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NEUROMUSCULAR ACTIVITY OF THE TRIETHYL ANALOGUE OF CHOLINE IN THE FROG

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Hunt & Taveau (1909) demonstrated the toxicity of triethylcholine chloride when injected into animals, and included muscular weakness amongst the symptoms of the poisoning. Keston & Wortis (1946) assumed that the toxicity was due to competition with choline and demonstrated in mice that the administration of choline protected the animal against the toxic action of its triethyl analogue. These authors also reported that triethylcholine blocked the response of frog gastrocnemius and rectus abdominis muscles to choline but not to acetylcholine. More recently, Bowman & Rand (1961) confirmed the protective action of choline and considered the possibility that triethylcholine might act by interfering with the synthesis of acetylcholine. They showed that in conscious rabbits triethylcholine produced a weakness of voluntary muscle resembling that of myasthenia gravis, being increased by exercise and decreasing again during a rest period. Similar results were obtained in the isolated rat diaphragm and in tibialis anterior muscle preparations in rabbits and cats; in each case the effect was reversed by choline. Bowman & Rand also showed that close arterial injection of acetylcholine produced a contraction of the tibialis anterior muscle of the cat during the paralysis following the administration of triethylcholine.

In view of these results, it seemed worth while to investigate the action of triethylcholine at the neuromuscular junction by the more precise techniques of intracellular recording; this paper is concerned with the results obtained from 'fast' muscle fibres of the frog.

METHODS

Sartorius and extensor longus digiti IV nerve–muscle preparations from English frogs were used during these experiments, which were carried out during the months March–April at room temperatures of 17–20° C.

Solutions. The Ringer's fluid had the following composition, expressed in m-mole/l.; NaCl 115; KCl 2·5; $CaCl_2$ 1·8; Na_2HPO_4 2·15; NaH_2PO_4 0·85. Solutions of triethylaminoethanol (triethylcholine, TEC; Ward, Blenkinsop) were prepared daily, in concentrations of 10 mg TEC in 1 ml. of Ringer's fluid. The effect of TEC at the neuromuscular junction was

tested after the addition of 0·5 ml. of this solution to the 25 ml. of Ringer's fluid in the muscle bath so that the final concentration of TEC was 200 μg/ml., which is approximately 1 m-mole/l.

In some experiments the mechanical response of the muscle was abolished with D-tubocurarine chloride (Burroughs Wellcome and Co.) in a concentration which permitted observation of end-plate potentials evoked by nerve stimulation. In such cases, the TEC solution was made up in the same Ringer-curare fluid so that addition of TEC to the muscle bath did not alter the curare concentration.

The experiments on the quantal response of the neuromuscular junction were carried out with a modified Ringer's fluid containing less Ca than normal and with additional Mg, in the manner described by del Castillo & Katz (1954*a*), the same modified Ringer's fluid being used to make up the solutions of TEC.

Intracellular records of e.p.p.s, miniature potentials and ACh potentials were obtained by methods similar to those described by Fatt & Katz (1951, 1952) and by del Castillo & Katz (1955).

Muscle twitches in response to low-frequency indirect stimulation were recorded either with an isotonic lever and smoked drum, or with an RCA transducer valve, the amplified output of which was used to drive a Record type penwriter.

RESULTS

The effect of TEC on miniature end-plate potentials

Intracellular recordings were made at neuromuscular junctions in the sartorius muscle and it was found that TEC reduced the amplitude of the miniature potentials without appreciably altering their frequency. Figure 1 shows a typical set of records made before and after TEC had been added to the bath, as well as a control series after the drug had been washed out from the preparation.

These results were consistent with a direct action of TEC on the acetylcholine-synthesis mechanisms, which appears to produce the transmitter substance in 'packets' of a fairly uniform size. A depression of this mechanism might well operate by reducing the number of ACh molecules contained in each 'packet', which would then produce a smaller miniature potential. Alternatively, the reduced size of the miniature potentials could have been due to a decreased sensitivity of the post-junctional membrane to ACh; and the next series of experiments were carried out to test this possibility.

TEC and acetylcholine sensitivity of the neuromuscular junction

The sensitivity of a single neuromuscular junction to acetylcholine can be tested by applying small quantities of it by iontophoresis from a micropipette, the tip of which is placed close to the junction, while at the same time a second micropipette placed intracellularly close to the end-plate is used to record the resulting depolarization. When the quantity of acetylcholine applied and the site of application do not change during the experi-

ment, an alteration in the amplitude of the depolarization reflects a change in the sensitivity to acetylcholine.

