J. Physiol. (1967), **191**, *pp.* 257–270
With 9 text-figures
Printed in Great Britain

NERVOUS FACTORS INFLUENCING THE MEMBRANE ACTIVITY OF INTESTINAL SMOOTH MUSCLE

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(*Received* 19 *September* 1966)

SUMMARY

The effects of various chemical agents on the spontaneous membrane activities and those electrically elicited in the smooth muscles of small intestine were investigated.

1. The effects of various chemicals on the spontaneously active membrane might be summarized as follows. (*a*) Cholinergic agents; atropine slightly hyperpolarized the membrane and reduced the amplitude of slow potential changes even in aged preparations. Prostigmine depolarized the membrane, and enhanced the amplitude and prolonged the duration of the slow potential changes. Atropine prevented the actions of prostigmine on the membrane. (*b*) Ba^{2+} depolarized the membrane, and enhanced the amplitude and prolonged the duration of the slow potential changes. The spike frequency was initially increased, then reduced. Atropine and tetrodotoxin partially prevented the action of Ba^{2+} on the membrane activities.

2. Effects of chemical agents on the membrane activity elicited by electrical stimulation might be summarized as follows. (*a*) Short pulse stimulation (0·5–1 msec) generated the spike as a direct response of the muscle cell membrane, then it was followed by slow depolarization, delayed hyperpolarization, i.e. the 'inhibitory potential', and post-inhibitory rebound successively. (*b*) The slow depolarization and the post-inhibitory rebound were reduced in amplitude by treatment with atropine, and enhanced by treatments with prostigmine and Ba^{2+}. Tetrodotoxin blocked all activities except the spike.

3. When repetitive stimulation (20 c/s) was applied to the membrane, the membrane hyperpolarized; then, after 3–5 sec, it gradually depolarized even if the stimulation was continued, and triggered spikes. The hyperpolarization always preceded depolarization. The duration and the amplitude of the delayed depolarization was proportionally increased by the increased intensity and duration of stimulation. Atropine and tetrodotoxin blocked the generation of the post-inhibitory rebound.

4. Effects of repetitive stimulation on the stored tissues were observed.

The responses to repetitive stimulation of the membrane of muscles which had been stored 50 hr at 4 °C, were the same as those observed in the fresh tissue. The response of the tissue which had been stored 100 hr was the same as that observed in the fresh tissue treated with tetrodotoxin, i.e. all activities except the spikes were blocked.

INTRODUCTION

There are two kinds of nervous plexuses in the intestinal wall. Auerbach's plexus is situated between the longitudinal and the circular muscles and is thought to be mainly motor; Meissner's plexus is in the submucous layer and thought to be mainly sensory. Therefore, nervous factors may be involved in the membrane activity of intestinal smooth muscle; they may either be spontaneous or elicited by external electrical stimulation. Since the spontaneously generated spike is coupled with a contraction this may provoke the activity of an intrinsic reflex arc.

Gillespie (1962*a*, *b*) has studied the membrane activity of smooth muscle cells in rabbit nerve-intestine preparations using micro-electrodes, and observed the effects of sympathetic and parasympathetic nerve stimulation. Bennett, Burnstock & Holman (1966*a*, *b*) observed the effect of transmural stimulation on the taenia coli and recorded an inhibitory potential, i.e. a transient hyperpolarization and block of the spike generation. They considered that the inhibition was partly due to the release of noradrenaline from perivascular nerves but mostly due to an unknown inhibitory transmitter. They also sometimes recorded an excitatory potential, i.e. a depolarization of the membrane which was blocked by atropine (Burnstock, Campbell, Bennett & Holman, 1963; Bennett *et al.* 1966*a*, *b*; Burnstock, Campbell & Rand, 1966). Suzuki & Inomata (1964) also recorded inhibitory potentials from taenia coli confirming the observations made by Burnstock *et al.* (1963).

Bülbring, Kuriyama & Tomita (1965) thought that part of the inhibitory potential might be myogenic. However, recently careful electrophysiological analysis (Bülbring & Tomita, 1966) showed that the inhibitory potential was mainly due to excitation of intrinsic inhibitory nerves.

