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The effects of saxitoxin and tetrodotoxin on nerve conduction in the presence of lithium ions and of magnesium ions

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1. It has been shown that nerve fibres from rat cauda equina will conduct action potentials after immersion in saline in which lithium chloride is substituted for sodium chloride.

2. Both saxitoxin and tetrodotoxin inhibit lithium-generated action potentials. The concentration of toxin needed to inhibit the lithium-generated action potentials is similar to that needed to inhibit sodium-generated action potentials.

3. If magnesium chloride is added to the saline to give a concentration of 10–15 mM there is usually a slight fall in amplitude of the compound action potential. Saxitoxin and tetrodotoxin now inhibit the action potential to a greater degree than in the absence of magnesium ions.

It is now well known that saxitoxin and tetrodotoxin abolish action potentials in nerve and muscle fibres by preventing the membrane permeability to sodium ions from increasing (Hille, 1968 ; see Kao, 1966). It is also known that lithium ions can successfully substitute for sodium ions in generating action potentials in some tissues (Overton, 1902), including frog nerve axons (Gallego & Lorente de Nó, 1951) and mammalian C fibres (Ritchie & Straub, 1957). The first part of the present publication describes experiments in which it was shown that saxitoxin and tetrodotoxin could abolish lithium-generated action potentials in mammalian myelinated nerve fibres. While these experiments were in progress a report was published showing that both toxins can inhibit inward lithium current at the nodes of Ranvier in frog nerve (Hille, 1968).

The second part of this paper describes an investigation of the potentiation of the action of saxitoxin and tetrodotoxin by magnesium ions. The experiments were done because of differences previously reported in the actions of these toxins at the neuromuscular junction. Some of the work reported had been done in the presence of curare (Furukawa, Sasaoka & Hosoya, 1959), but part of the evidence for pre-synaptic action came from experiments in which $MgCl_2$ had been added to the bath to prevent the end-plate potential from generating a propagated response in the muscle (Kao & Nishiyama, 1965). There was a possibility that in the latter experiments the magnesium ions enhanced the pre-synaptic action of the toxins, and if so,

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post-synaptic effects might have gone unnoticed. The present paper describes the potentiation of the actions of these toxins on nerve conduction in the presence of magnesium ions; the effects at the neuromuscular junction are described in the following paper (Evans, 1969).

Methods

The experiments were done on the large myelinated nerve axons of the cauda equina of the rat, obtained in the manner described previously (Evans, 1968). Fine filaments were selected from the cauda equina and set up in a Perspex triple chamber. Each end of the filament was in mineral oil (liquid paraffin s.g. 0·83–0·87, shaken with 0·9% NaCl to saturate the oil with water). The ends lay on platinum wire stimulating and recording electrodes. The central length of the filament passed through a chamber separated from the oil-filled chambers by seals of Vaseline (in the early experiments) or silicone stopcock grease (in the later experiments). This central chamber contained an earthing electrode and was perfused with physiological saline at room temperature (18°–22° C). The capacity of the chamber was about 0·7 ml. and the saline flow rate was usually 1–2 ml./min.

The composition of the standard physiological saline was (mM): NaCl 150, KCl 5·6, $CaCl_2$ 2·2, glucose 5·6. It was buffered to pH 7·4 by the addition of Tris hydrochloride buffer 2–5 mM. In some experiments NaCl was replaced by LiCl. In other experiments a saline was prepared with the NaCl replaced by $MgCl_2$ 100 mM. This was mixed with the standard physiological saline in the appropriate proportions to obtain a fluid in which some NaCl was replaced by an osmotically equivalent amount of $MgCl_2$. All the salines were gassed with oxygen.

The saxitoxin (STX, mussel poison from *Mytilus californianus*) was generously supplied by Dr. E. J. Schantz. The stock solution was 10 mg/l. in 0·01 N HCl and this was stable when refrigerated. It was prepared for use by mixing a small volume with an equal volume of 0·01 N NaOH before diluting with the appropriate saline to a concentration in the range 1–10 μg/l. Tetrodotoxin (TTX, Sankyo Co. Ltd.) was dissolved in distilled water at a concentration of 10 mg/l. The excess citric acid present in the commercial product kept the solution in the range pH 4–5 and this was stable when refrigerated. It was diluted with the appropriate saline before use to a final concentration generally in the range 3–10 μg/l.

