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The effects of hyoscine and anticholinesterases on cholinergic transmission to the smooth muscle cells of the avian gizzard

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1. Intracellular recordings were made from smooth muscle cells of the pigeon and chick gizzards. Hyoscine (10^{-6} g/ml.) blocked, within 2 min, the excitatory junction potentials (E.J.P.s) evoked by transmural stimulation. As the amplitudes of E.J.P.s decreased, their durations also decreased. Hyoscine had no effect on the inhibitory junction potentials (I.J.P.s) evoked by transmural stimulation.

2. Initially, physostigmine or neostigmine ($10^{-7} - 5 \times 10^{-6}$ g/ml.) caused a marked increase in the amplitudes of E.J.P.s, without affecting their time courses. Sometimes, anticholinesterases caused a single stimulus to evoke multiple E.J.P.s.

3. After several minutes in the presence of high concentrations ($10^{-6} - 5 \times 10^{-6}$ g/ml.) of anticholinesterases the muscle cells were depolarized by about 15–20 mV, and thus the amplitudes of I.J.P.s evoked by transmural stimulation were larger than under control conditions. E.J.P.s summed at much lower stimulation frequencies than normal, an observation which indicated that high concentrations of anticholinesterases increased the durations of the E.J.P.s. Repetitive stimulation evoked a membrane depolarization which persisted for as long as 10 sec after stimulation was stopped.

4. These results are discussed in terms of the action of the drugs at the nerve-smooth muscle junction and on ganglion cells in the myenteric plexus.

Bell (1967) made an electrophysiological study of the effects of atropine and physostigmine on transmission to the guinea-pig vas deferens. Atropine increased the rate of decay of excitatory junction potentials (E.J.P.s) recorded from the smooth muscle cells in response to postganglionic stimulation of the hypogastric nerve. In some, but not all cells physostigmine markedly prolonged the time course of the E.J.P. Bell concluded that the E.J.P.s in these cells were partly due to a direct action of acetylcholine on the muscle cell membrane.

In a brief report Hidaka, Kuriyama & Toida (1968) described an increase in the amplitudes of E.J.P.s recorded from the guinea-pig intestine in the presence of physostigmine, but did not mention any change in the time course of the junctional events.

A recent histochemical study (Bennett, 1969a) has shown that the smooth muscle of the gizzards of the pigeon and the chick is densely innervated by cholinergic fibres. Electrophysiological studies (Bennett, 1969b, c) have indicated that the smooth muscle cells of the avian gizzard may be affected by excitatory cholinergic nerves and by inhibitory nerves which release an unidentified transmitter. Noradrenergic fibres seen in the smooth muscle of the avian gizzard appear to be associated only with the vasculature (Bennett, 1969a). The present study is largely concerned with the effects of hyoscine and anticholinesterases on E.J.P.s, recorded intracellularly from smooth muscle cells of the pigeon and chick gizzards, in response to stimulation of intrinsic nerve fibres.

Methods

White leghorn chicks between 1 day and 12 weeks old, and adult pigeons were used. The preparations usually consisted of muscle strips taken from the serosal surface of the lateral muscle (see Bennett, 1969b), together with the myenteric plexus. In some experiments the nerve plexus was carefully stripped from the underlying muscle, in order to obtain a preparation which was free from ganglion cells. As described elsewhere (Bennett, 1969b), a histological or histochemical examination of the tissue was made at the end of the experiment in order to check that all ganglion cells had been removed from the preparation. The intrinsic nerves in the muscle were stimulated with transmural electrodes, which consisted of two platinum wires set 2–3 mm apart in a rubber block. The tissue was pinned to the block in such a way that one electrode lay under the cut surface of the tissue; the other electrode was pressed lightly against the upper, serosal surface of the preparation.