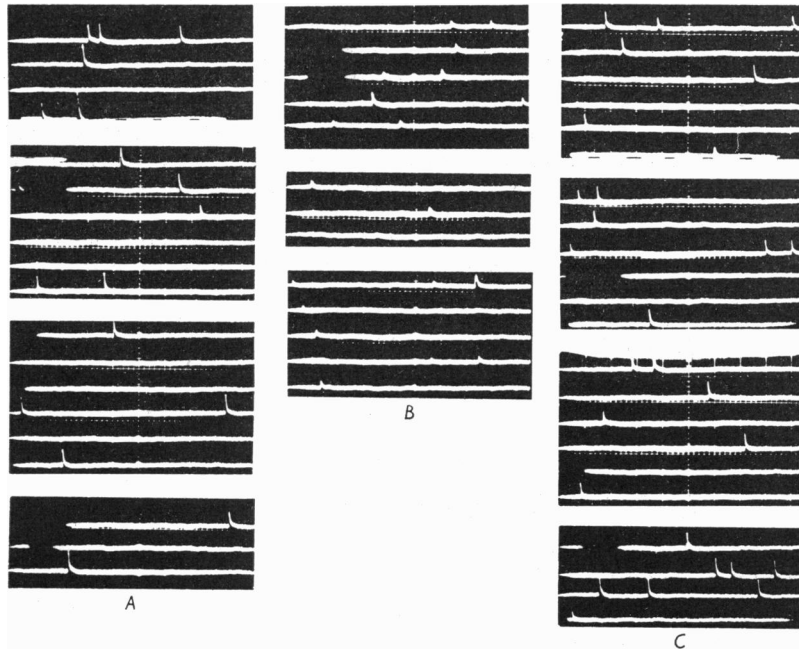

Fig. 1. Miniature potentials recorded at one end-plate in a sartorius muscle. *A*, before adding TEC; *B*, 2 min after the addition of TEC (200 μg/ml.); *C*, 5 min after washout of the TEC. Vertical distance between traces = 1 mV.

The results of two such experiments are shown in Fig. 2. In the presence of TEC the acetylcholine potentials, and the miniature end-plate potentials also recorded, are reduced in amplitude. The decrease in the size of the miniature potentials therefore appears to be a post-junctional effect of TEC, resembling that of curare, and not to be due to any change in the amount of acetylcholine released in each packet. In the concentrations used in these experiments TEC did not alter the resting membrane potential.

Effect of TEC on the mechanical response of a nerve–muscle preparation

In view of the depression of sensitivity of the neuromuscular junction to acetylcholine by TEC, it was expected that the drug would have a blocking action on the nerve–muscle preparation. Confirmation of this was sought by recording the contractions of an extensor longus digitorum IV muscle produced by slow single-shock indirect stimulation. It was found (Fig. 3) that in presence of TEC the twitch height in response to each stimulus was greater than that obtained in the control periods before and

after TEC. A similar effect was observed in the sartorius muscle. Other experiments on the same preparations after curare had been given showed that in fact TEC would restore conduction after a complete block had been achieved with curare (Fig. 4). It was clear, therefore, that the over-all action of TEC on the neuromuscular junction was one of facilitation, in spite of its depressant action on the sensitivity of the end-plate to acetylcholine.

Two possible mechanisms were considered to explain the increase in the twitch tension occurring with TEC, both of which depend on an increase in the depolarization at the end-plate in the presence of TEC. Thus, in the case of a junction with an end-plate potential insufficient in amplitude to excite the adjacent muscle membrane, TEC might act by increasing the

