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Differences between the effects of saxitoxin (paralytic shellfish poison) and tetrodotoxin on the frog neuromuscular junction

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1. End-plate potentials (e.p.p.) have been recorded from the neuromuscular junctions of frog sartorius and extensor longus dig. IV muscles, using intracellular micropipettes. Either curare or $MgCl_2$ were present in the Ringer solution, to keep the e.p.p. amplitude below the threshold for a muscle action potential and contraction.

2. It has been shown that saxitoxin (paralytic shellfish poison) usually caused a progressive reduction in the amplitude of the e.p.p. Occasionally, when it was applied in the presence of $MgCl_2$, the e.p.p. disappeared abruptly.

3. Tetrodotoxin usually caused the e.p.p. to disappear abruptly. Occasionally, when applied in the presence of curare, the e.p.p. declined progressively for a short time before disappearing abruptly.

4. It is concluded that at the frog neuromuscular junction the preferential site of action of saxitoxin is at the nerve terminals, but tetrodotoxin preferentially blocks nerve conduction at a site proximal to the junction.

5. It is suggested that this preparation would be a convenient and reliable test object for distinguishing saxitoxin from tetrodotoxin.

Saxitoxin (STX) and tetrodotoxin (TTX) are known to be two different substances, yet their effects are remarkably similar. They both cause muscular paralysis by blocking action potentials in motor axons as well as through a direct action on the electrically excitable muscle membrane. Their action is to block the inward movement of sodium ions through electrically excitable membranes without altering the potassium permeability, thus inhibiting the conduction of action potentials without changing the resting potential. On most tissues their effective concentrations are similar and, in general, it is almost impossible to distinguish between them (see Kao, 1966 ; Evans, 1969a).

A few differences have been reported, the most striking being in their effects on nerves of the puffer fish *Spheroides maculatus* and the newt *Taricha torosa*. Both these species, together with some related species, produce tetrodotoxin (tarichatoxin) and are very resistant to it. The resistance is fairly specific, desheathed nerves of these species are readily blocked by STX but resist concentrations of TTX 1,000 to 20,000 times greater than that needed to block a frog nerve (Kao, 1967). Other, less

striking, differences that have been reported include a difference in the dose-response curves when survival times of mice are plotted against doses greater than 1 LD50 (McFarren & Bartsch, 1960) and a greater tendency to hypotension, hypothermia and vomiting in animals affected by TTX than in those paralysed by STX (see Kao, 1966).

Furukawa, Sasaoka & Hosoya (1959) showed that when the sartorius nerve-muscle preparation from a frog or toad was exposed to low concentrations of TTX, the end-plate potential (e.p.p.), recorded with an intracellular micropipette in the muscle, failed abruptly. They reasoned that TTX was acting pre-synaptically to cause an all-or-nothing failure of conduction in the motor axon, a view which is supported by a number of more recent publications. Nishiyama & Kao (1964) reported that STX, in the presence of curare, causes the e.p.p. of the frog sartorius nerve-muscle preparation to decline progressively. Although this effect is typical of a post-synaptic blocking agent, like curare, they had evidence that STX does not act post-synaptically.

Some of the evidence which suggested a pre-synaptic site of action came from experiments in which magnesium, and not curare, was used to control neuromuscular transmission. There was therefore a possibility that the site of action of these toxins near the neuromuscular junction might depend on the agent used to control transmission. Some support for this hypothesis came from a discovery that magnesium, in concentrations similar to those used to control neuromuscular transmission, potentiated the action of these toxins in blocking conduction of impulses along axons (Evans, 1969b). The effects of STX and TTX on the frog neuromuscular junction were therefore re-investigated, both in the presence of curare and of magnesium. The results reported in this paper have shown that the choice of neuromuscular depressant has only a small influence on the effects of these toxins and that there is a definite qualitative difference in their actions at the neuromuscular junction in the frog.

The paralytic shellfish poison used in these experiments was a sample purified from poisonous mussels, *Mytilus californianus*. The name saxitoxin was originally applied to pure paralytic shellfish poison obtained from the clam *Saxidomus giganteus*. It is now thought that the poisons from both of these sources are identical (Schantz, Mold, Howard, Bowden, Stanger, Lynch, Wintersteiner, Dutcher, Walters & Riegel, 1961). The name saxitoxin is therefore used in this paper in place of the older term paralytic shellfish poison, and is taken as including mussel poison.