The duration of stimulating pulses was 0·2 msec in all experiments; the strengths of stimuli were varied between 50 and 90 V, the latter strength being sufficient to elicit maximal nerve-mediated responses in most preparations. The tissue was mounted in a bath through which a salt solution (composition: (mM) NaCl 150, $NaHCO_3$ 20, KCl 5·0, $CaCl_2$ 5·0, $MgCl_2$ 5·0, with glucose 2 g/l.) (Ginsborg, 1960), flowed. The bath temperature was kept at $38° \pm 1°$ C and the stock bottle of salt solution was constantly bubbled with 95% oxygen and 5% carbon dioxide. Under these conditions the tissue was quiescent, but vigorous oxygenation of the fluid in the bath caused the tissue to undergo spontaneous contractions. There were no changes in pH of the bathing fluid associated with the different levels of oxygenation, thus it seems feasible that anoxia was responsible for the suppression of the contractions of the tissue; slight anoxia has previously been shown selectively to block the action potential generating mechanism of the smooth muscle cells of the avian gizzard while leaving junctional transmission unimpaired (Bennett, 1969b). All the present experiments were carried out under the above conditions of sub-optimal oxygenation, so that the effects of drugs on junctional transmission could be studied without the complication of action potential firing. Intracellular electrical events were recorded with glass microelectrodes with resistances of 50–80 mΩ. The recording system was conventional.

Drugs used were hyoscine hydrobromide, neostigmine methylsulphate and physostigmine sulphate. They were injected into the bath; concentrations cited refer to the final concentration of salt in the bath.

Results

Bennett (1969b) suggested that the excitatory junction potentials recorded from the smooth muscle cells of the avian gizzard were due to stimulation of cholinergic nerve fibres. Figure 1 demonstrates the effect of hyoscine (10^{-6} g/ml.) on E.J.P.s evoked, in a single smooth muscle cell of the pigeon gizzard, by transmural stimulation. Within 2 min the E.J.P.s were blocked. Control experiments, in which intrinsic nerves were stimulated repetitively in the absence of hyoscine, showed that there was no decrease in the amplitudes of E.J.P.s under these conditions. In all experiments with hyoscine, the durations of E.J.P.s decreased with decrease in amplitude. Some cells showed only inhibitory junction potentials (I.J.P.s) in response to transmural stimulation (Bennett, 1969b); hyoscine had no effect on these I.J.P.s. In other experiments, block of E.J.P.s by hyoscine revealed I.J.P.s in the same cell.

Physostigmine and neostigmine ($10^{-7} - 5 \times 10^{-6}$ g/ml.) caused initially a marked increase in E.J.P. amplitude without affecting duration. Figure 2 illustrates the effect of physostigmine (10^{-7} g/ml.). After about 2 min there was a four-fold increase in the amplitudes of the fully facilitated (Bennett, 1969b) E.J.P.s, but there was no change in their durations. In this experiment the myenteric plexus had been removed from the preparation in order to preclude any ganglionic effects of