Most of the cauda equina filaments tested consisted almost entirely of large myelinated nerve fibres. These gave a homogeneous compound action potential which was conducted at 21–48 m/sec at room temperature (18°–22° C). The compound action potential was usually large when the preparation was first set up, the amplitude occasionally being over 40 mV; it then generally deteriorated to a variable extent, but usually settled down to a reasonably steady value after 1 or 2 hr. The results are expressed in mV peak amplitude of this compound action potential, measured with a graticule from the display on a Tektronix 565 oscilloscope. As long as the responses were greater than about 1 mV the short-term random variation in amplitude was barely detectable, using a repetition rate of 1 or 3/sec, and the peak amplitudes could be measured with an accuracy of $\pm 2\%$. Representative responses were photographed on Polaroid film. In a few of the later experiments the area under the compound action potential was integrated electronically and simultaneous measurements made of this integral and of the peak amplitude of the response. When the results were plotted graphically few differences were seen

between these two sets of data, indicating that in these experiments the peak amplitude of the compound action potential was a reliable indicator of the relative numbers of conducting nerve fibres.

Results

Action of the toxins when sodium is replaced by lithium

When the myelinated fibres of the rat cauda equina were immersed in a saline in which the sodium had been replaced by lithium they could continue to conduct action potentials for at least an hour. The amplitude of the compound action potential declined more rapidly than in sodium saline, but there was no serious diminution during a 5–10 min test period. Control experiments showed that if the sodium was replaced by Tris the conducted responses failed in 3 min and if the sodium was replaced by potassium the action potentials disappeared in 45 sec. In both cases the responses reappeared when the flow of standard sodium saline was resumed.

When the nerves were conducting action potentials in lithium saline, these were abolished by saxitoxin and by tetrodotoxin. In an attempt to compare the relative susceptibility of lithium- and sodium-generated action potentials to the toxins, experiments were done in which the toxin concentration was selected to block only some of the fibres, producing a partial depression of the compound action potential. When a partial block had been achieved in sodium saline the perfusion fluid was changed to lithium saline containing the same concentration of toxin. No consistent differences were seen on replacing sodium ions with lithium. In some experiments the responses increased slightly in lithium but in others a slight fall occurred. In general it seemed that lithium-generated action potentials and sodium-generated action potentials were equally susceptible to saxitoxin and tetrodotoxin.

The results from one of these experiments are shown in Fig. 1, which is a graph of peak amplitudes of the responses plotted against time. The inset to the right of

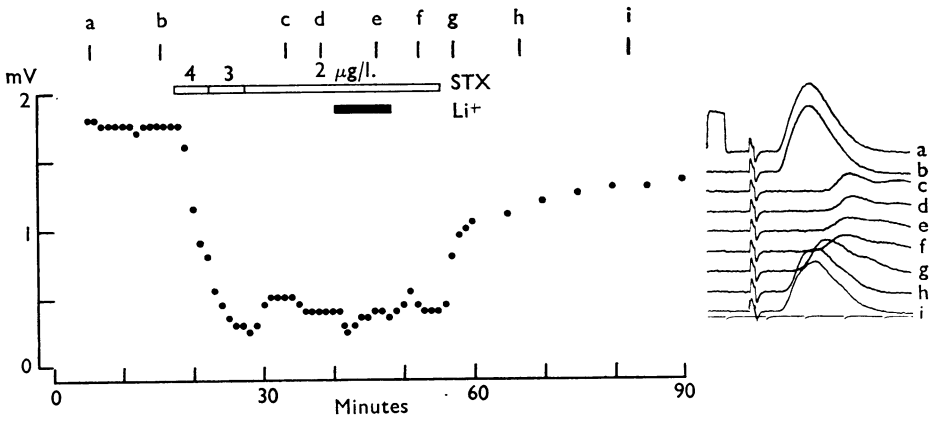

FIG. 1. Graph of peak amplitudes of compound action potentials conducted in cauda equina nerve. During the time marked by the open rectangle the perfusion fluid contained saxitoxin at concentrations of 4, 3 or 2 μg/l. as indicated above. During the period marked by the filled rectangle the sodium in the perfusion fluid was replaced by lithium. The inset on the right-hand side shows representative action potentials photographed at the times marked a–i above the graph. The calibrating marks in the inset show 1 mV and millisec intervals.