The present experiments were undertaken to investigate mainly the excitatory nervous factors influencing spontaneous activity as well as membrane activities of the small intestine triggered by field stimulation. From the results it may be concluded that, in physiological conditions, the spontaneous activity is influenced by spontaneously released chemical transmitters. By studying the influence of atropine and tetrodotoxin, the membrane activity evoked by transmural electrical stimulation could be classified as myogenic or neurogenic responses. The possible mechanism of the actions of the nervous network is discussed.

METHODS

The experimental methods in the present study were the same as those described in the previous paper (Kuriyama, Osa & Toida, 1967).

The tissue used for the present experiment was mainly the longitudinal muscle of the jejunum of the guinea-pig. The drug concentrations used will be described in Results.

For extracellular stimulation of the tissue, two Ag–AgCl electrodes were used (diameter 1 mm), one electrode being placed on the tissue and the other at a distance of 3–4 cm from the tissue.

RESULTS

Effects of various chemicals on the spontaneously discharging smooth muscle cells

Cholinergic agents. As already described in the previous paper (Kuriyama *et al.* 1967), when the tissue was bathed in Krebs solution for several hours, the membrane activities deteriorated. In this condition, the effects of tetrodotoxin (10^{-7} g/ml.) and of atropine (10^{-6} g/ml.) were observed,

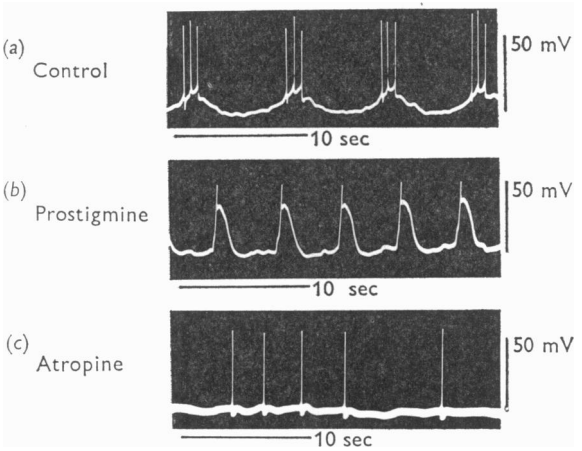

Fig. 1. Effects of prostigmine (5×10^{-5} g/ml.) and atropine (5×10^{-6} g/ml.) after pretreatment with prostigmine on the membrane activities of the longitudinal muscle of jejunum. *a*, Control; *b*, effect of prostigmine (after 10 min); *c*, effect of atropine in the presence of prostigmine (after 10 min); *b* and *c* records were taken from the same cell.

using the longitudinal muscle of jejunum after 8 hr in Krebs solution. Both agents changed the spike configuration and restored it to that of a fresh preparation, but the slow potential change was suppressed.

Differences between tetrodotoxin and atropine are as follows. (1) Tetrodotoxin does not change the membrane potential but atropine slightly hyperpolarizes the membrane. (2) The maximum rate of rise of the spike is restored by treatment with atropine after the tissue has been bathed by

modified Krebs solution for several hours; in contrast, the effect of tetrodotoxin on it is weaker. (3) Tetrodotoxin is stronger than atropine in blocking the generation of the slow potential change.

Figure 1 shows the effects of prostigmine (5×10^{-5} g/ml.) and atropine (5×10^{-6} g/ml.) In normal Krebs solution the spike appeared on a slow depolarization (*a*). After treatment with prostigmine, the spike configuration was completely changed. The amplitude of the slow component was augmented (sometimes exceeding 35 mV), its duration was 2–3 sec, so that a spike with an elevated plateau potential appeared (*b*). When the prostigmine concentration was raised up to 10^{-4} g/ml., the membrane depolarized