Methods

The experiments were done on isolated nerve-muscle preparations from frogs *Rana temporaria* and *R. pipiens*. In one experiment tissue from *R. esculenta* was used, but the results were atypical and this species was not used again. No differences were noted between the first two species. Most of the experiments carried out were on the sartorius nerve-muscle preparation. The extensor longus dig. IV preparation was used for several experiments and gave similar results.

The nerve-muscle preparation was fixed in a conventional Perspex chamber mounted above the base of a Zeiss sliding micromanipulator. A high input impedance probe, based on a field effect transistor circuit of Fein (1964), was held in one of the tool carriers and glass micropipettes were mounted in a silver, silver-chloride

electrode plugged into the f.e.t. probe. The micropipettes were made in a vertical puller (Scientific and Research Instruments Ltd.) and filled slowly with KCl 200 g/l. by the technique of Caldwell & Downing (1955). Micropipettes with resistance in the range 7–20 MΩ and low tip potential were selected to record the intracellular end-plate potentials (e.p.p.). These responses were displayed in a conventional manner on an oscilloscope, from which photographic records were made for subsequent measurement. At every recorded end-plate the all-or-nothing nature of the e.p.p. was confirmed by reducing the nerve stimulus strength to threshold. A few muscle fibres showed evidence of multiple innervation and were not used in the experiments.

The muscle was irrigated by a Ringer solution composed of (mM): NaCl 110; KCl 2·5; $CaCl_2$ 1·8; glucose 5·6; Tris hydrochloride buffer (pH 7·4) 2–5. This was gassed with oxygen and the flow rate through the chamber was 2–3 ml./min. To prevent the muscle from twitching when the nerve was stimulated, the e.p.p. was reduced to below the threshold of propagated muscle action potentials. In about half the experiments this was done by adding tubocurarine chloride (Tubarine miscible, Burroughs Wellcome & Co.) 1–3 mg/l. to the Ringer solution. In the rest of the experiments the e.p.p. was controlled by replacing part of the NaCl in the Ringer solution with an isosmotic quantity of $MgCl_2$, generally 9–13 mM. Tetrodotoxin (TTX) and saxitoxin (STX, mussel poison) were prepared from stock solutions as described in the previous paper (Evans, 1969b).

Results

Resting potentials in the presence of the toxins

When the muscle fibres were first penetrated by the micropipettes, the resting membrane potential was usually about −90 mV if a clean penetration had been effected. Small changes in the recorded potential, either increasing or decreasing by a few mV, were commonly seen during the first few minutes after penetration. These changes, plus the small baseline changes due to amplifier drift, alterations in microelectrode tip potential and Ag-AgCl reference potentials, made it impracticable to look for very small changes in resting membrane potential. If saxitoxin or tetrodotoxin did affect the resting potential the change so produced cannot have been greater than 2 mV. Figure 1(f) shows a record of resting potential and it can be seen that the application of TTX (vertical signal line) cannot be correlated with any definite change of potential.

End-plate potentials in the presence of tetrodotoxin

TTX was added to the Ringer solution, which contained either tubocurarine or $MgCl_2$, in amounts ranging between 1 and 10 μg/l. The minimum effective concentration was usually in the range 2–6 μg/l. TTX produced a loss of the e.p.p. after a latent period that varied from less than a minute to more than 15 min, depending partly on the concentration of toxin.

When $MgCl_2$ was used to depress neuromuscular transmission, TTX had no effect on the e.p.p. amplitude up to the time when the e.p.p. was suddenly blocked. Figure 1 shows records taken in one such experiment, and should be read from below upward, starting at the bottom of column (a), which shows the control e.p.p. responses. TTX 5 μg/l. was applied to the muscle during the time marked by the