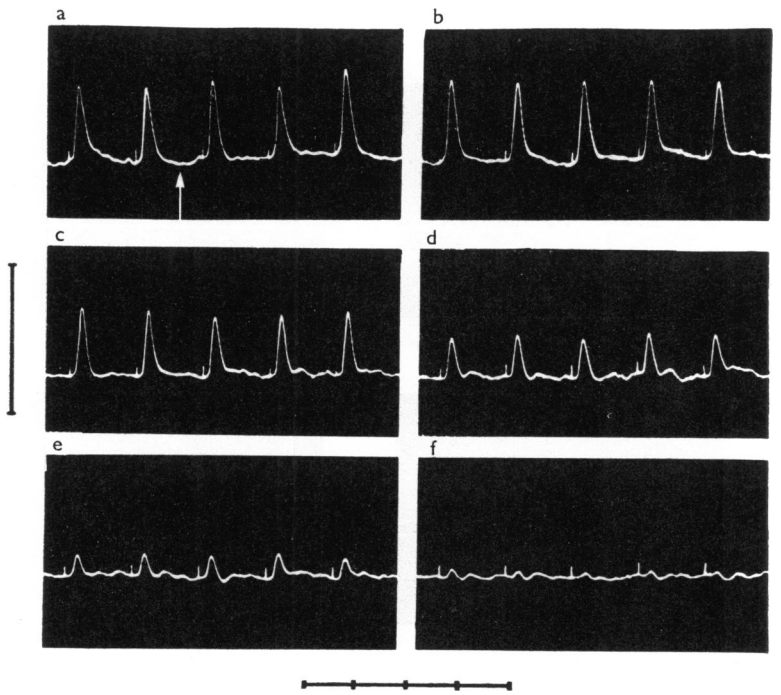

FIG. 1. Pigeon gizzard preparation with myenteric plexus intact. Consecutive records from a continuous intracellular recording in the same smooth muscle cell. Recording electrode about 4 mm from stimulating electrode. Time interval between records 20 sec. Transmural stimulation at a frequency of 0·8 pulses/sec; pulse duration 0·2 msec; pulse strength 90 V (maximal). At arrow in (a), hyoscine (10^{-6} g/ml.) injected into the bath. Note that in the presence of hyoscine, E.P.J. amplitudes and durations decreased. Slight hyperpolarization in (f) may have been due to stimulation of inhibitory nerves. Figure retouched. Vertical calibration, 30 mV; horizontal calibration 4 × 1 sec.

the anticholinesterase. The microelectrode was inserted into a cell which was about 1 mm or less to the side of the stimulating electrode against the serosal surface of the preparation.

Occasionally both anticholinesterases had an effect in addition to that described above. The record shown in Fig. 3 was taken from an experiment in which the recording electrode was about 1·5 cm from the upper stimulating electrode; the myenteric plexus had not been removed from the preparation. At first, neostigmine (5×10^{-7} g/ml.) had the usual effect of causing an increase in amplitude of the E.J.P., without affecting its time course. Subsequently, the E.J.P. became complex, in that a single stimulus evoked what appeared to be multiple E.J.P.s, with various degrees of summation. Such multiple E.J.P.s were only seen in preparations with the myenteric plexus intact and only then when the distance between recording and stimulating electrode was large (1 cm or more).

In the presence of high concentrations of physostigmine or neostigmine ($1-5 \times 10^{-6}$ g/ml.), the period during which the amplitude and, occasionally, the form of the E.J.P. were modified was followed by membrane depolarization. A systematic comparison of muscle membrane potentials before and after the application of the anticholinesterases was not made. However, it appeared, from those measurements that were made, that the normal membrane potential of 55–70 mV was reduced to 40–50 mV. In depolarized muscle the amplitudes of the E.J.P.s

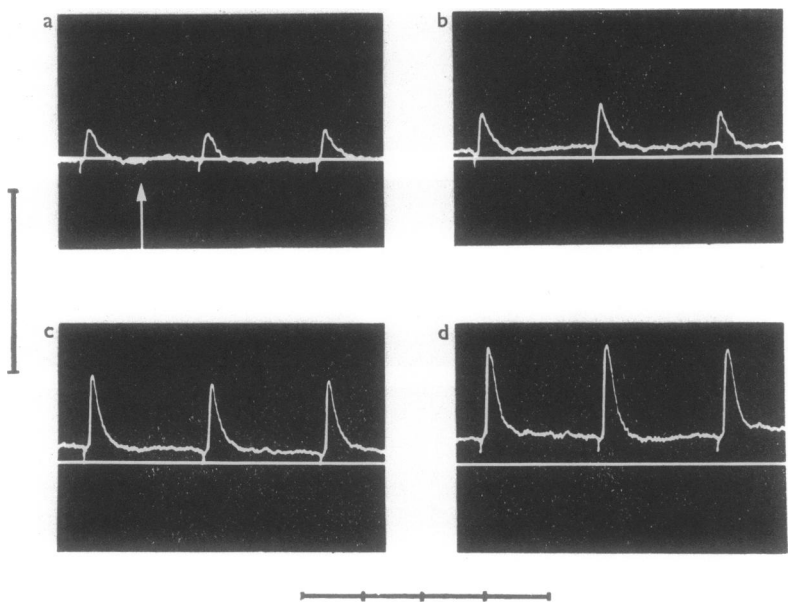

FIG. 2. Chick gizzard preparation without myenteric plexus. Consecutive records from a continuous intracellular recording from one smooth muscle cell. Recording electrode about 1 mm from stimulating electrode. Time interval between records 20 sec. Transmural stimulation at a frequency of 0·5 pulses/sec; pulse duration 0·2 msec; pulse strength 80 V (maximal). At arrow in (a), physostigmine (10^{-7} g/ml.) injected into the bath. Note that with increasing E.J.P. amplitude there was no change in duration. There was a slight depolarization of the membrane in the presence of the anticholinesterase. Figure retouched. Vertical calibration, 40 mV; horizontal calibration 4 × 1 sec.

