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Investigation of the spasmogenic effect of manganese on the guinea-pig isolated ileum preparation

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1. The mechanism of the manganese-induced spasm of the guinea-pig ileum was investigated using agents known to modify nerve function. The spasm was reduced by cooling, tetrodotoxin, procaine, *Botulinus* toxin (Type A), hyoscine and pempidine. It was potentiated by mipafox.

2. In the presence of manganese, the release of acetylcholine from the ileum was greatly increased.

3. Tetrodotoxin prevented the manganese-induced increase in acetylcholine output from the ileum but had no significant effect on the spontaneous acetylcholine output.

4. It is suggested that the manganese-induced spasm of the ileum results from an action on intramural cholinergic nerves.

During an investigation of the effect of manganese on the responses of the guinea-pig isolated ileum preparation to various drugs, it was found that manganese itself, in concentrations ranging from 4 to 64 μM produced a marked spasm of the ileum. This paper presents the results of experiments by which the mechanism of this spasmogenic effect was investigated. A preliminary account of these experiments was communicated to the British Pharmacological Society at the Cardiff Meeting (Schnieden & Weston, 1968).

Methods

Guinea-pigs of either sex and weighing 250–450 g were killed by stunning and bleeding. A segment of ileum 2·5 cm in length and situated about 15 cm proximal to the ileo-caecal junction was used. The tissue was set up in a 10 ml. organ bath containing Krebs solution maintained at 37° C and bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Tension changes of the ileum were recorded with a force displacement transducer and pen recorder; the tissue was connected to the transducer with terylene thread.

The transducer was supported in a rack work X-block which allowed the resting tension of the ileum to be changed smoothly. The load was initially fixed at 0·5 g but the ileum stretched, lowering the tension in the system. The tissue was washed with Krebs solution at 4 min intervals and the load was adjusted to 0·5 g after each wash. After six to twelve washes, a fairly stable baseline was achieved and the rate of relaxation did not exceed the equivalent of 0·05 g/hr.

Experimental design. Experiments were designed to test the hypothesis that the manganese-induced spasm of the ileum was of indirect (neurogenic) origin. In these experiments an attempt was made to inactivate selectively nervous tissue in the ileum. In order to assess the degree and specificity of this inactivation two controls were used. Changes in the twitch response of the ileum produced by transmural electrical stimulation (Paton, 1957) were assumed to reflect changes in the function of intramural cholinergic nerves, while changes in the contraction produced by a constant dose of bradykinin in the ED50 range were assumed to reflect changes in the reactivity of smooth muscle cells (Gershon, 1967). Preliminary experiments showed that the responses to transmural stimulation, the bradykinin ED50 and 64 μM manganese, were of comparable magnitude.

Constant responses were obtained to transmural stimulation, to bradykinin and to manganese, after which the ileum was exposed to one of the following treatments for a specific equilibration period before drug responses were re-examined. This equilibration period was 15 min for cooling, and 30 min for tetrodotoxin, procaine, hyoscine and pempidine. In all these instances, the treatment was continued for the remainder of the experiment. The equilibration period for *Botulinus* toxin (Type A) was 20 min, and the periods for mipafox 10^{-5} g/ml. and 10^{-4} g/ml. were 75 min and 90 min respectively. After treatment with either *Botulinus* toxin or mipafox the tissue was washed with Krebs solution at 4 min intervals (eight washes) before drug responses were re-examined.

Use of mipafox as an anticholinesterase. Harry (1962) showed that mipafox 10^{-5} g/ml. inhibited all cholinesterase activity in the guinea-pig ileum. However, Davison (1953) found that prolonged incubation with mipafox 10^{-4} g/ml. was necessary for complete cholinesterase inhibition in rat brain suspensions. Therefore, in some of the experiments described in this paper, the effect of both concentrations of mipafox (10^{-5} g/ml. and 10^{-4} g/ml.) was assessed.

Electrical stimulation of the ileum. Rectangular pulses of 0·1 msec duration were delivered at a frequency of 3/min, the luminal electrode being made positive. The voltage used was slightly greater than that which just produced a maximum response (this was assessed in each preparation). A stimulation period of 2 min was used after which the tissue was washed with Krebs solution. Exposure to bradykinin occurred 2 min later.

Dose cycle. For spasmogens other than manganese a 4 min dose cycle was adopted. Drug contact time was 25 sec and the Krebs solution was changed at 25 sec and 2 min. For manganese an 8 min cycle was used. Contact time was 4 min and the Krebs solution was changed at 4 min and 6 min.

Collection of spasmogen. Collections were made from ileum pretreated with mipafox.

(1) *Experiments with manganese.* Spasmogen was allowed to accumulate for periods of 10 min; the bath fluid was then removed for assay and replaced with fresh Krebs solution. The spontaneous release of spasmogen was always determined before and 44 min (twelve changes of the Krebs at 4 min intervals) after the 10 min exposure to manganese.