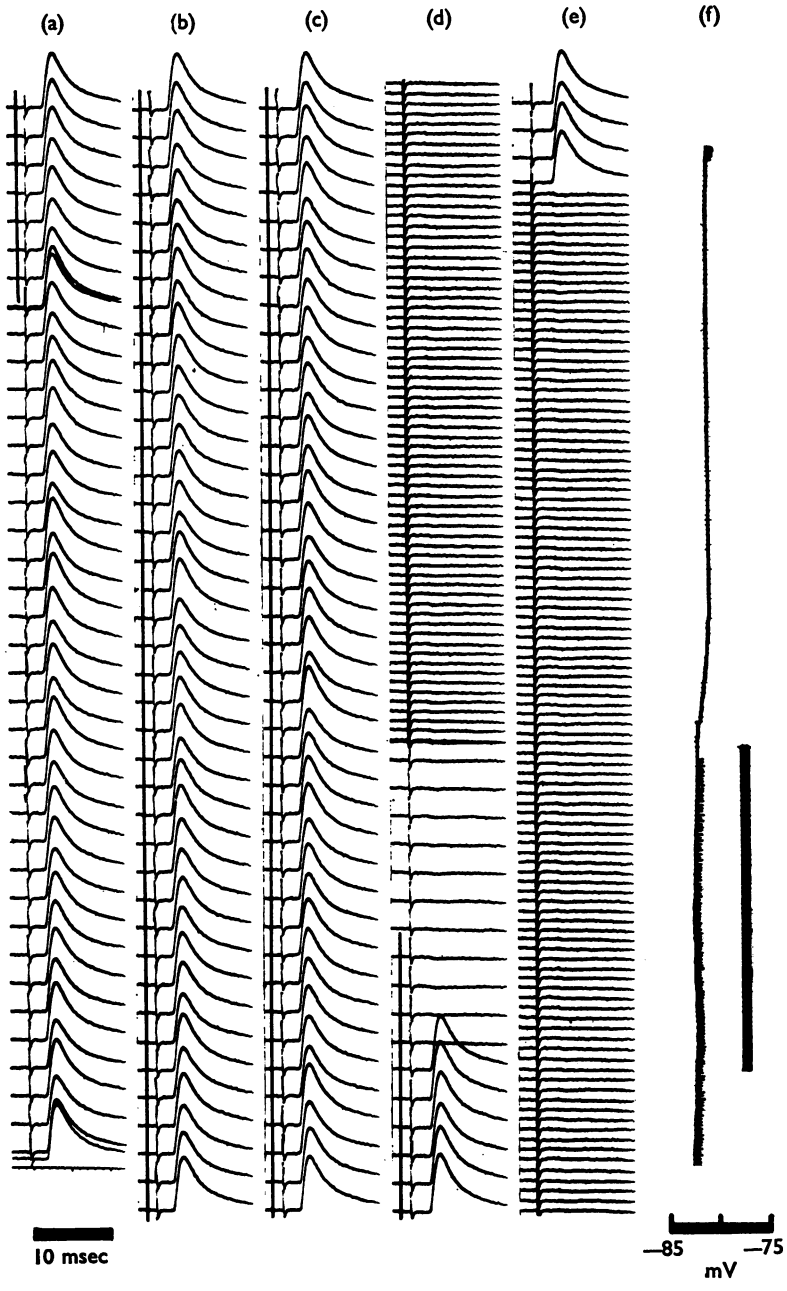

FIG. 1. (a)–(e): End-plate potential (e.p.p.) responses recorded from a frog sartorius muscle bathed in Ringer solution containing $MgCl_2$ 11·1 mM. The records commence at the bottom of column (a) and read upwards to the end at the top of column (e). The signal mark, which starts near the top of column (a) and ends in column (d), indicates the presence of tetrodotoxin 5 μg/l. in the Ringer solution. The responses were evoked every 4 sec (the film was moved more slowly during columns (d) and (e) while awaiting recovery of responses). Column (f) is the record of muscle membrane potential during the time covered in columns (a)–(e), with the period of exposure to TTX marked by the vertical signal line. The small deflections on this record are the e.p.p. responses, with amplitudes curtailed by the slow response of the potentiometric recorder.

black line running up the left-hand edge of the records, starting near the top of column (a) and ending near the bottom of column (d). It can be seen that there was no change in e.p.p. amplitude until after more than 6 min in toxin, when the responses disappeared abruptly. The toxin was discontinued after the disappearance of the responses, and the film moved on slowly (columns (d) and (e)) until the e.p.p. recovered abruptly to its full amplitude, after washing for nearly 12 min.

