts & Kuperman (1959) have shown that two types of back response can be detected in mammals after treatment with neostigmine analogues. The first can be initiated by post-synaptic events and the second is due to a pre-synaptic action. These effects have not been detected in frog muscle preparations, but the possibility that in our experiments neostigmine potentiated the action of gallamine by a similar additive action cannot be excluded. The failure of neostigmine to affect the response after tetraethylammonium would be explained if the effect of tetraethylammonium were already maximal.

It is interesting to note that triethylcholine, which has structural similarities to gallamine, has been reported by Roberts (1962) to produce repetitive firing in frog muscle and also appears to increase the amount of acetylcholine released by a single stimulus.

SUMMARY

1. The action of gallamine and tetraethylammonium bromide on frog neuromuscular transmission was investigated by extra- and intra-cellular electrical recording techniques.

2. Gallamine produced a typical competitive shift of the carbachol dose-depolarization curve.

3. The carbachol dose-depolarization curves produced in the presence of tetraethylammonium showed some shift to the right but were also associated with an additional post-synaptic depressant effect.

4. End-plate potentials seen after gallamine or tetraethylammonium were longer in duration than those seen after tubocurarine.

5. After tetraethylammonium and sometimes after gallamine a single nerve stimulus led to repetitive post-synaptic responses.

6. Neostigmine and eserine potentiated or produced a repetitive response to gallamine.

7. The repetitive post-synaptic responses seen after gallamine or tetraethlyammonium were associated with synchronous antidromic activity in the motor nerve.

8. These findings are discussed in relation to possible mechanisms of action on the pre-synaptic nerve terminals.

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