the graph illustrates some representative responses photographed at the times marked by lines above the graph. During the time marked by the white rectangle the nerve was in a solution containing saxitoxin, initially 4 μg/l., then 3 μg/l. and finally 2 μg/l., which maintained a partial conduction block with the responses reduced to about 25% of the control level. During the 8 min marked by the black rectangle the perfusion fluid was changed from saxitoxin 2 μg/l. in sodium saline to the same concentration of toxin in lithium saline. It can be seen that there was no significant change in the proportion of blocked nerve fibres when the sodium was replaced by lithium. When the nerve was returned to toxin-free sodium saline the response slowly recovered toward their original amplitude.

Figure 2 shows the results from a similar experiment in which tetrodotoxin was used to block conduction in the majority of the fibres. The toxin was applied at a concentration of 5 μg/l. to establish a partial block, which was maintained with 3 μg/l. In this experiment the partially blocked response showed a small further reduction in amplitude when the sodium was replaced by lithium.

Potentiation of toxin action by magnesium

The addition of even small amounts of magnesium to the perfusion saline resulted in some decrease in the peak amplitude of the compound action potentials in the cauda equina filaments. This was accompanied by a slight lengthening of the latent period, indicating a fall in conduction velocity. These effects were seen within a minute or two of adding the magnesium and were fully reversed within a similar

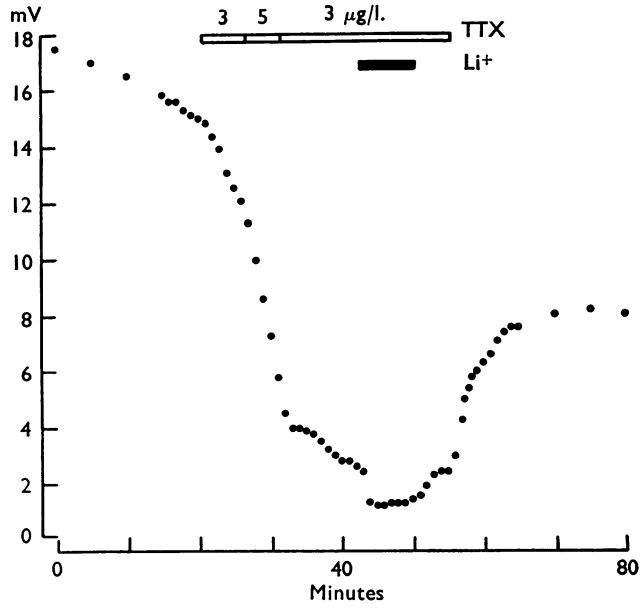

FIG. 2. Graph of peak amplitudes of compound action potentials conducted in cauda equina nerve. During the time indicated by the open rectangle the perfusion fluid contained tetrodotoxin at concentrations of 3, 5 and 3 μg/l. as shown above. During the period marked by the filled rectangle the sodium in the perfusion fluid was replaced by lithium.