In a few experiments the e.p.p. responses fell in discrete steps when exposed to TTX and did not disappear all at once like the responses in Fig. 1 did. Figure 2 shows a graph of the peak amplitudes of the e.p.p. responses from an experiment on a muscle exposed to TTX 6·2 μg/l. There was little, if any, effect for the first 12 min, then the responses fell in a series of well defined steps to a low value. On washing out the toxin the e.p.p. recovered abruptly, pausing for a short time at one of the intermediate levels. This type of response to TTX was seen both in the presence of curare and of $MgCl_2$.

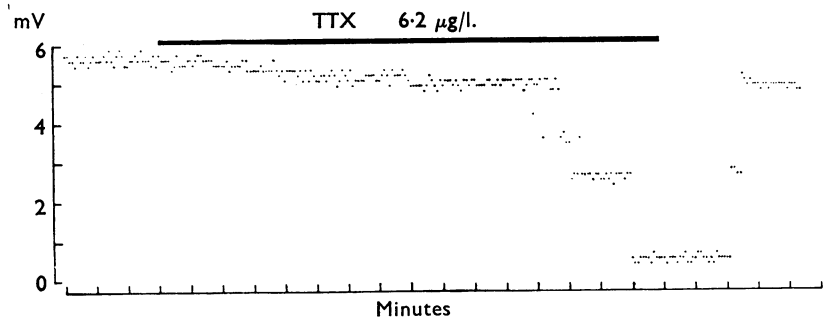

FIG. 2. Graph of peak amplitudes of e.p.p. responses recorded from a frog sartorius muscle bathed in Ringer solution containing curare 2·5 mg/l. The responses were evoked at 5 sec intervals and each one is shown as a separate point. The signal line above the graph indicates the presence of TTX 6·2 μg/l. in the Ringer solution.

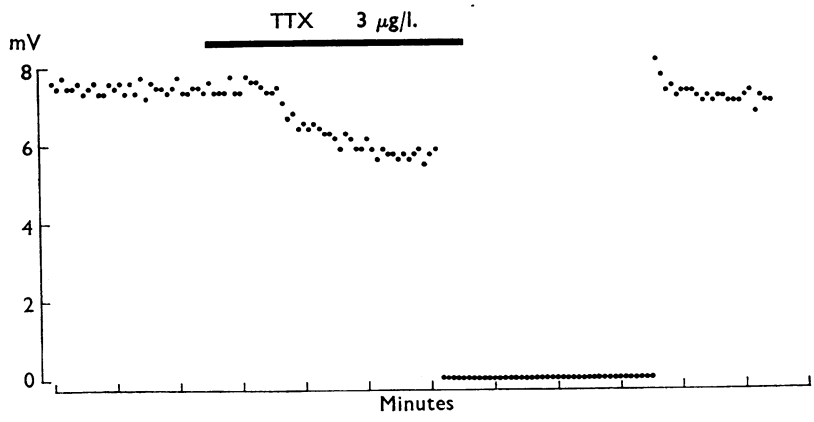

FIG. 3. Graph of peak amplitudes of e.p.p. responses recorded from a frog ext. long. dig. IV muscle in Ringer solution containing curare 2 mg/l. The amplitude of each response is shown separately at 5 sec intervals. The signal line above the graph indicates when TTX 3 μg/l. was present in the Ringer solution.