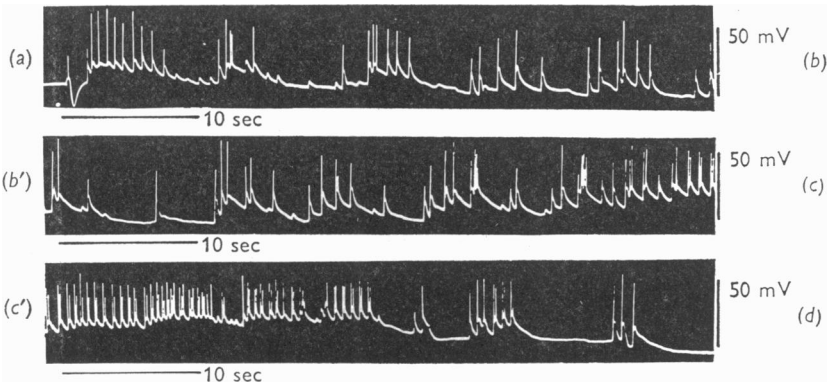

Fig. 2. An example of the effect of Ba^{2+} (0·5 mM) on the membrane activity of the longitudinal muscle of jejunum. A white spot indicates extracellular stimulation (5 msec pulse duration) after 10 min exposure. Slow depolarizations with continuous spike discharges appeared, and ceased again even in the presence of Ba^{2+}. Continuous record.

by more than 15 mV and the generation of the spike and the slow component were blocked; sometimes only slow oscillatory potentials remained. Treatment with atropine abolished the slow depolarization and only spikes appeared (Fig. 1*c*).

Barium ion. By treatment with 1 mM-Ba^{2+}, the membrane at first depolarized by 5–10 mV and the spike frequency was increased. Subsequently, a gradual decrease of the spike frequency and a tremendous prolongation of the depolarization phase were observed as reported by several investigators (Burnstock & Prosser, 1960; Bortoff, 1961; Suzuki, Nishiyama & Okamura, 1964; Bülbring & Kuriyama, 1963). The duration of the prolonged slow depolarization was usually 1–3 sec, but in some experiments it exceeded 10 sec. When the Ba^{2+} concentration was less than 1 mM, the membrane was less depolarized and the spike frequency was often increased.

Figure 2 shows an example of the effect of Ba^{2+} (0·5 mM) on the membrane activity, recorded continuously. During the quiescent period, at the beginning of the top record, a single electrical stimulus evoked a spike, a slow depolarization and a delayed hyperpolarization as described previously (Kuriyama *et al.* 1967). After the delayed hyperpolarization, spikes and slow depolarizations appeared continuously for more than 1 min. Two types of slow potentials could be observed: the first type was a slow fluctuation of the membrane potential of long duration, and the second type was directly related to the spike.

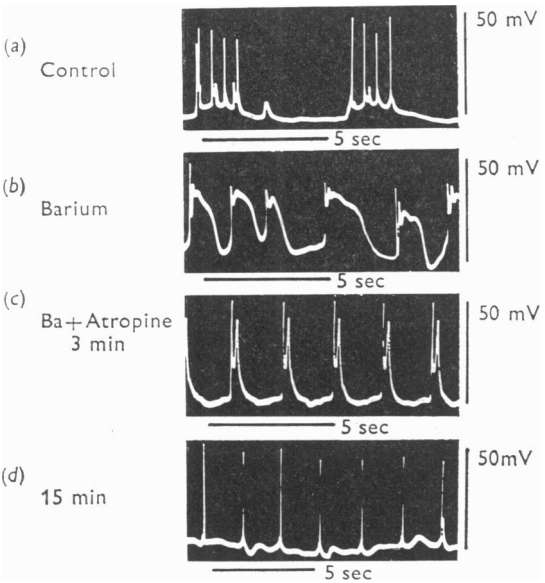

Fig. 3. Effects of atropine (5×10^{-5} g/ml.) on the membrane activities produced by pretreatment with Ba^{2+} (0·5 mM). *a*, Control; *b*, treatment with $BaCl_2$ (after 10 min); *c*, treatment with atropine after pre-treatment with $BaCl_2$ (after 3 min); *d*, after 15 min.

The slow depolarization, elevated by treatment with Ba^{2+}, was reduced by treatment with atropine. Figure 3 shows the effect of atropine (5×10^{-5} g/ml.) on the membrane activities under pre-treatment with Ba^{2+} (0·5 mM). By treatment with Ba^{2+}, the slow and prolonged depolarizations developed (*b*). Atropine drastically changed the membrane activities, i.e. it reduced the plateau phase (*c*), and finally abolished the slow depolarization (*d*), indicating that the increased membrane activities of the smooth muscle might be due to increased nervous activity, releasing the chemical transmitter.