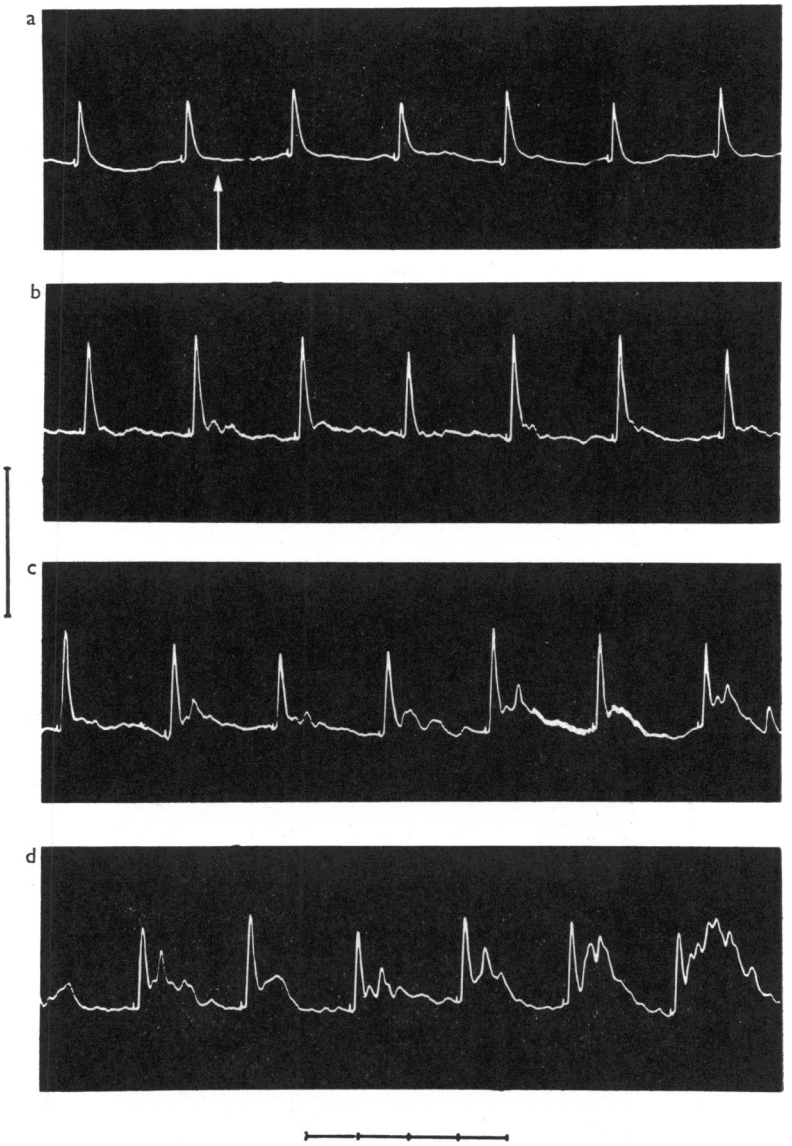

FIG. 3. Chick gizzard preparation with myenteric plexus intact. Consecutive records from a continuous intracellular recording from one smooth muscle cell. Recording electrode about 1·5 cm from the stimulating electrode. Time interval between records 20 sec. Preparation stimulated transmurally at a frequency of 0·5 pulses/sec; pulse duration 0·2 msec; pulse strength 90 V (maximal). At arrow in (a), neostigmine (5×10^{-7} g/ml.) injected into the bath. Note that the amplitude of the control response was initially increased and that subsequently simple E.J.P.s in response to single stimuli became multiple (c, d). The various components of the multiple event appeared to sum (d), giving rise to a prolonged membrane depolarization, but the duration of the initial E.J.P.s was unaffected. Figure retouched. Vertical calibration 30 mV; horizontal calibration 4×1 sec.

were less than those obtained under control conditions, in spite of the fact that nerves were stimulated maximally without action potential firing. Hyoscine (10^{-6} g/ml.) partially antagonized the membrane depolarization caused by anticholinesterases. In depolarized muscle, inhibitory junction potentials became much more prominent; their amplitudes increased with the degree of depolarization (Fig. 4).