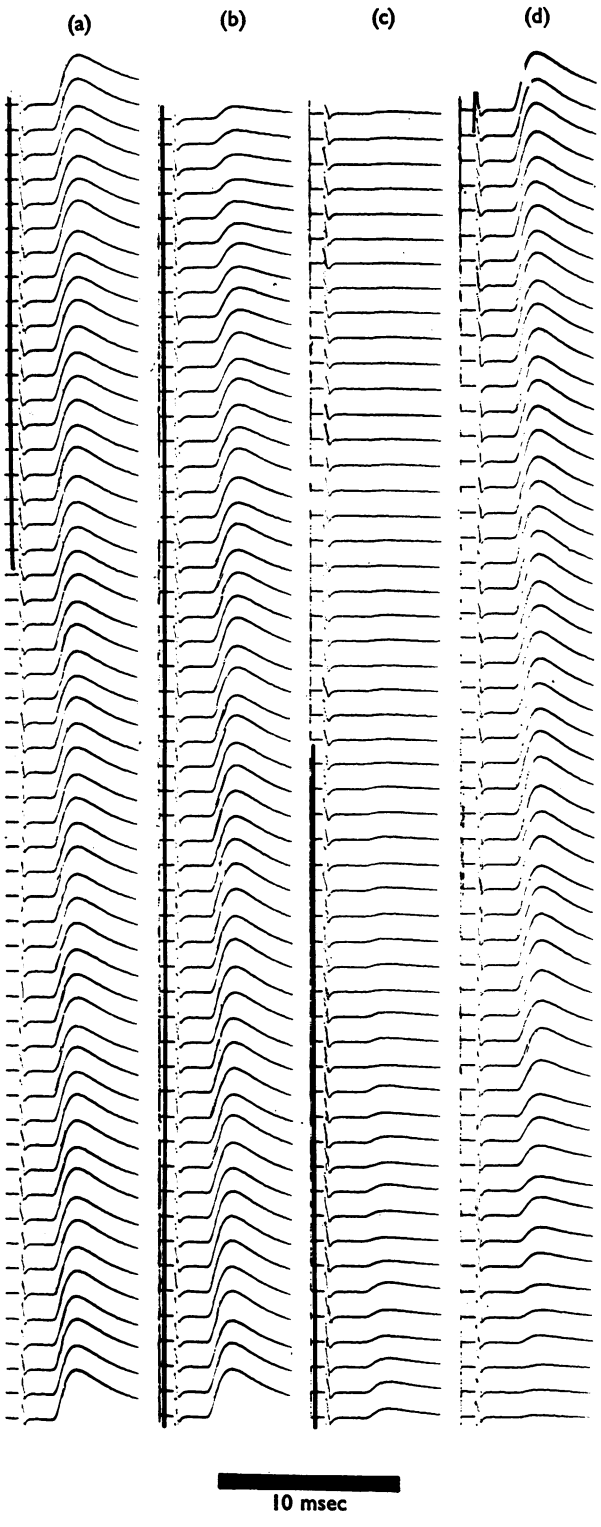

FIG. 4. End-plate potentials recorded from a frog sartorius muscle in Ringer solution containing $MgCl_2$ 9·5 mM. The records commence at the bottom of column (a) and read upwards. The signal mark, from the upper part of column (a), through (b) to the middle of (c), indicates the presence of saxitoxin 5 μg/l. in the Ringer solution. The responses were evoked every 3 sec.

In the presence of curare, but not when $MgCl_2$ was used, the type of behaviour illustrated in Fig. 3 was occasionally encountered. The neuromuscular junction from which these records were taken was irrigated for 4 min with TTX 3 μg/l. After a short latent period the e.p.p. began to decline gradually in amplitude before disappearing abruptly, when it had fallen to 76% of the control height. When the TTX was washed out, the e.p.p. recovered abruptly and fully to the control amplitude.

End-plate potentials in the presence of saxitoxin

STX was added to the Ringer solution, which contained either tubocurarine or $MgCl_2$, in amounts between 1 and 20 μg/l. The minimum effective concentration was usually in the range 1·5–6 μg/l. STX differed from TTX in its effects. STX

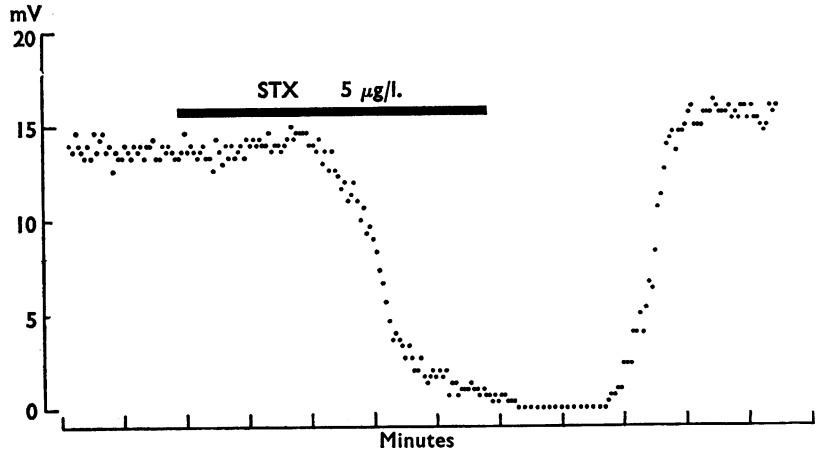

FIG. 5. Graph of peak amplitudes of e.p.p. responses shown in Fig. 4, each one being plotted as a separate point. The signal line above the graph indicates the presence of STX 5 μg/l. in the Ringer solution.