Membrane activities elicited by electrical stimulation

Bennett *et al.* (1966*a*) reported that the transmural stimulation of the taenia coli evoked inhibitory potentials which blocked the spike generation during the hyperpolarizing period. The present experiment confirmed the above observation.

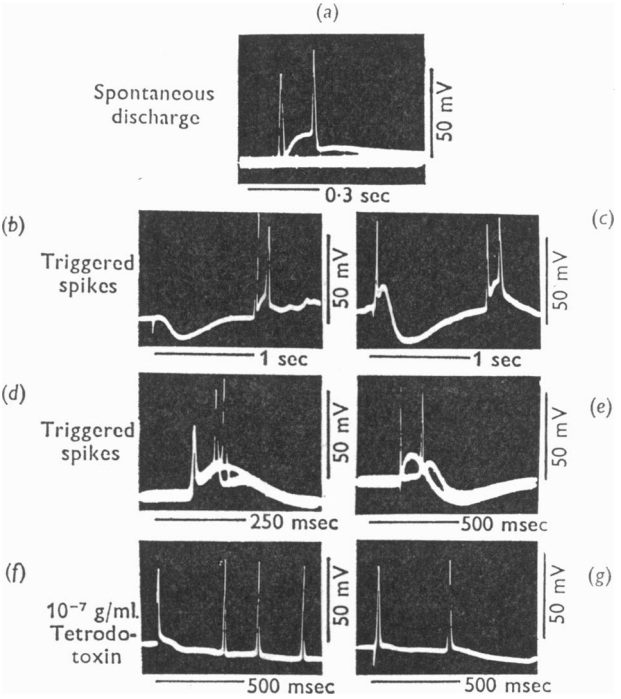

Fig. 4. Spontaneous activity of the longitudinal muscle of jejunum and that evoked by the extracellular short pulse stimulation (1 msec). *a*, Twin spike generated spontaneously; *b*, *d*, *f* are produced by anodal stimulation (1 msec); *c*, *e*. *g* are produced by cathodal stimulation (1 msec); *b–e* are recorded from the cells without any treatment; *f* and *g* are recorded after treatment with tetrodotoxin (10^{-7} g/ml.). For detailed description see text.

Thresholds to generate the spike, the slow depolarization and the inhibitory potential by the electrical stimulation were different. With current pulses of less than 1 msec duration, the threshold for the inhibitory potential was lower than that of the slow depolarization and the spike. This tendency was detected in both cathodal and anodal stimulation. However, the anodal stimulation was more effective than the cathodal one.

Figure 4 shows the spontaneous activity of jejunum and that evoked

by the extracellular short pulse stimulation (1 msec). The first spike was generated spontaneously from the resting potential level but the second arose from the slow potential changes which followed the first spike. The falling phase of the second spike partially cancelled out the slow depolarization (*a*). Similar twin spikes were seen when the membrane was stimulated electrically (*b–e*). Extracellular anodal stimulation evoked neither the spike nor the slow potential but it evoked the inhibitory potential and the post-inhibitory rebound (*b*). In contrast, extracellular cathodal stimulation evoked the spike, the slow depolarization, the inhibitory potential and the rebound (*c*). The faster sweep of records (*d*) showed the spike which was triggered by the electrical stimulation and the spike evoked on the slow