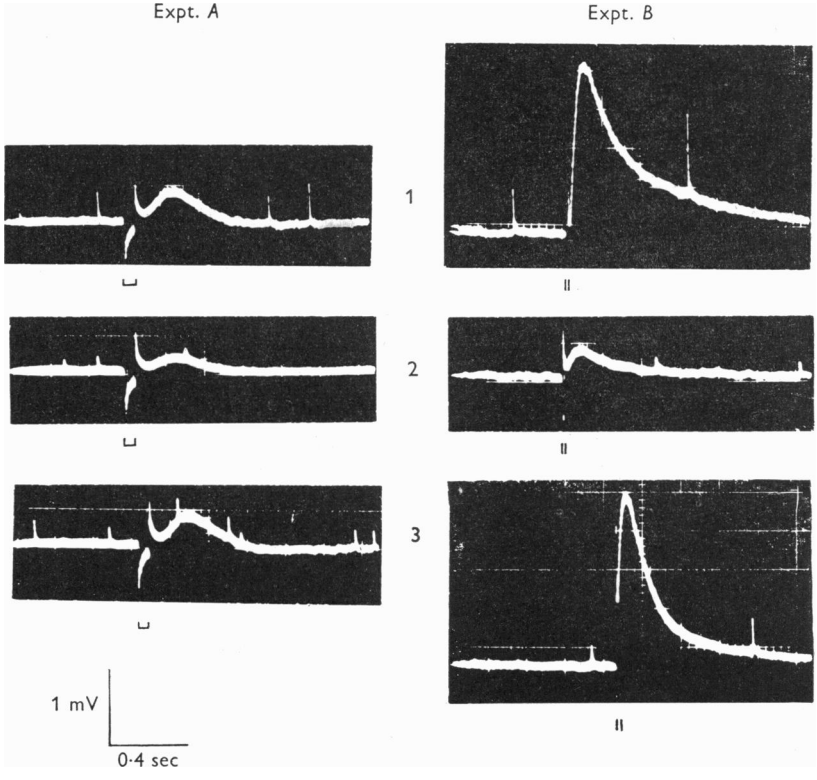

Fig. 2. Recordings of potential changes at two end-plates of m. sartorius in response to iontophoretic application of acetylcholine. Reading from above downwards in each experiment the records are of the response (1) before TEC, (2) 2 min later, in the presence of TEC (200 μg/ml.), and (3) 2 min after one change of Ringer's fluid in the muscle bath. The duration and amplitude of the ACh pulse were kept constant throughout each experiment, and the moment of application of the ACh is indicated by the mark under each trace. Calibration, 1 mV and 0·4 sec.

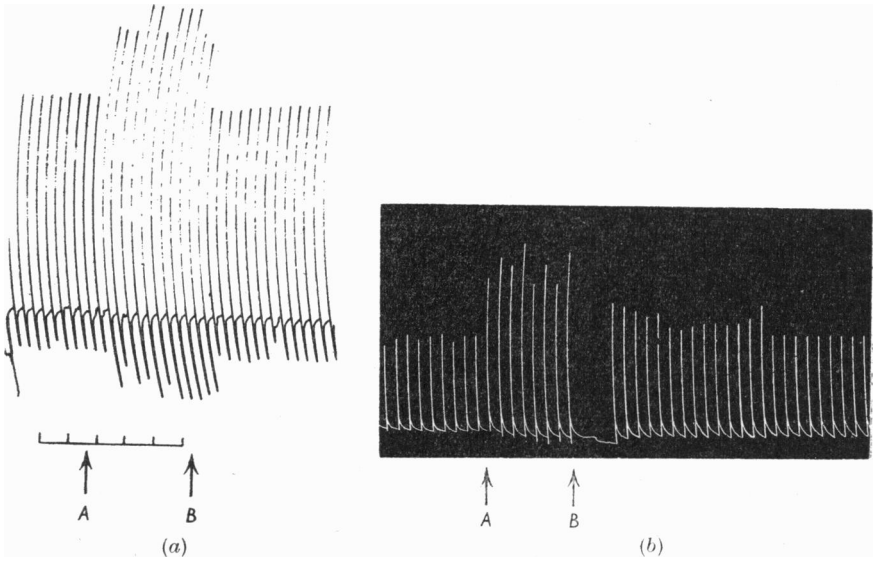

Fig. 3. (*a*) Isometric twitches of m.ext. l. dig. IV evoked by supramaximal nerve stimuli applied at a rate of 3/min. At *A*, TEC added to the bath, in a final concentration of 200 μg/ml. At *B*, muscle bath washed out. Time marker 1 min. (*b*) Isotonic twitches of m. sartorius evoked by supramaximal nerve stimuli applied at a rate of 3/min. At *A*, TEC added to the bath, in a final concentration of 200 μg/ml. At *B*, muscle bath washed out.