(2) *Experiments without manganese.* In these experiments, the effect of tetrodotoxin on spontaneous spasmogen release was investigated. Two adjacent pieces of ileum were set up in Krebs solution. Every 10 min the bath fluid was removed

for assay and replaced with fresh Krebs solution. At time = 40 min, one preparation was exposed to tetrodotoxin 10^{-7} g/ml. A 30 min equilibration period was allowed, during which the bath fluid was removed every 10 min but discarded; 10 min collections were then continued until time = 110 min.

Assay of the spasmogen. The primary assay tissue was the guinea-pig ileum pretreated with mipafox 10^{-5} g/ml. and in the presence of tetrodotoxin 10^{-7} g/ml. Only preparations which failed to respond to a challenge concentration of manganese 10 μM in the presence of tetrodotoxin were used for assay purposes. Several of the samples were also assayed for depressor activity on the rat blood pressure preparation (Straughan, 1958). Spasmogenic activity was assessed in terms of acetylcholine; 16 point assays were conducted on both tissues according to a Latin square design using a 4 min dose cycle.

Criteria used to identify the spasmogen as acetylcholine. (1) The log. concentration: effect lines of bath fluid samples and acetylcholine did not deviate from parallelism in the assays on the ileum ($P > 0{\cdot}1$) or in those on the rat blood pressure ($P > 0{\cdot}1$). (2) The slopes of the log. concentration: effect lines of bath fluid samples and of acetylcholine on the rat blood pressure were shallower than those on the ileum. (3) Spasmogenic activity on the ileum of bath fluid samples and of acetylcholine was abolished after exposure to hyoscine 10^{-8} g/ml. (4) Spasmogenic activity was also abolished by boiling in N/6 NaOH but was unaffected by boiling in N/6 HCl. (5) Desensitization of the ileum to 5-hydroxytryptamine (5-HT) (Brownlee & Johnson, 1963) abolished further responses of the ileum to 5-HT but did not affect responses to bath fluid samples.

Drugs and solutions. The Krebs solution used had the following composition (mM): Na^+, 143; K^+, 5·93; Ca^{++}, 2·55; Mg^{++}, 1·2; Cl^-, 125; HCO_3^-, 25; SO_4^{--}, 1·2; $H_2PO_4^-$, 1·18; dextrose, 11·1.

The drugs used were acetylcholine chloride, *Botulinus* toxin (Type A), synthetic bradykinin, hyoscine hydrobromide, manganese chloride, mipafox (N,N′-diisopropylphosphorodiamidic fluoride), nicotine hydrogen tartrate, pempidine hydrochloride, procaine hydrochloride, tetrodotoxin citrate and 5-hydroxytryptamine creatinine sulphate. All concentrations are expressed in terms of the weight of base/ml. of bath fluid with the exception of manganese chloride, which is expressed in terms of molarity. Drug dilutions were made using Krebs solution, except for manganese chloride when double distilled water was used. The volume of manganese chloride solution added to the bath never exceeded 0·2 ml.

Statistical methods. Student's *t* test (two-tailed) was used to measure the probability of differences between mean responses arising by chance. All measurements of variation stated are of standard errors.

Results

Manganese-induced spasm of the ileum

A record of the tension changes induced in the ileum by 16 μM manganese is shown in Fig. 1. The tension rose slowly to a maximum in 3–4 min and then declined despite the continued presence of manganese. The amplitude of spontaneous activity was, however, increased. The magnitude of the manganese-induced spasm was dose-dependent (Fig. 2). No tachyphylaxis was observed during six successive exposures of the tissue to 64 μM manganese.

Analysis of the mechanism of the manganese-induced spasm

Several agents were used to assess whether the spasm resulted from a direct (myogenic) or indirect (neurogenic) action. At the same time, the specificity of these agents and the extent to which they acted in the desired manner was assessed by eliciting responses considered to be either purely direct or purely indirect in nature. The paradigm of direct stimulation selected was the response to an ED50 of bradykinin while the paradigm of indirect stimulation was usually the electrically induced twitch response of the ileum.

Effect of cooling. Cooling the ileum to 20° C for 15 min reduced the maximum spasmogenic effect of manganese to $6 \pm 4\%$ of its previous amplitude. Twitch responses were abolished but the response to bradykinin was unaffected (Fig. 3A).

Effect of tetrodotoxin. In the presence of tetrodotoxin 10^{-7} g/ml. the maximum spasmogenic effect of manganese was reduced to $6 \pm 6\%$. Twitch responses were completely abolished but the bradykinin response was unaffected (Fig. 3B).