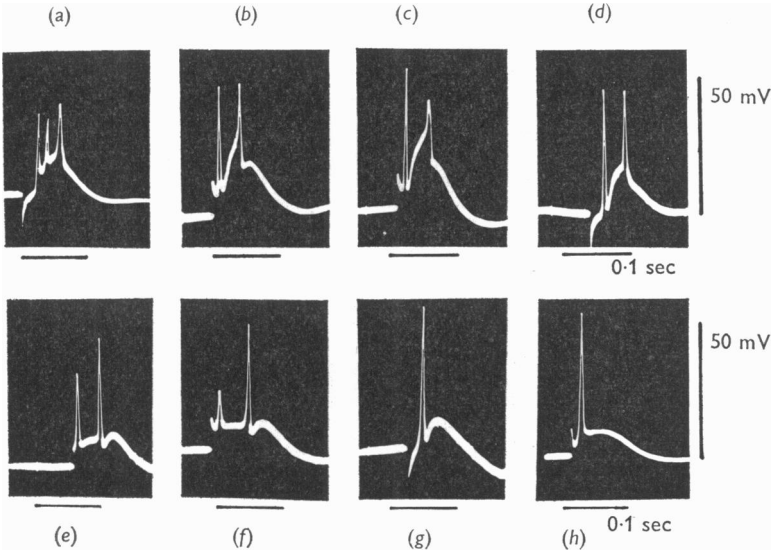

Fig. 5. Relation between slow depolarization and spike. One stimulating electrode was placed on the tissue and the other at a distance of 3 cm from the tissue. The amplitude of the slow depolarization triggered by single stimulation varies in individual cells (1msec stimulus duration). *a*, *d*, *g* are evoked by cathodal stimulation, *b*, *c*, *e*, *f*, *h* by anodal stimulation.

potential. Three successive records were superimposed. The slow potential was partially cancelled out when the spike was triggered. In (*e*), two successive records were superimposed. The spikes appeared with different latencies and were followed by the slow depolarization and the inhibitory potential. These records indicate that the slow depolarization might be triggered by the preceding spike, although it could be triggered by the direct field stimulation. However, the inhibitory potential was directly

triggered by the field stimulation, because the crest times of the hyperpolarization appeared at the same time on both the records (*e*) regardless of when the spike and the slow potential were elicited. This observation might indicate that the generation mechanisms of the slow depolarization and of the inhibitory potential are different. Treatment with 10^{-7} g/ml. of tetrodotoxin blocked the generation of the slow depolarization and of the inhibitory potential and, consequently, the post-inhibitory rebound could not be observed (*f* and *g*).

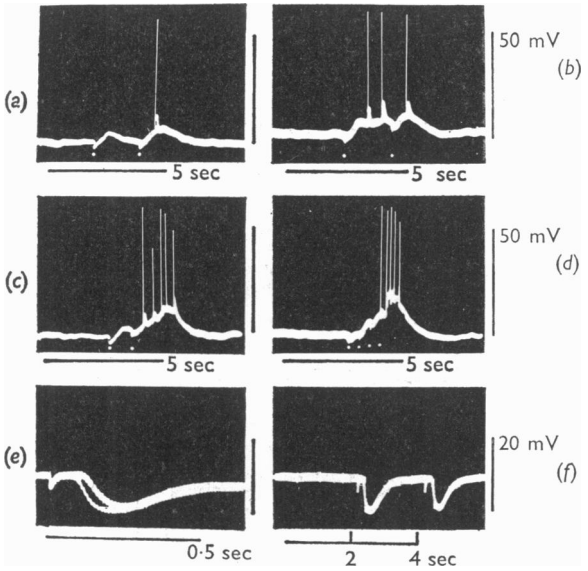

Fig. 6. Responses of the membrane to extracellular short pulse stimulation (0·5 msec). *a–d* are recorded from cells in which the slow depolarization is dominant, and *e* and *f* are recorded from cells in which the inhibitory potential is dominant. White spots indicate the points of stimulation.

The amplitude of the slow depolarization and the inhibitory potential varied with the individual cell; in some cells the depolarization was more dominant than the hyperpolarization and in the other cells the dominance was reversed.

Figure 5 shows the relation between the slow depolarization and the spike. The shapes of the slow depolarization triggered by single stimuli varied in individual cells and when the slow depolarization reached the firing level the spike was triggered, which partially cancelled out the process of the slow depolarization (*b–d*).

Figure 6 shows the responses of the different cell membranes to the extracellular short pulse stimulation (0·5 msec). (*a–d*) were recorded from cells in which the slow depolarization was dominant and (*e*) and (*f*) were

recorded from cells in which the inhibitory potential was dominant. With increased stimulus frequencies the depolarization was enhanced and the number and frequency of spike generation was increased (*a–d*).