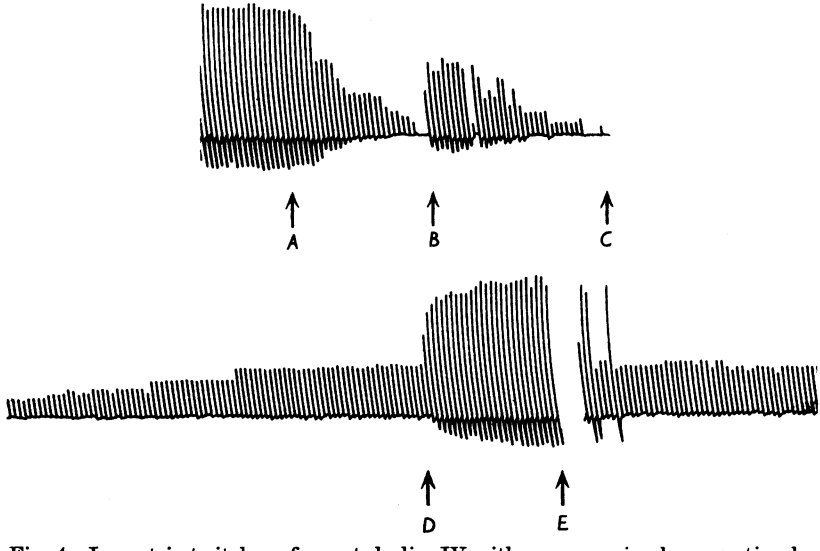

Fig. 4. Isometric twitches of m.ext. l. dig. IV with supramaximal nerve stimulation at a rate of 3/min. At *A*, tubocurarine added (3 μg/ml.). At *B*, TEC added (200 μg/ml.). At *C*, muscle bath washed out and recording continued in lower trace. At *D*, TEC added (200 μg/ml.) during recovery from curarization. At *E*, muscle bath washed out.

depolarization and so bring the muscle fibre into action. Alternatively, when transmission is already effective an increase in end-plate depolarization by TEC could give rise to multiple firing of the muscle fibre and an increase in twitch tension by summation of contractions.

TEC and the end-plate potential

Further information regarding the nature of the facilitatory action of TEC was sought by making intracellular recordings of end-plate potentials in a partly curarized sartorius muscle. It was found that addition of TEC to the bath increased the amplitude of the end-plate potential by a factor of about three. Thus, as shown in Fig. 5, recordings made at 10 sec intervals after the addition of TEC show a gradual increase in end-plate potential which finally stabilized at the value shown in the fourth record and which returned to normal (for the particular concentration of curare used) after the TEC had been washed out.

In order to confirm that this increase in end-plate potential occurred at the same time that acetylcholine sensitivity was decreased, intracellular recordings were made of the responses of the same end-plate to nerve stimulation and to iontophoretic application of acetylcholine, before and after addition of TEC. The records of this experiment (Fig. 6) show that a threefold increase in amplitude of the end-plate potential occurs with a 25% reduction in acetylcholine sensitivity. It will be noticed that this reduction in acetylcholine sensitivity is smaller than that recorded in the experiments of Fig. 2, although the same concentration of TEC was used throughout. It is suggested that this difference may be due to the use of curare in the experiment of Fig. 6, so that the end-plate potential could be recorded, and that TEC has a smaller effect on the sensitivity of an end-plate, some of whose receptors are already blocked by molecules of curare.

In another experiment on the uncurarized ext. l. dig. IV muscle, intracellular recordings were made of action potentials in the muscle in response to nerve stimulation and it was found that the addition of TEC produced a multiple response in the muscle fibres when single stimuli were applied to the motor nerve (Fig. 7). This experiment was repeated on three further preparations and on each occasion repetitive responses were observed after the addition of TEC. In another experiment, electrical recordings were made from the whole ext. l. dig. IV muscle, the surface of the Ringer's fluid being used as an electrode, and in this case also repetitive responses were obtained after TEC. In view of these results it is thought that this type of response was not due to mechanical damage to the muscle fibres by the intracellular electrodes. The probability of its occurrence will, of course, depend upon the threshold of the muscle fibres from which recordings are

made and if, as is suggested in the following section, TEC increases acetylcholine output, it will also depend on the previous rate of acetylcholine release and the size of any increase produced by TEC. The increase in twitch tension after TEC may therefore be due to an increase in the number of active muscle fibres and also to an increase in the tension developed in individual fibres by repetitive firing.