The response of smooth muscle cells of the pigeon and chick gizzards to repetitive stimulation of intrinsic cholinergic nerves has been described previously (Bennett, 1969b). Figures 5a, b and c demonstrate facilitation and summation of E.J.P.s under control conditions. When the muscle was depolarized by high concentrations of anticholinesterases, the characteristics of these responses were markedly altered. Figures 5d, e and f demonstrate typical responses to transmural stimulation in the presence of physostigmine (10^{-6} g/ml.). Pulses at low frequencies evoked, initially, small E.J.P.s and large I.J.P.s, but as the E.J.P.s facilitated and summed, the

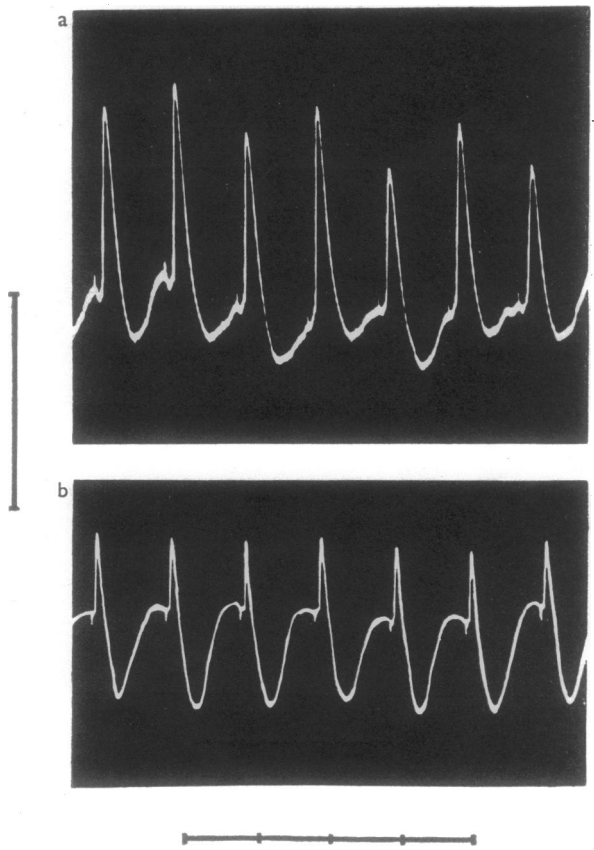

FIG. 4. Pigeon gizzard preparation with myenteric plexus intact. In (a) and (b), recording electrode about 2 mm from stimulating electrode. (a) Responses in one cell to transmural stimulation (1·0 pulses/sec; pulse duration 0·2 msec; pulse strength 90 V (maximal)) under normal conditions. The diphasic response was due to simultaneous stimulation of excitatory and inhibitory nerve fibres. (b) Responses in another cell in the same preparation to transmural stimulation (1·0 pulses/sec; pulse duration 0·2 msec; pulse strength 90 V (maximal)) in tissue depolarized by neostigmine (10^{-6} g/ml.). The inhibitory responses were larger and the excitatory responses smaller than under normal conditions. Figure retouched. Vertical calibration 20 mV; horizontal calibration 4 × 1 sec.

membrane hyperpolarization due to stimulation of inhibitory nerves was converted to a depolarization. In depolarized muscle, summation of E.J.P.s occurred at much lower stimulation frequencies (0·8–1 pulses/sec) than under control conditions (3–5 pulses/sec) a finding which indicates that the durations of the E.J.P.s were increased. Following repetitive stimulation, the membrane potential remained depolarized for periods of up to 10 sec ; the level of depolarization reached and the time required for the membrane to repolarize increased with the frequency of stimulation, up to