In (*e*) and (*f*), the inhibitory potential was recorded. In contrast to the slow depolarization, facilitation was not observed and the first inhibitory potential always showed a shorter latency and higher amplitude than the subsequent ones. When the stimulus frequency was higher than 1 c/s, the inhibitory potentials summated. However, already at a frequency of 1 c/s the membrane gradually depolarized and then generated a spike. Thus, the slow depolarizations became gradually larger and more dominant than the inhibitory potential, which did not facilitate. The latency of the

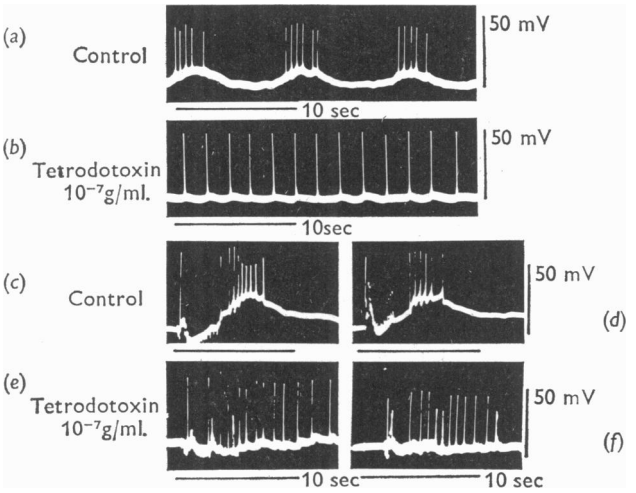

Fig. 7. Effect of tetrodotoxin (10^{-7} g/ml.) on the spontaneous discharges (*a*, *b*) and also on the membrane activity elicited by repetitive stimuli (*c–f*). 0·5 msec pulse 20 c/s. *c*, *e* are produced by cathodal stimulation, *d*, *f* by anodal stimulation.

inhibitory potential was 50–80 msec and the time to the crest was 120–280 msec. These variations were obtained with different impalements, but individual cells produced nearly constant successive responses. The more the intensity of stimulation was increased the more the amplitude and the slope of the hyperpolarization could be increased, but the latencies and the crest times remained the same. These properties are analogous to those observed in the hypogastric nerve vas deferens preparation (Burnstock, Holman & Kuriyama, 1964), in which increased stimulation of the hypogastric nerve enhanced the amplitudes and the maximum rates of rise of the junction potential but did not affect its latency and crest time.

Effect of repetitive stimuli on the membrane and drug action on it

As described previously, the spontaneous spike discharges in physiological condition appeared as a train of discharges with the slow depolarization. A similar spike discharge could be recorded as post-inhibitory rebound when repetitive stimulation was applied. Figure 7 shows the effect of tetrodotoxin (10^{-7} g/ml.) on the spontaneous discharges (*b*) and also on the membrane activity elicited by repetitive stimuli (0·5 msec pulse, 20 c/s) (*e*, *f*). When repetitive stimuli were applied to the tissue (*c*, *d*), the first stimulus elicited a spike and then, with or without a preceding slow depolarization, the membrane was hyperpolarized. When the stimulation was stopped, the membrane was depolarized and repetitive spikes were triggered. The amplitudes and the number of the spikes depended on the amplitude and the duration of the slow depolarization. The time course of

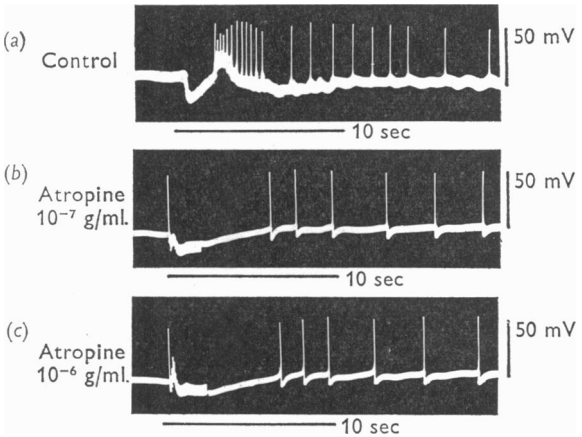

Fig. 8. Effect of atropine (10^{-7} g/ml. and 10^{-6} g/ml.) on the membrane activity elicited by repetitive stimuli (pulse duration 0·5 msec, frequency 20/sec, duration 2 sec). *a*, *b* are produced by cathodal stimulation and *c* by anodal stimulation.

the slow depolarization was nearly the same as that observed in spontaneous discharges (cf. *c*, *d*, *a*). The polarity of stimulation did not affect it. When the duration of the repetitive stimulation exceeded several seconds, the hyperpolarized membrane became gradually depolarized even during the period of stimulation and spikes were generated. Tetrodotoxin (10^{-7} g/ml.) completely abolished both the initial and the post-inhibitory rebound depolarization. Nevertheless, spikes appeared during and also after the cessation of the repetitive stimulation (*e*, *f*).