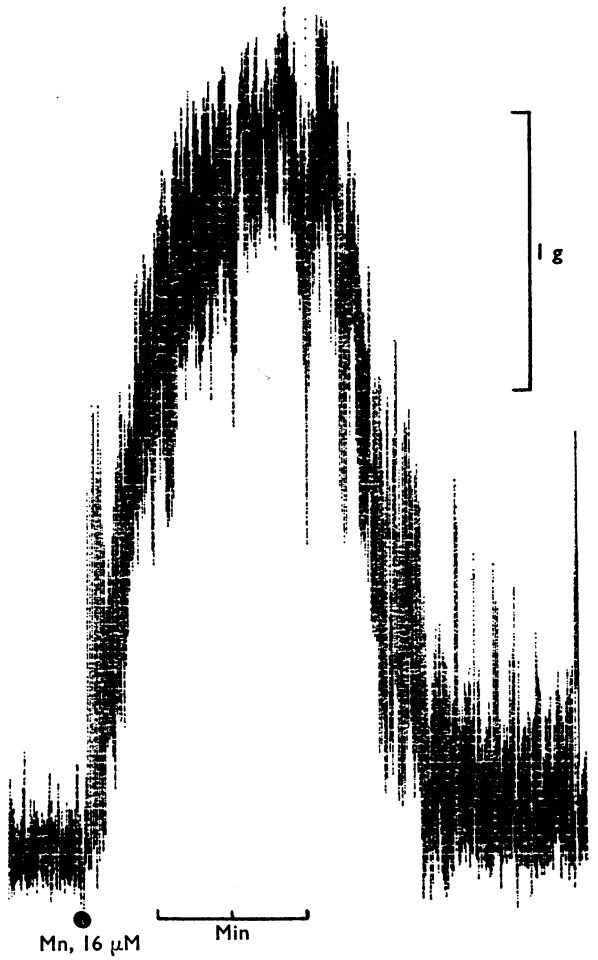

FIG. 1. Guinea-pig ileum. Time-course of the spasmogenic effect of manganese (Mn). The response to 16 μM Mn is shown.

Effect of procaine. In the presence of procaine 10^{-4} g/ml. the maximum spasmogenic effect of manganese was reduced to $50 \pm 5\%$. Twitch responses were reduced to $22 \pm 7\%$ and the bradykinin response was unaffected (Fig. 3C).

Effect of Botulinus toxin (Type A). Exposure of the ileum to *Botulinus* toxin (Type A) 8×10^3 MLD mouse/ml. for 20 min resulted in a reduction of the maximum spasmogenic effect of manganese to $15 \pm 6\%$. The twitch response was reduced to $8 \pm 4\%$ but the bradykinin response was unaffected (Fig. 4A). These effects on the control responses were assessed about 1 hr after the initial exposure of the preparation to the toxin.

Effect of hyoscine. In the presence of hyoscine 10^{-7} g/ml. the maximum spasmogenic effect of manganese was reduced to $36 \pm 6\%$. The twitch response was reduced to $6 \pm 1{\cdot}5\%$ but the bradykinin response was unaffected (Fig. 4B).

Effect of pempidine. In the presence of pempidine 2×10^{-5} g/ml. the maximum spasmogenic effect of manganese was reduced to $61 \pm 6\%$. Neither the twitch response nor the response to bradykinin was affected (Fig. 4C). All experiments with pempidine were also controlled with nicotine 10^{-6} g/ml. The effect of nicotine was reduced to $8 \pm 4\%$.

Effect of mipafox. Incubation of the ileum with mipafox 10^{-5} g/ml. for 75 min resulted in a leftward shift of the acetylcholine log. concentration : effect curve. There was no change in the slope or maximum response. In these conditions, the

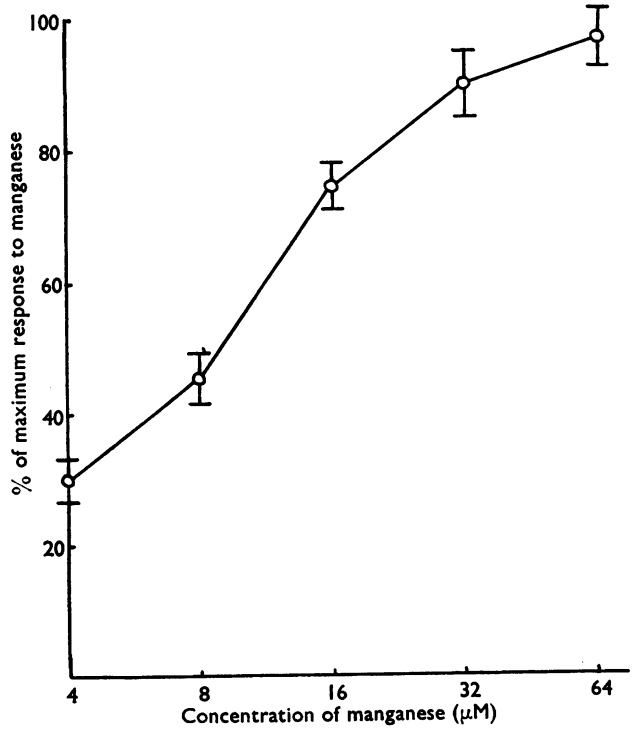

FIG. 2. Guinea-pig ileum. Log. concentration : effect curve of manganese (Mn). Means of forty-two experiments with the standard errors are plotted. The maximum spasmogenic effect of Mn was approximately equivalent to that of an ED50 of bradykinin.

manganese log. concentration : effect curve was also shifted to the left with a large increase in the maximum response (Figs. 5A & 5B) although the time-course of the spasm was unaffected. These experiments with mipafox were also controlled by the bradykinin and the twitch responses. The response to bradykinin was unaffected but the twitch response was potentiated (Fig. 5C and 5D).