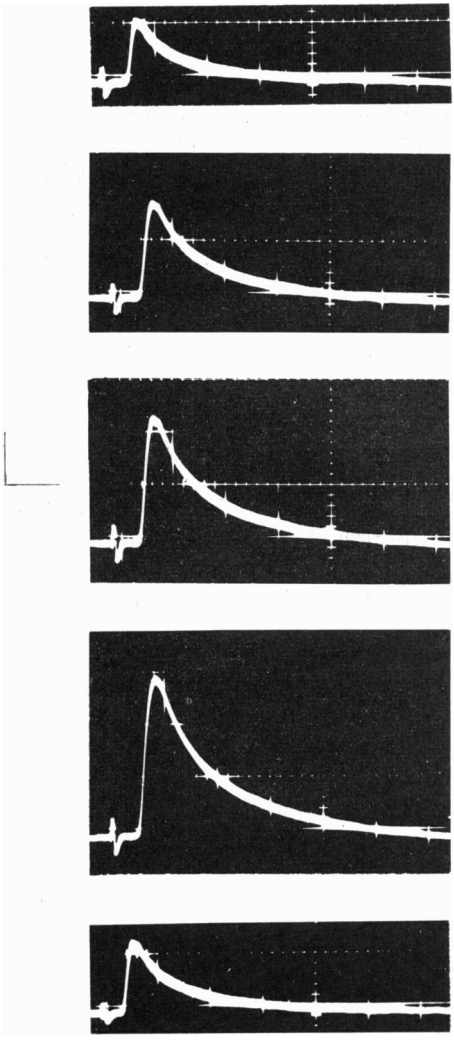

Fig. 5. End-plate potentials in a partly curarized sartorius muscle, before the addition of TEC (trace 1), 1, 2 and 3 min after 200 μg/ml. TEC (traces 2–4), and 3 min after washing out with the original Ringer's curare solution (trace 5). Calibration, 5 mV and 5 msec.

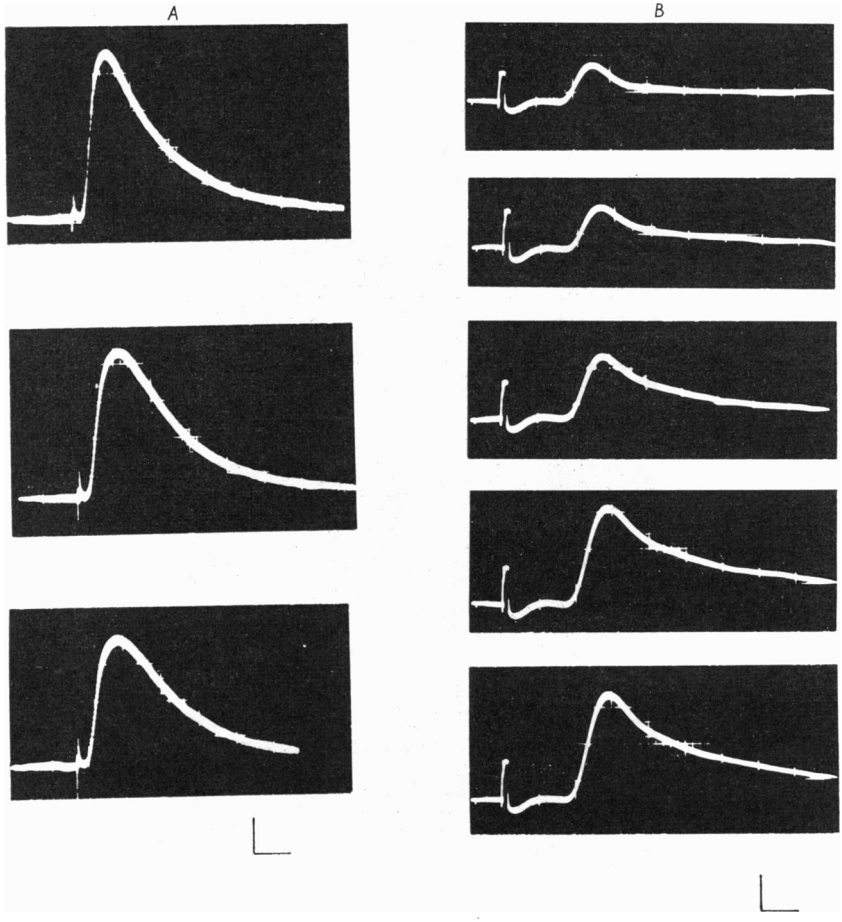

Fig. 6. Curarized m.ext. l. dig. IV. Acetylcholine potentials (*A*) and end-plate potentials (*B*) with TEC added between traces 1 and 2 in each series. Calibration, 2 mV and 0·2 sec (*A*); 2 mV and 2 msec (*B*). Traces *A* 2 and *A* 3 recorded 1 and 1½ min respectively after addition of TEC. Traces *B* 2, 3, 4 recorded at 2, 2½ and 3 min respectively after addition of TEC.