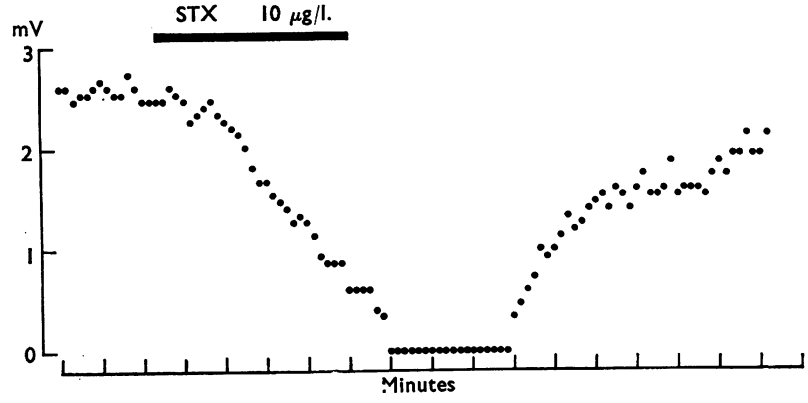

FIG. 6. Graph of peak amplitudes of e.p.p. responses recorded from a frog sartorius muscle in Ringer solution containing curare 3 mg/l. Each response is shown as a separate point at 10 sec intervals. The signal line above the graph indicates the presence of STX 10 μg/l. in the Ringer solution.

almost always caused the amplitude to decline gradually and the recovery on washing was usually graded.

This is shown by the records in Fig. 4, again to be read from below upwards starting at the bottom of column (a). In this experiment the Ringer solution contained $MgCl_2$ 9·5 mM. The vertical black line marks the application of STX 5 μg/l. for 5 min. After a latent period the e.p.p. responses started to decline slowly to zero. When the STX was washed out the responses recovered progressively. The results of this experiment are shown graphically in Fig. 5, from which it is clear that there were no abrupt steps in either the decline or recovery of the responses.

Comparable results were obtained from experiments in which tubocurarine was used in place of $MgCl_2$, as illustrated in Fig. 6. This shows the results from an experiment in which the neuromuscular junction was in tubocurarine 3 mg/l. and then exposed to STX 10 μg/l. for about 4·5 min. It can be seen that both the depression and the subsequent recovery during washing were smoothly graded.

Thirty-one experiments with STX, in the presence of either curare or $MgCl_2$, were plotted as graphs. Two of these graphs showed a sudden loss of the e.p.p. responses with abrupt recovery after washing, instead of the graded changes usually seen. In both cases the Ringer contained $MgCl_2$ and the abrupt block occurred after long exposure to low concentrations of STX (1 μg/l. for 11 min and 3 μg/l. for 3·5 min). One of these neuromuscular junctions responded to higher concentrations of STX, in a shorter time, with a graded depression of the e.p.p. responses. In the other case a neighbouring junction showed a graded depression when exposed to STX 4 μg/l. In four other experiments (two in curare and two in $MgCl_2$) a block occurred gradually, but the e.p.p. appeared to recover abruptly after washing.

Nerve conduction in the presence of toxins

In a few experiments, after exposure to sufficient toxin to cause the e.p.p. to disappear, the micropipette was withdrawn from the muscle fibre and placed on an intra-muscular branch of the nerve. In all cases the presence of action potentials showed that the nerve fibres were still conducting from the periphery as far as the main intra-muscular branches.

Discussion

Furukawa *et al.* (1959) recorded end-plate potentials from frog and toad sartorius muscles in the presence of tetrodotoxin, and their results are fully in agreement with those reported here. It is now generally accepted that TTX has an all-or-nothing effect on the e.p.p., causing its abrupt disappearance through a conduction block in the motor nerve, without altering the sensitivity of the post-synaptic end-plate receptors to acetylcholine (Katz & Miledi, 1967 ; see also Kao, 1966).