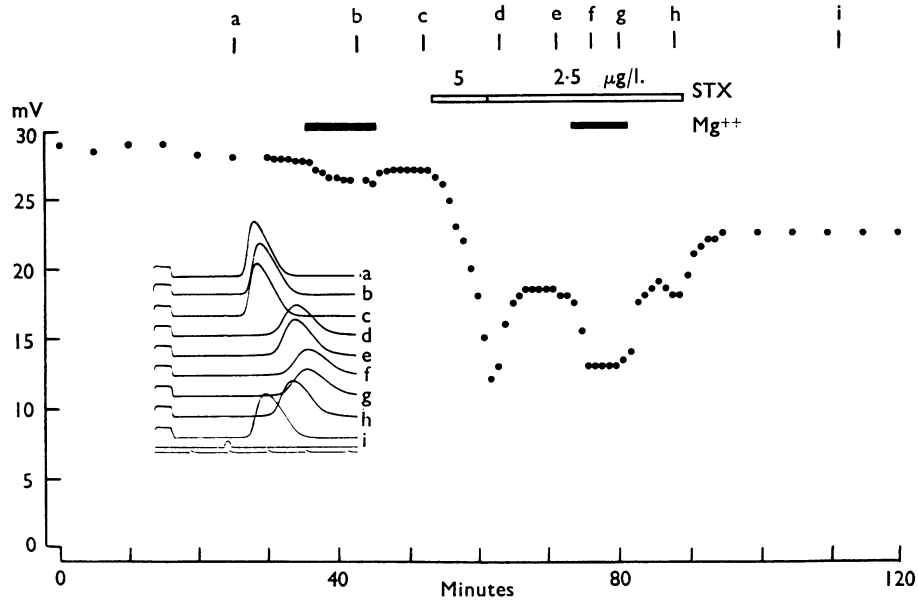

FIG. 3. Graph of the peak amplitudes of compound action potentials conducted in cauda equina nerve. During the time indicated by the open rectangle the perfusion fluid contained saxitoxin at concentrations of 5 and 2·5 μg/l. as shown above. During the periods marked by the filled rectangles part (22·5 mM) of the NaCl was replaced by 15 mM $MgCl_2$. The inset in the graph shows representative action potentials photographed at the times marked a–i above. The calibrating marks in the inset show 5 mV pulses, a stimulus mark and 1 m-sec intervals.

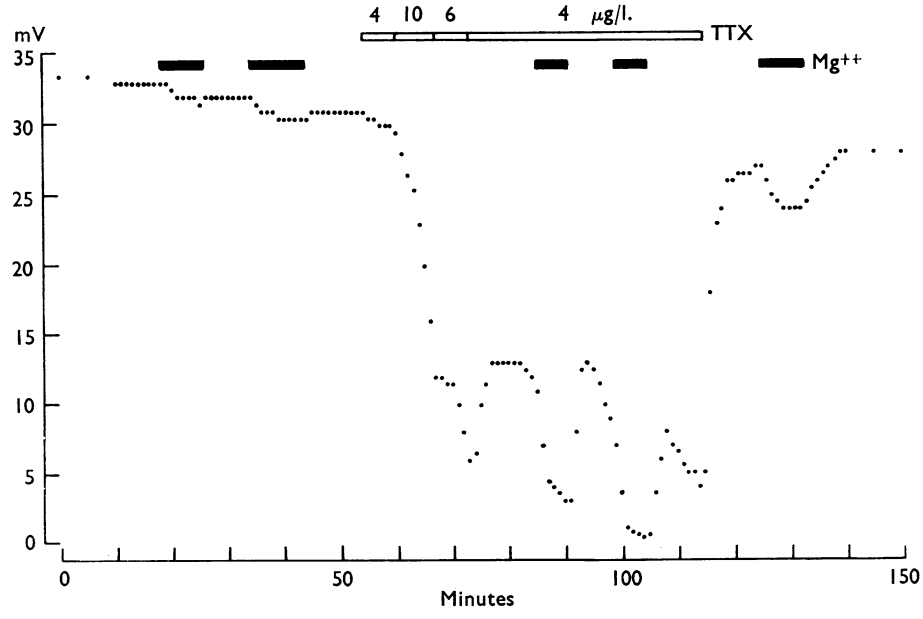

FIG. 4. Graph of the peak amplitudes of compound action potentials conducted in cauda equina nerve. During the time indicated by the open rectangle the perfusion fluid contained tetrodotoxin at concentrations of 4, 10, 6 and 4 μg/l. as shown above. During the periods marked by the filled rectangles part (15 mM) of the NaCl was replaced by 10 mM $MgCl_2$.

time after restoring the magnesium-free saline. When the magnesium ion concentration was 10 mM (replacing 15 mM sodium) the reduction in amplitude varied in different preparations from about 3 to 20%.

In some of the experiments there was little evidence of interaction between the magnesium and the toxin. The reduction in the amplitude of the responses brought about by simultaneous application of magnesium and toxin approximately equalled the sum of the reductions with separate application.

In about half the experiments there was evidence of a synergism between magnesium and the toxins. Figure 3 illustrates one such experiment in which the addition of $MgCl_2$ 15 mM before applying the toxin (first black rectangle) reduced the response by about 4%. In the presence of saxitoxin 2·5 μg/l., after a short exposure to 5 μg/l. (white rectangle), the same concentration of magnesium produced a much greater fall in amplitude. The inset on the graph shows a few of the individual responses photographed at the times marked by lines above the graph.