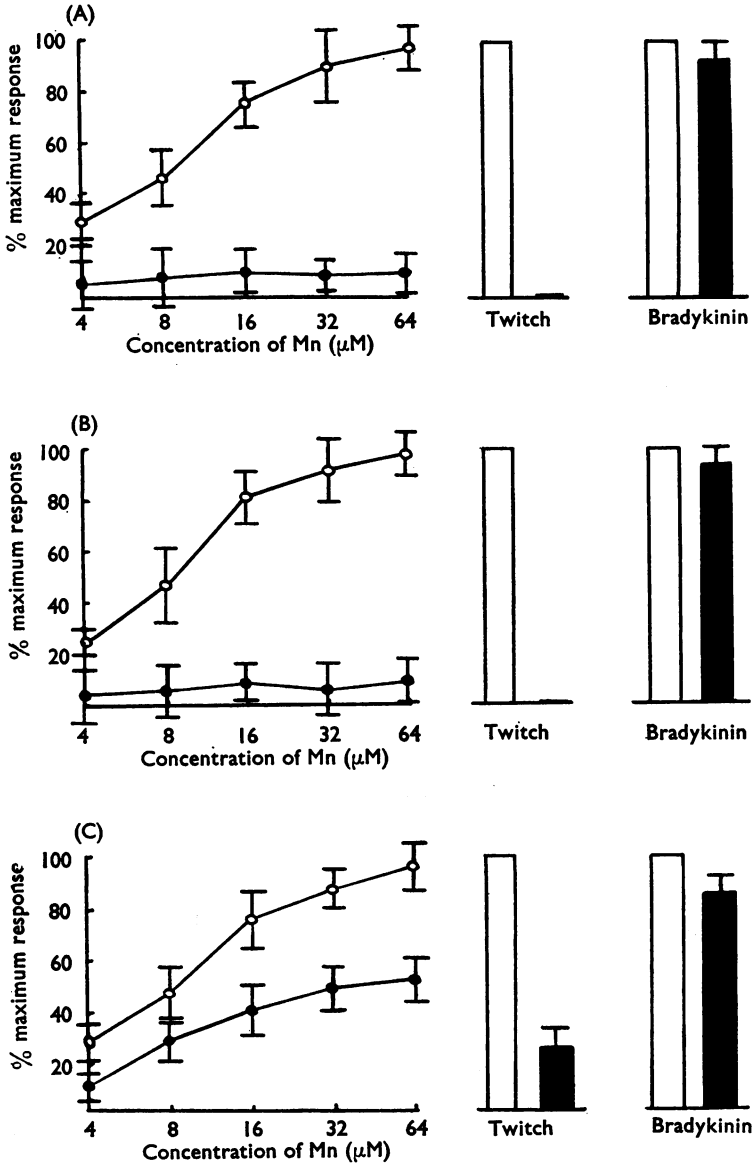

FIG. 3. Guinea-pig ileum. Influence of various agents on the manganese (Mn) log. concentration : effect curve and on responses to indirect (twitch) and direct (bradykinin) stimulation. Left: Mn log. concentration : effect curve ; middle: twitch response ; right: bradykinin response. Open circles and columns, Control responses ; black circles and columns, responses after A: cooling to 20° C ; B: tetrodotoxin, 10^{-7} g/ml. ; C: procaine, 10^{-4} g/ml. The Mn maximum, control twitch response and control bradykinin response are each expressed arbitrarily as 100%. These three responses were in fact comparable in magnitude. Means of at least six experiments with the standard errors are plotted.

Acetylcholine release from the ileum

Manganese was found to cause a release of spasmogen from the ileum and this spasmogen exhibited the properties of acetylcholine using the criteria given in **Methods**. It was therefore assayed in terms of acetylcholine. The effects of 10 min exposures to manganese (8 μM & 64 μM) on the amount of acetylcholine released from the ileum after treatment with mipafox 10^{-5} g/ml. are summarized in Fig. 6.