Nishiyama & Kao (1964) briefly reported that saxitoxin caused a gradual reduction in e.p.p. amplitude in the curarized sartorius preparation. One normally associates this type of effect with a progressive post-synaptic block of the end-plate receptor sites, yet in a later paper (Kao & Nishiyama, 1965) they presented evidence that STX was acting pre-synaptically without significantly reducing the post-synaptic sensitivity of the end-plate.

TTX and STX are known to have identical actions on most biological preparations (see Kao, 1966; Evans, 1969a). A few exceptions are known, which have been described briefly in the introductory paragraphs. In most other preparations it is virtually impossible to distinguish STX from TTX and therefore it was interesting to note the reported differences in their effects on the e.p.p. However, it was also noted that some of these experiments had been done in the presence of curare, while others had been done with $MgCl_2$ in the Ringer solution. Curare acts post-synaptically at the receptor sites in the end-plate, whereas Mg^{++} acts pre-synaptically to reduce transmitter release. A suspicion that the apparent differences between STX and TTX effects at the neuromuscular junction might have been due to the choice of curare or $MgCl_2$ as a neuromuscular depressant was strengthened by finding that $MgCl_2$ potentiates the actions of these toxins in blocking conduction along axons (Evans, 1969b). For this reason it was thought advisable to re-investigate the effects of both STX and TTX on the e.p.p. in frog muscle in the presence of curare and of $MgCl_2$.

The experiments reported here have confirmed that there is a qualitative difference between the effects of STX and TTX on the e.p.p. at the neuromuscular junctions in frog sartorius and ext. long. dig. IV muscles. Whether the experiments were done in the presence of curare or of $MgCl_2$ made only a minor difference to the results. TTX tends to cause an abrupt disappearance of e.p.p. responses. This is the only effect seen in the presence of $MgCl_2$, while in the presence of curare it is occasionally preceded by a slight decline in e.p.p. amplitude. On the other hand, STX almost always causes the e.p.p. to decline gradually, irrespective of the use of curare or $MgCl_2$. Exceptions have only been seen twice in the present series of experiments, and in both cases low concentrations of STX were being applied in the presence of $MgCl_2$.

The nerve-muscle preparation from *Rana temporaria* or *R. pipens* therefore seems to be a very useful preparation on which STX can be qualitatively distinguished from TTX. The only other preparations which allow a clear distinction, a desheathed nerve from *S. maculatus* or *T. torosa*, are not so readily available and due to the short length of the nerves are more difficult to use (Kao, 1967). The frog nerve-muscle preparation is now being used to investigate the toxin(s) from a sample of mussels (*Mytilus edulis*), believed to be derived from the plankton dinoflagellate *Gonyaulax tamarensis* and responsible for an outbreak of paralytic shellfish poisoning at Newcastle upon Tyne (Ingham, Mason & Wood, 1968).

There is good evidence that both toxins act pre-synaptically at the neuromuscular junction. Miniature end-plate potentials can be recorded even in the presence of quite high concentrations of the toxins and the end-plate remains sensitive to the depolarizing action of applied acetylcholine (Furukawa *et al.*, 1959; Elmqvist & Feldman, 1965; Kao & Nishiyama, 1965). In order to explain the qualitative differences between the effects of these toxins on the e.p.p. one must assume that the presynaptic sites of action are slightly different. TTX would appear to have a preferential axonal blocking action at a short distance from the motor nerve terminals. STX appears to reduce the release of transmitter when an impulse is sent down the nerve, but in the absence of impulses it does not reduce the quantal release of transmitter responsible for the m.e.p.p. Therefore it probably acts at the motor nerve terminals by progressively reducing their invasion by the action potential.