Figure 4 shows a similar experiment in which tetrodotoxin and magnesium were applied separately and in combination. During the control period the addition of $MgCl_2$ 10 mM (black rectangles) had little effect on the responses. Tetrodotoxin was then applied (white rectangle) at various concentrations until a fairly stable partial block was established at 4 μg/l. Now the addition of the same concentration of magnesium resulted in a severe block that almost abolished the responses. This effect is shown twice, together with the recovery that occurred when tetrodotoxin in magnesium-free saline was perfused. After washing the nerve with toxin-free saline for 10 min the responses had recovered almost to their pre-toxin level, but reapplication of magnesium at this time caused a much greater depression than during the pre-toxin period. Evidently the fibres remained unusually susceptible to the action of magnesium for some time after the toxin appeared to have been washed out. This effect diminished with further washing. This experiment was one in which peak amplitudes and integrated areas of the responses were measured simultaneously. For the sake of clarity, and to conform in style with the three other illustrations, only the peak amplitudes are shown in Fig. 4. The plot of integrated areas was almost identical except during the final test with magnesium in the post-toxin washing period. Here the fall in peak amplitude was due largely to an increased temporal dispersion of individual action potentials, which resulted in a spreading of latencies that lowered the peak out of proportion to the numbers of fibres actually blocked. It is not clear why this effect should be so pronounced in the post-toxin period.

Effect of calcium

Experiments were done on two nerve filaments to examine the ability of these toxins to block conduction in the presence of increased concentrations of calcium. The $CaCl_2$ concentration was increased from its usual amount (2·2 mM) up to 10 mM, but no interactions with the toxins were noted.

Discussion

It is now well established that tetrodotoxin acts by preventing the increase in sodium conductance that is necessary for the generation of action potentials in vertebrate nerve and muscle. It does this without significant effect on potassium

conductance (many references in Kao, 1966). For this reason there has been a general belief that tetrodotoxin is a specific inhibitor of sodium ion fluxes through nerve and muscle cell membrane, but this view is not strictly accurate. The toxin probably blocks the channels in the membrane through which the sodium ions must pass to generate action potentials. In experimental conditions a few other ions, including lithium, can be substituted successfully for sodium as inward charge carriers, and tetrodotoxin blocks their entry also. Hille (1968) has shown that both tetrodotoxin and saxitoxin prevent lithium ions from carrying action current in the nodes of Ranvier of frog axons. The present experiments suggest that this is also true for mammalian nerves. The experiments have shown too that lithium-generated action potentials are blocked by both toxins at the same concentrations that block sodium-generated action potentials. This is what one would expect if they act on the membrane channels rather than on the ions. Moore, Narahashi & Shaw (1967) have suggested that one molecule of tetrodotoxin will block one channel in the membrane, and Hille (1968) has supported this view.

Some of the early reports on the actions of these toxins suggested that there could be differences between their effects at the neuromuscular junction. Furukawa *et al.* (1959) reported that tetrodotoxin had a presynaptic action at the junction, causing an abrupt loss of the end-plate potential (e.p.p.) by blocking conduction along the motor axon. This has been confirmed by later workers (see Kao, 1966). On the other hand, saxitoxin in the presence of curare leads to a gradual diminution in the amplitude of the e.p.p. (Nishiyama & Kao, 1964). Application of curare itself causes the e.p.p. to decline gradually due to competitive inhibition of chemical transmission at the post-synaptic end-plate receptors. The similar effect seen by Nishiyama & Kao suggested that saxitoxin might also act at the neuromuscular junction. However Kao & Nishiyama (1965) later presented evidence that saxitoxin was acting pre-synaptically at the junction. Some of this evidence was based on experiments in which $MgCl_2$ 10 mM was added to the saline to prevent the e.p.p. from generating an action potential in the muscle. It seemed possible that if the presence of magnesium potentiated pre-synaptic actions of saxitoxin, Kao & Nishiyama could have missed a post-synaptic action.

The experiments described in this paper have shown that magnesium, in concentrations similar to those used to depress the neuromuscular junction, can potentiate the action of saxitoxin and tetrodotoxin in blocking conduction along axons. However, the effect is seldom as great as the example shown in Fig. 4 and sometimes it is insignificant. Other experiments, reported in the following paper (Evans, 1969), in which the e.p.p. of the frog neuromuscular junction has been recorded in the presence of either curare or magnesium, and then exposed to these toxins, have shown that in fact the choice of neuromuscular blocking agent makes only a marginal difference to the effects produced by the toxins.

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