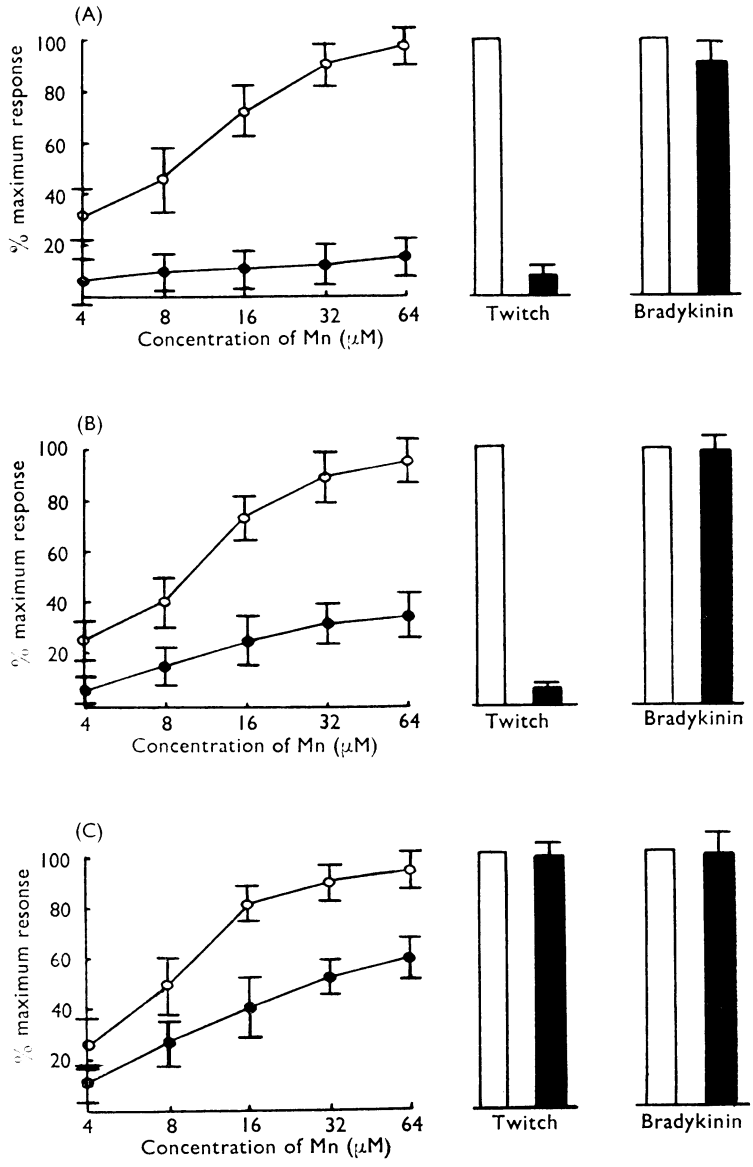

FIG. 4. Guinea-pig ileum. Influence of various agents on the manganese log. concentration: effect curve and on responses to indirect and direct stimulation expressed as in Fig. 2. Open circles and columns, Control responses; black circles and columns, responses after A: *Botulinus* toxin (Type A), 8×10^3 MLD mouse/ml.; B: hyoscine, 10^{-7} g/ml.; C: pempidine, 2×10^{-5} g/ml. Means of at least six experiments with the standard errors are plotted.

It can be seen that manganese produced a concentration-dependent increase in acetylcholine output from the ileum. The initial resting release of acetylcholine was found to be 62 ± 6 ng acetylcholine/g wet weight of ileum. This figure is similar to that found by Johnson (1963). In the presence of 8 μM manganese, the acetylcholine release was 115 ± 9 ng acetylcholine/g wet weight of ileum and in the presence of 64 μM manganese it was 290 ± 16 ng acetylcholine/g wet weight of ileum. There was no significant difference between the initial control determination of resting acetylcholine release and further control determinations made after manganese was washed out.

After incubation of the ileum with mipafox 10^{-4} g/ml., a resting release of 55 ± 10 ng acetylcholine/g wet weight of ileum was detected (five experiments). In the presence of manganese (8 μM and 64 μM), the acetylcholine release was 111 ± 12 ng acetylcholine/g wet weight of ileum (five experiments) and 295 ± 21 ng acetylcholine/g wet weight of ileum (five experiments) respectively.