Acetylcholine release after TEC

In view of the simultaneous reduction in the size of the spontaneous miniature potential, and the increase in the end-plate potential evoked by a nerve impulse, it appeared that TEC increases the number of acetylcholine quanta released by a single nerve action potential. (For a review of the evidence for the quantal composition of the end-plate potential reference may be made to Katz (1958).)

To obtain more direct evidence on this point, use was made of the action of a high-Mg, low-Ca Ringer's solution which causes the ACh

quanta released by the nerve impulse to be reduced to a small and easily determined number (del Castillo & Katz, 1954*b*). An end-plate in the ext. l. dig. IV muscle was chosen which responded to each nerve stimulus with a small number of quanta and in which there was a certain proportion of failures to respond. When TEC was added to the bath the failure rate was reduced to zero and the number of quanta released by

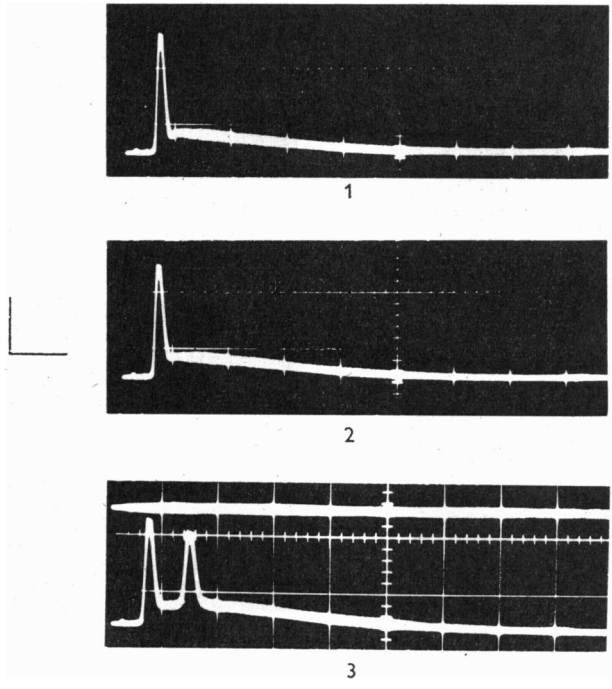

Fig. 7. Uncurarized m.ext. l. dig. IV. Indirect stimulation, intracellular recordings of action potentials from the same muscle fibre (1) before TEC, (2) immediately after the addition of TEC, and (3) one minute later. Calibration 50 mV and 5 msec.

each nerve impulse was increased (Fig. 8). Similar recordings were made at three other junctions; Table 1 lists the ranges in the numbers of quanta released in each case and the changes occurring after TEC. In each case there was an increase in the minimum number of quanta released as well as an increase in the range of quanta released. In the case of the first junction (Table 1) counts were made of the failure rate in the responses to three batches of 50 stimuli each, applied to the nerve at a rate of 30/min and allowing 5 min rest in between each batch. Out of each of the 50 possible responses, there were 11 failures. After TEC there were no failures to respond to a similar number of test stimuli.