Figure 8 shows the effect of atropine (10^{-6} g/ml.) on the membrane activity elicited by repetitive stimuli (pulse duration 0·5 msec, frequency

20/sec and duration 2 sec). In Fig. 9(*a*), during the period of stimulation, the membrane was hyperpolarized at first, then gradually depolarized to the level before the stimulation. When the stimulation was stopped, the membrane rapidly depolarized below the resting potential level and repetitive spikes were generated on the slow depolarization. After the depolarization had passed, the spike discharge continued with low frequency. Atropine abolished the delayed depolarization with the high frequency spike discharge (*b*, *c*). This observation might indicate that the delayed depolarization following repetitive stimulation was due to nervous factors influencing the membrane activities.

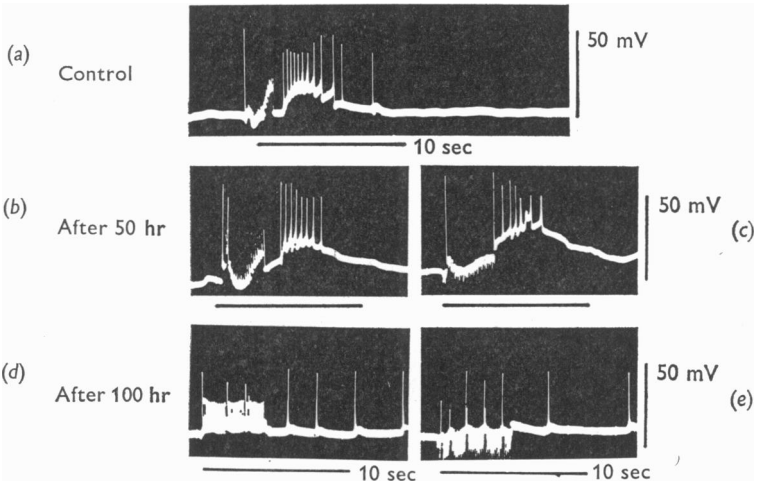

Fig. 9. Effects of the repetitive stimuli (pulse duration 1 msec, frequency 20 c/s, stimulus duration 1·5–3 sec) to the fresh tissue (*a*), tissue stored for 50 hr (*b*, *c*) and tissue stored for 100 hr (*d*, *e*). *a*, *b*, *d* are produced by anodal stimulation and *c*, *e* by cathodal stimulation.

Effect of repetitive stimuli on the stored tissue

An interesting property of smooth muscle is that membrane activity can be recorded even after the tissue has been kept in cold store (4° C) for more than a week. However, it was thought that the myenteric plexus might be damaged much earlier than the muscle, and that the membrane activity could be investigated after elimination of the nervous factors. The cut ends of the excised jejunum were tied off to prevent the outflow of the secretions from the intestinal tube and it was kept at 4° C in oxygenated Krebs solution; the solution was changed every day to a freshly oxygenated one.

When the tissue was rewarmed to 36° C after the cold storage of 50 ± 2 hr at 4° C, the membrane gradually hyperpolarized from 42 mV (20

impalements) to 68 mV (20 impalements which were recorded after 30 min in Krebs solution at 36° C) and it remained high for about 1 hr then gradually depolarized to a level below the membrane potential of the fresh preparation (51 mV, 30 impalements). However, after several hours, the membrane was further depolarized and the spike configuration deteriorated more quickly than in the fresh preparation. After 1 hr at 36° C the repetitive stimulation elicited exactly the same response in the tissue which was stored for 50 hr (*b*, *c*) as that recorded from the fresh preparation shown in (*a*). However, after the tissue had been stored for 100 hr (*d*, *e*) the membrane activity recorded in response to the repetitive stimuli after rewarming for 1 hr was different from the fresh preparation, and similar to that observed after tetrodotoxin treatment in the fresh preparation, as shown in Fig. 7 (*e*, *f*). The spikes appeared during the stimulation and no delayed depolarization was observed. However, when the tissue was perfused in Krebs solution for several hours the delayed depolarization elicited by the repetitive stimuli was partially restored.