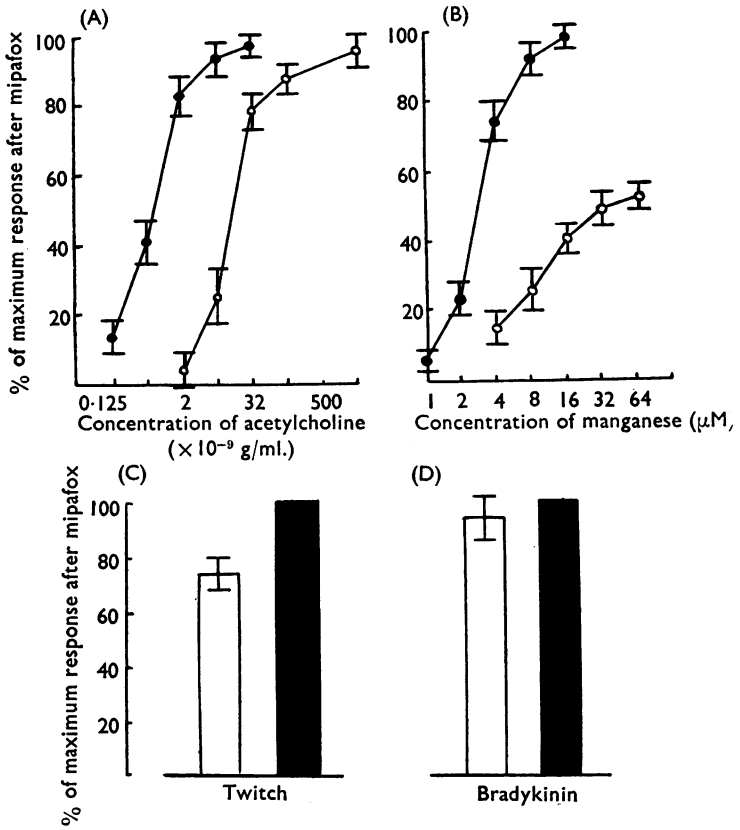

FIG. 5. Guinea-pig ileum. Influence of 75 min pretreatment with mipafox 10^{-5} g/ml. on the log. concentration : effect curves of acetylcholine (ACh) and manganese (Mn) and on the paradigms of indirect and direct stimulation. Log. concentration : effect curves of (A) ACh and (B) Mn. The two responses were in fact comparable in magnitude. C, Twitch response ; D, bradykinin response. All maximum responses after mipafox pretreatment arbitrarily expressed as 100%. Open circles and columns, Control responses ; black circles and columns, responses after mipafox. Means of at least six experiments with the standard errors are plotted.

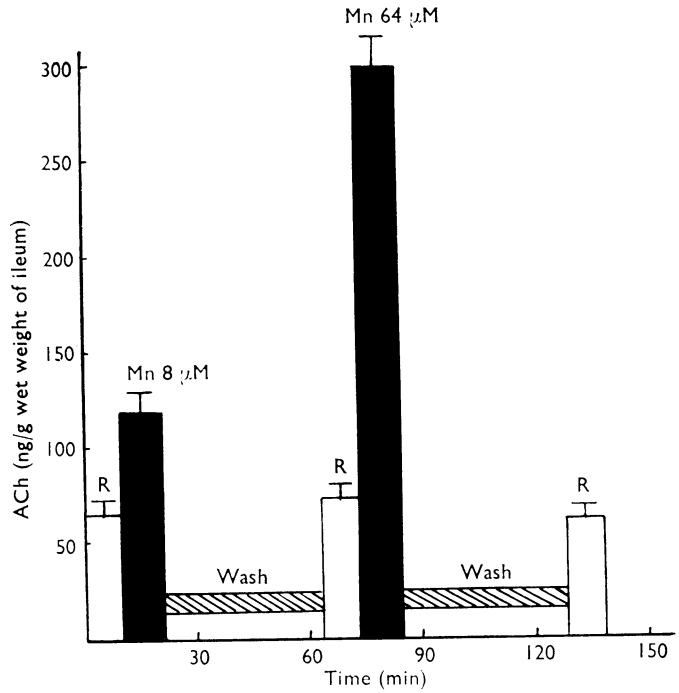

FIG. 6. Guinea-pig ileum pretreated with mipafox, 10^{-5} g/ml. The effect of manganese (Mn) on acetylcholine (ACh) output. ACh was allowed to accumulate for periods of 10 min ; the bath fluid was then removed for assay and replaced with fresh Krebs solution. The spontaneous release of ACh (R) was always determined before, and 44 min after, the 10 min exposure to Mn. Means of six experiments with the standard errors are plotted.

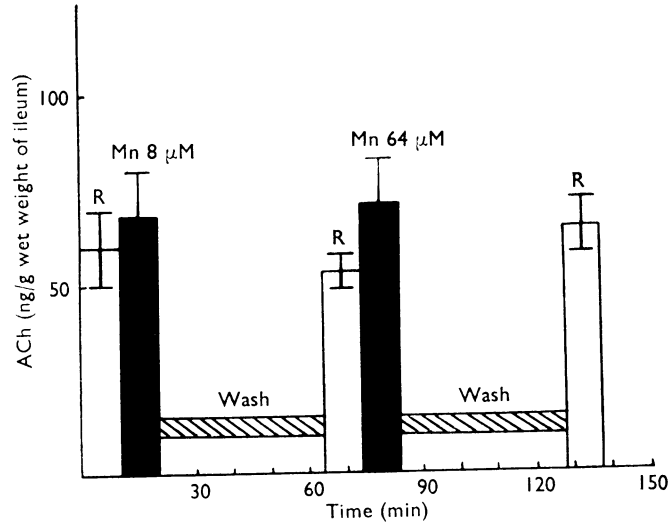

FIG. 7. Guinea-pig ileum pretreated with mipafox, 10^{-5} g/ml. The effect of tetrodotoxin on the manganese-induced acetylcholine release. Details as for Fig. 6, but tetrodotoxin 10^{-7} g/ml. present throughout the experiment and for 30 min beforehand.