The action of TTX on the motor nerve cannot be very far from the terminals, because even when the e.p.p. is blocked by the toxin, action potentials can still be recorded from the intramuscular branches of the nerve. The connective tissue sheath around peripheral nerve is a very effective barrier against the penetration of these toxins (Evans, 1964, 1968). The pre-terminal part of the motor axon is enclosed in the sheath of Henle, but this does not seem to be such a serious barrier to the toxins. The stepwise reduction in the e.p.p. which was sometimes seen with TTX (Fig. 2) was probably due to the toxin first blocking one branch of a motor terminal, as at 'c' in Fig. 7. This caused the e.p.p. to drop abruptly to an intermediate level, depending on the degree of activity in adjacent end-plates, until these too failed when the axon became blocked at 'a' or 'b' and the e.p.p. disappeared abruptly. This view has been expressed by Furukawa *et al.* (1959) and by Katz & Miledi (1968).

The slight decline in the e.p.p. sometimes seen when TTX was applied in the presence of curare can be explained if it is assumed that the TTX can also reduce progressively the conduction of the nerve impulse into the terminals 'd'. Even in the presence of curare this action would still be incomplete by the time the TTX effected a conduction block at 'a', 'b' or 'c'. In the presence of $MgCl_2$ this terminal action would not have started by the time the axonal block was established, because of the potentiating effect of Mg^{++} on the axonal action (Evans, 1969b). A terminal action of this type has been illustrated by Katz & Miledi (1968). Their Fig. 5 shows an e.p.p. declining progressively as TTX is applied microelectrophoretically to an endplate. Localization of the application in this way allows one to see the terminal effect before the toxin is able to diffuse along the nerve and produce a conduction block at one of the nodes of Ranvier.

Whereas TTX appears to act predominantly on the axon, at the nodes, STX seems to have a preferential action at the nerve terminals 'd', producing a progressive reduction in the amount of transmitter released by the arrival of a nerve impulse. It can be postulated that it causes incomplete invasion of the terminals by the action potential; in a partial block only a few microns of the terminal are affected and electrotonic spread over such a short distance will be sufficient to release a reduced quantity of transmitter. As the STX action spreads back along the unmyelinated

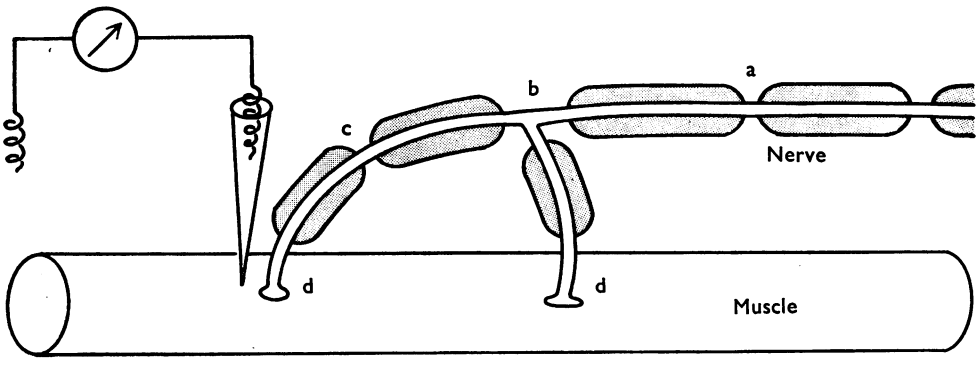

FIG. 7. Diagrammatic representation of the probable pattern of a motor axon innervating two neuromuscular junctions on a muscle fibre. An intracellular micropipette is shown near one junction. Further details are discussed in the text.

terminal, electrotonic conduction into the blocked terminal will become less and less effective. Transmitter release will fall to zero when a length of terminal has been blocked comparable with the " length constant " of the nerve at this point (Katz & Miledi, 1968). It is well known that STX can block conduction in peripheral nerves, and the two exceptional experiments mentioned in the results serve as a reminder that low concentrations of STX in the presence of $MgCl_2$ may be able to block the pre-terminal nodes if allowed time to penetrate, even though the concentration is too low to affect the terminals. When higher concentrations are applied the greater accessibility of the terminals to STX allows a graded block to become fully established before an adequate concentration has been able to build up at the less accessible nodes.

I am grateful to Dr. E. J. Schantz for supplying the saxitoxin from *Mytilus californianus*. It is a pleasure to acknowledge the technical assistance of Mrs. W. Matthews and Miss J. M. Tulloch. Apparatus for the experiments was purchased with the aid of grants (5-RO5-TW-00249 and 00355) from the U.S. Public Health Service.

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