Figure 9 shows the effect of the repetitive stimuli (pulse duration 1 msec, frequency 20 c/s and stimulus duration 1·5–3 sec) to the fresh tissue (*a*), tissue stored for 50 hr (*b*, *c*) and tissue stored for 100 hr (*d*, *e*). All tissues were excised from the same guinea-pig.

DISCUSSION

When extracellular field stimulation was applied to the tissue, two types of slow depolarization could be observed, i.e. an early depolarization and a delayed depolarization. Both were blocked by treatment with atropine or tetrodotoxin. They therefore might be caused by the release of acetylcholine from the nerve terminals.

The slow depolarization appearing immediately after the stimulation might be due either to the direct stimulation of the nerve terminal or to the firing of the nerve fibre caused by current flow from the muscle fibres surrounding the nerve fibre. In some instances the slow depolarization appeared without spike generation and the latency to elicit the slow potential was only about 10 msec. In other instances the slow depolarizations appeared with the spike which was generated with varying latencies after the stimulation. The spike in the smooth muscle cells in this tissue appeared synchronously with the neighbouring cells and these spikes might have added up to currents sufficiently intense to influence the nerve fibres closely located within the muscle bundles.

The interpretation of the generation mechanism of the delayed depolarization is more difficult. If the delayed depolarization were due to release of chemical transmitter from nerve terminals directly excited by the electrical

stimulation, the latency would not have been as long as that observed. Moreover, there was no delayed depolarization when extracellular stimulation was applied to the ablated longitudinal muscle. It might be related to a peripheral reflex at the level of the myenteric plexus or the postive feedback system in the intestinal nerve network (Hukuhara, Yamaguchi & Nakayama, 1958). Electron-microscopic observation has shown that an efferent nerve fibre, in some cases, penetrates the smooth muscle cell with close contact of nerve and muscle membrane. It has also been reported that the visceral afferent nerve endings are distributed closely to, or make contact with, the smooth-muscle cell membrane (Yamauchi, 1964).

Burnstock *et al.* (1963, 1966) studied the membrane activities in response to the transmural stimulation and they recorded the inhibitory potential which hyperpolarized the membrane and blocked the spike generation. Chronic treatment with reserpine only partially blocked the inhibitory potential. Also in untreated preparations only a part of the inhibitory potential was generated by the perivascular sympathetic stimulation. Therefore they concluded that the inhibitory potential was mainly due to an unknown chemical transmitter which hyperpolarized the membrane and blocked the spike generation, and that only a minor part was due to catecholamines. The inhibitory potential recorded from the jejunum in the present experiment has the same properties as that in the taenia coli.

In the vas deferens repetitive stimuli to the hypogastric nerve enhanced the amplitude of the junction potential, but in the jejunum repetitive stimuli never enhanced the amplitude of the inhibitory potential. Furthermore, the amplitude of the second inhibitory potential is always smaller than the first. Interaction could be observed between the depolarization caused by acetylcholine and the hyperpolarization caused by inhibitory transmitter which were both released simultaneously by the field stimulation. The inhibitory potential appeared more dominantly and often reduced the duration of the depolarization. However, repetitive stimuli gradually enhanced the amplitude of the depolarization, but not that of the hyperpolarization. Consequently, the membrane at first hyperpolarized, then gradually depolarized and finally triggered spikes even when the stimulation was continued. Repetitive stimuli (20/sec) relaxed the tissue at first; then after 3–5 sec the tension developed, i.e. a diphasic action on the tension development could be seen by the repetitive stimulation of the intestine (see also Campbell, 1966). However, the distribution of the excitatory and inhibitory nerves in the intestine may not be homogeneous. This might modify the shape of the diphasic tension development.

As a conclusion, the normal membrane activities of the smooth muscle in this experimental condition may be influenced by the release of acetylcholine from the nerve terminals and, when extracellular field or trans-

mural stimulation are applied to the tissue, varying influences of the excitatory and inhibitory nervous factors might modify the membrane activities of the smooth-muscle cells of the intestine.

We are very grateful to Dr E. Bülbring and Dr T. Tomita for criticizing the manuscript, and the Asahi newspaper company and U.S. Army research and development group (Far East) (No. DA-CRD-AG-S92-544-67-G56) for grants to support this work.

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