Effect of tetrodotoxin on acetylcholine release

Tetrodotoxin in a concentration (10^{-7} g/ml.) which almost completely abolished the manganese-induced spasm (Fig. 3B) had no significant effect on spontaneous acetylcholine release from the ileum (Fig. 8). In contrast, the same concentration

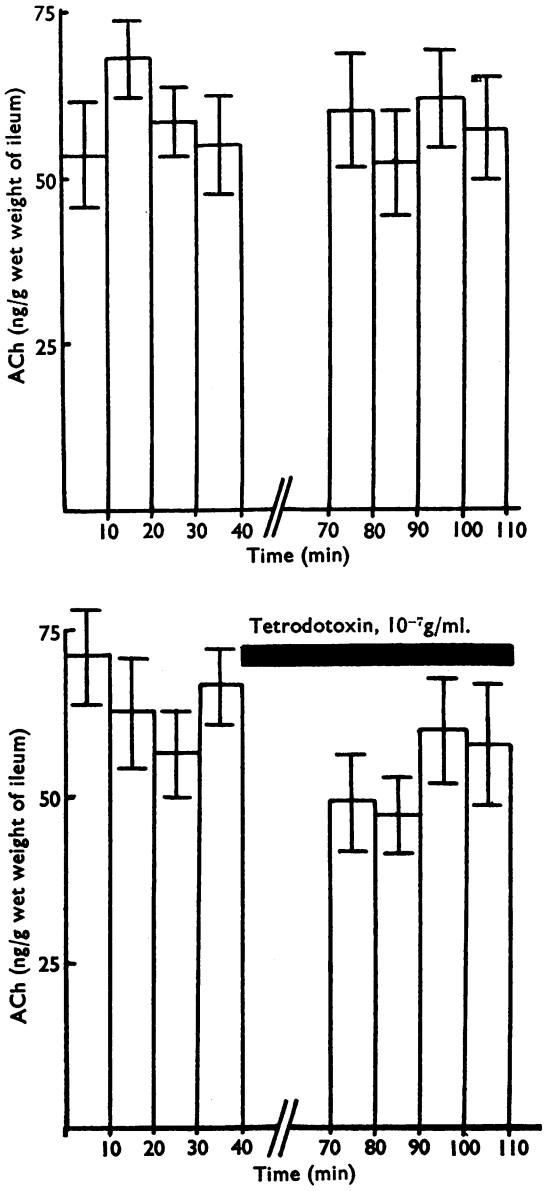

FIG. 8. Guinea-pig ileum pretreated with mipafox, 10^{-5} g/ml. The effect of tetrodotoxin on spontaneous acetylcholine release. Two adjacent pieces of ileum were set up in Krebs solution. Every 10 min the bath fluid was removed for assay and replaced with fresh Krebs solution. At time=40 min, one preparation (lower diagram) was exposed to tetrodotoxin, 10^{-7} g/ml. A 30 min equilibration period during which the bath fluid was removed every 10 min but discarded, was allowed. 10 min collections were then continued. Means of six experiments with the standard errors are shown.

of tetrodotoxin prevented the large manganese-induced increase in acetylcholine output from the ileum (Fig. 7).

Discussion

Cooling a tissue to remove neuronal influences has been exploited by several workers (Ambache, 1946; Blair & Clark, 1956; Innes, Kosterlitz & Robinson, 1957). After cooling to 20° C, the maximum effect of manganese was reduced to $6 \pm 4\%$. Kao (1966) has reviewed the evidence that tetrodotoxin is a specific inactivator of the sodium conductance changes which occur during nervous activity and this drug was used by Gershon (1967) to denervate functionally several mammalian smooth muscle preparations. In our experiments, tetrodotoxin reduced the maximum effect of manganese to $6 \pm 6\%$. Feldberg & Lin (1949) showed that local anaesthetics acted on nerve plexuses in smooth muscle rather than on the muscle itself. In the presence of procaine the maximum effect of manganese was reduced to $50 \pm 5\%$. The experiments of Ambache (1949), Burgen, Dickens & Zatman (1949) and Ambache & Lessin (1955) have shown that *Botulinus* toxin is able to paralyse cholinergic nerve endings by prevention of acetylcholine release. Harry (1962) confirmed these findings and demonstrated the occurrence of a marked reduction in acetylcholine output from the ileum following transmural stimulation in the presence of the toxin. Exposure of the ileum to *Botulinus* toxin for 20 min reduced the maximum effect of manganese to $15 \pm 6\%$. The use of muscarinic receptor blocking agents to remove cholinergic components of drug action on smooth muscle has been exploited by Gaddum & Hameed (1954), Gaddum & Picarelli (1957) and Kosterlitz & Robinson (1958). Hyoscine reduced the maximum effect of manganese to $36 \pm 6\%$.

The above observations show that a large proportion, if not all, of the spasmogenic effect of manganese is produced by an action on intramural cholinergic nerves. This conclusion is confirmed by the potentiation of the spasm after treatment of the ileum with mipafox, an irreversible cholinesterase inhibitor. In addition, a greatly increased output of acetylcholine was detected from mipafox-treated ileum after exposure to manganese. Assays of this acetylcholine were conducted on pieces of ileum which, in the presence of tetrodotoxin, failed to respond to a test concentration of 10 μM manganese; the use of tetrodotoxin and the manganese test in these assays eliminated any possibility of a spasm resulting from the presence of low concentrations of manganese in the samples for assay.

In addition to reducing the manganese-induced spasm of the ileum, tetrodotoxin prevented the large increase in acetylcholine output detected after exposure to manganese. This observation suggests that this increased output of acetylcholine is responsible for the spasm of the ileum following exposure to manganese.