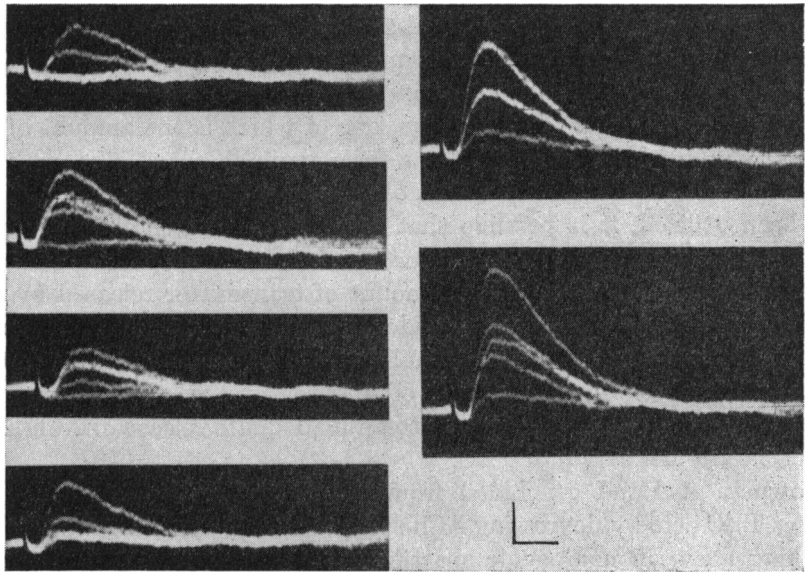

Fig. 8. End-plate potentials in m.ext. l. dig. IV reduced to the quantal level by low-Ca, high-Mg Ringer's solution. Records 1–4 show the response to single nerve stimuli before TEC, five traces being superimposed in each record. Records 5 and 6 show the response after the addition of TEC (200 μg/ml.) Calibration, 1 mV and 2 msec.

TABLE 1. The effect of TEC (200 μg/ml.) on the acetylcholine quantal level and the end-plate potential failure rate

	Without TEC				With TEC			
Expt. no.	No. of stimuli	No. of failures	Minimum quantum number	Maximum quantum number	No. of stimuli	No. of failures	Minimum quantum number	Maximum quantum number
1	50	11	0	4	50	0	1	9
2	43	10	0	4	44	0	3	10
3	35	0	1	4	35	0	12	18
4	35	0	1	2	35	0	4	6

DISCUSSION

A direct comparison of these results with those of Bowman & Rand (1961) is not possible owing to the difference in the species investigated, and to the different techniques employed. It is possible, however, to speculate on the mode of action of TEC as a neuromuscular blocking agent in the frog. It is clear that because of the increased output of ACh per nerve impulse there will be an initial facilitatory action on neuromuscular transmission, shown in myographic recording by an increase in the twitch tension. This effect was observed in the frog, and although they did

not report it in their paper, Bowman & Rand noted a similar increase in tension at the start of their stimulation experiments of the cat tibialis muscle and the rat diaphragm (personal communication).

The ability of a motor nerve ending to support this increased ACh output will depend on two main factors, one of which is the amount of preformed ACh in the nerve ending in a form available for release, while the other factor is the rate of synthesis of ACh. Once the local store of ACh has been utilized, it is possible that the synthesis mechanism is unable to supply ACh fast enough to support the increased output, so that after prolonged nerve stimulation the amount of transmitter released by each nerve impulse would decrease. Under these conditions the reduction in ACh sensitivity of the post-junctional membrane by TEC would no longer be offset by an increased quantity of ACh released, so that the eventual neuromuscular block would be due to an inadequate release of ACh plus a direct curare-like action of TEC.

Bowman & Rand concluded from their results that in mammalian tissues TEC acts by depressing ACh synthesis; they suggested its use as a blocking agent in neurogenic spastic states, since it would be effective against very active neuromuscular junctions while sparing those which are relatively inactive. However, if the mode of action of TEC is not on the synthesis but on the release of ACh, as the present experiments suggest, an eventual neuromuscular block would occur when the rate of release exceeded that of synthesis, but this would be preceded by an initial phase of increased neuromuscular activity and hence an increase in the degree of spasticity.

It is not clear why TEC should alter the probability of release of ACh quanta, although, since Ca and Mg ions are involved in this part of neuromuscular transmission, it is possible that TEC exerts its effects by a modification of the numbers of these ions present at the site of release of ACh. In this connexion it is perhaps of interest to note the work of Roepke & Welch (1936) on the cationic exchange of choline, and certain of its analogues, with calcium, as well as the more recent work by Schubert (1952) on complex ion formation between calcium and ethanolamine.

SUMMARY

1. Triethylcholine has been shown to have two effects on neuromuscular transmission, one being post-junctional and depressant, the other pre-junctional and facilitatory.

2. The post-junctional activity resembles that of curare, reducing the response to acetylcholine without depolarization.

3. TEC acts at a pre-junctional site to facilitate transmission by increasing the number of acetylcholine quanta released by the nerve action

potential. In this respect, TEC resembles calcium; the possibility is considered that some form of cationic exchange mechanism is operating and that TEC acts by altering the local concentration of Ca ions.

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