An interesting aspect of the effect of manganese is the gradual reduction of the spasm despite the continuing presence of the ion (Fig. 1). Nachmansohn (1940) showed that 20 μM manganese increased the activity of eel acetylcholinesterase (AChE) some fourteen-fold. However, the time course of the manganese-induced spasm was not altered after treatment of the ileum with mipafox, which suggests that the reduction of the spasm is independent of the activity of AChE.

Repetitive stimulation of cholinergic nerves in several tissues has been shown to have an adverse effect on acetylcholine output. Perry (1953) showed that the acetyl-

choline output from the perfused superior cervical ganglion of the cat declined rapidly during preganglionic stimulation. Similarly, the rate of acetylcholine release from phrenic nerve endings in the rat diminished as duration of stimulation was increased (Straughan, 1960). Paton (1957) showed that the amount of acetylcholine released per stimulus from the guinea-pig ileum fell as stimulation frequency was increased. At the same time the ileum was unable to maintain its contractile response to a given frequency of stimulation. Such observations suggest that the reduction of the manganese spasm despite the continued presence of the ion may be due to a reduced rate of acetylcholine output as a result of repetitive nerve stimulation.

Acetylcholine has been shown to be released spontaneously from the guinea-pig isolated ileum preparation (Johnson, 1963) and at the end-plate region of resting amphibian muscle (Fatt & Katz, 1952). More recently, Katz & Miledi (1967) have shown that tetrodotoxin failed to prevent the spontaneous release of acetylcholine from nerve endings in amphibian and mammalian muscle ; moreover, electrical stimulation of neurone terminals in the presence of tetrodotoxin resulted in the appearance of a normal muscle action potential in these preparations. The results reported in this paper show that tetrodotoxin had no significant effect on the spontaneous acetylcholine release from the guinea-pig ileum. The failure of tetrodotoxin to alter the spontaneous transmitter release from both spinal motor nerve endings and autonomic cholinergic nerve endings suggests that there may be a common transmitter release mechanism in such nerves. In addition, tetrodotoxin could prove a useful tool in distinguishing between those drugs which act on neurone cell bodies or their axons and those which act on neurone terminals.

In contrast to the above observations, Ogura, Mori & Watanabe (1966) have reported that tetrodotoxin produced a significant reduction in the spontaneous acetylcholine output from the guinea-pig ileum. In their experiments, eserine was used to prevent acetylcholine breakdown. It is interesting to compare the values quoted in the literature for experiments in which eserine or mipafox have been used to allow measurement of spontaneous or electrically-induced release of acetylcholine from the ileum. With eserine, the release of very large quantities of acetylcholine has been reported (Paton, 1957 ; Schaumann, 1957 ; Paton, 1963 ; Ogura *et al.*, 1966 ; Paton & Zar, 1968). In contrast, when mipafox has been used as an anticholinesterase in similar situations, quantities of acetylcholine some 10–50 times smaller have been obtained (Harry, 1962 ; Johnson, 1963 ; Schnieden & Weston, this paper). Carlyle (1963) found evidence that eserine itself induced acetylcholine release from the guinea-pig tracheal chain preparation and there are many other reports of a neurone-stimulating action of eserine (Werner & Kuperman, 1963). Such an action could well account for the difference between our observations and those of Ogura *et al.* (1966) concerning the effect of tetrodotoxin on spontaneous acetylcholine release from the ileum.

Fatt & Ginsborg (1958) and Hagiwara & Naka (1964) have suggested that manganese (1–10 mM) is able to prevent the entry of Ca^{++} into crustacean and barnacle muscle cells. There are several reports that similar concentrations of manganese (0·5–10 mM) reduced the responses of various mammalian isolated smooth muscle preparation to acetylcholine (Nonomura, Hotta & Ohashi, 1966), to noradrenaline and to 5-HT (Su & Bevan, 1967) and to K^+ (Imai & Takeda, 1967). Such concentrations of manganese are in the same range as the concentration of

Ca^{++} in Krebs solution (2·5 mM). On the basis of these experiments it has been suggested that manganese, in millimolar concentrations, is able to prevent Ca^{++} entry into mammalian smooth muscle cells (Nonomura *et al.*, 1966; Bülbring & Tomita, 1968). The spasm of the ileum reported in this paper was produced by micromolar concentrations of manganese and it seems unlikely therefore that prevention of Ca^{++} movement can account for this spasm.

This effect of manganese is not specific for this ion alone. Eichler and Lippert (1966) have shown that spasms of the guinea-pig ileum can be produced by cobalt, nickel, copper and zinc. In addition, Ba^{++} (100–500 μM) has long been known to be a potent spasmogen and the action of this metal has been shown to possess a considerable neurogenic component (Ambache, 1946; Ambache & Lessin, 1955; Gershon, 1967; Paton & Zar, 1968). It seems possible that all these ions may share a common mode of action and further study of their effects could well provide useful information concerning nerve and muscle excitation processes.

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