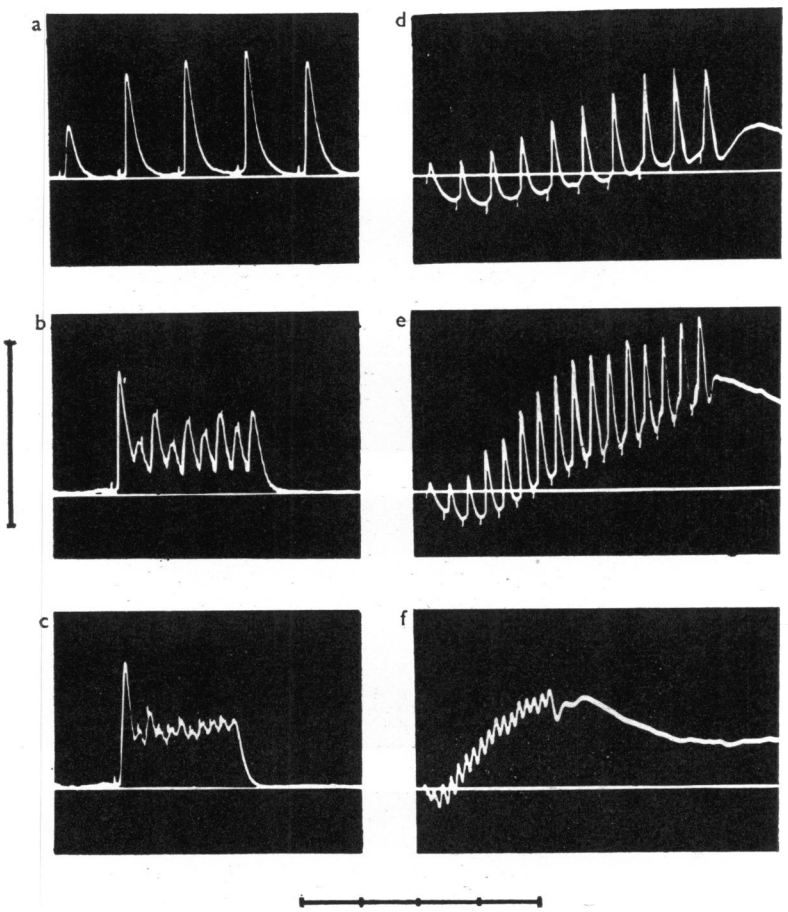

FIG. 5. (a), (b), (c), Pigeon gizzard preparation without myenteric plexus. Intracellular records from one smooth muscle cell. Recording electrode about 2 mm from stimulating electrode. Control conditions ; responses to transmural stimulation (pulse duration 0·2 msec ; pulse strength 90 V (maximal)) at frequencies of (a) 1·0 pulse/sec, (b) 3·5 pulses/sec, (c) 5·3 pulses/sec. Note facilitation of E.J.P.s at low stimulation frequencies (a) and summation of E.J.P.s at higher stimulation frequencies (b, c). Note that the time course of repolarization of a single E.J.P. was the same as the time course of membrane repolarization following summed E.J.P.s. (d), (e), (f), Pigeon gizzard preparation with myenteric plexus intact ; intracellular recording from one smooth muscle cell. Recording electrode about 2 cm from the stimulating electrode. Muscle depolarized by physostigmine (10^{-6} g/ml.). Responses to transmural stimulation (pulse duration 0·2 msec, pulse strength 90 V (maximal)), at frequencies of (d) 1·0 pulses/sec, (e) 1·5 pulses/sec, (f) 4·0 pulses/sec. Note the persistent membrane depolarization following the cessation of stimulation. Figure retouched. Vertical calibration 40 mV in (a), (b), (c), 20 mV in (d), (e), (f). Horizontal calibration 4×1 sec in (a), (b), (c) ; 4×2 sec in (d), (e), (f).

about 4 pulses/sec. At this frequency the E.J.P.s were decreased in amplitude (Fig. 5f), and the membrane depolarization attained was less than that with lower frequencies (Fig. 5e).

During the period of membrane depolarization due to physostigmine or neostigmine ($1-5\times10^{-6}$ g/ml.), multiple E.J.P.s in response to single stimulating pulses were not seen (Fig. 5d). When the drug-containing bath fluid was replaced with drug-free salt solution, the muscle cells repolarized slightly, and inhibitory responses were then less prominent (Fig. 6). Under these conditions, repetitive stimulation evoked E.J.P.s which still summed at lower frequencies than before the exposure to anticholinesterases (Fig. 6), but the level of depolarization reached was less and the rate of repolarization was greater than in the presence of physostigmine or neostigmine ($1-5\times10^{-6}$ g/ml.) (Fig. 5, d, e, f). Moreover, multiple E.J.P.s were now seen with low frequency stimulation (Fig. 6a), but only in preparations

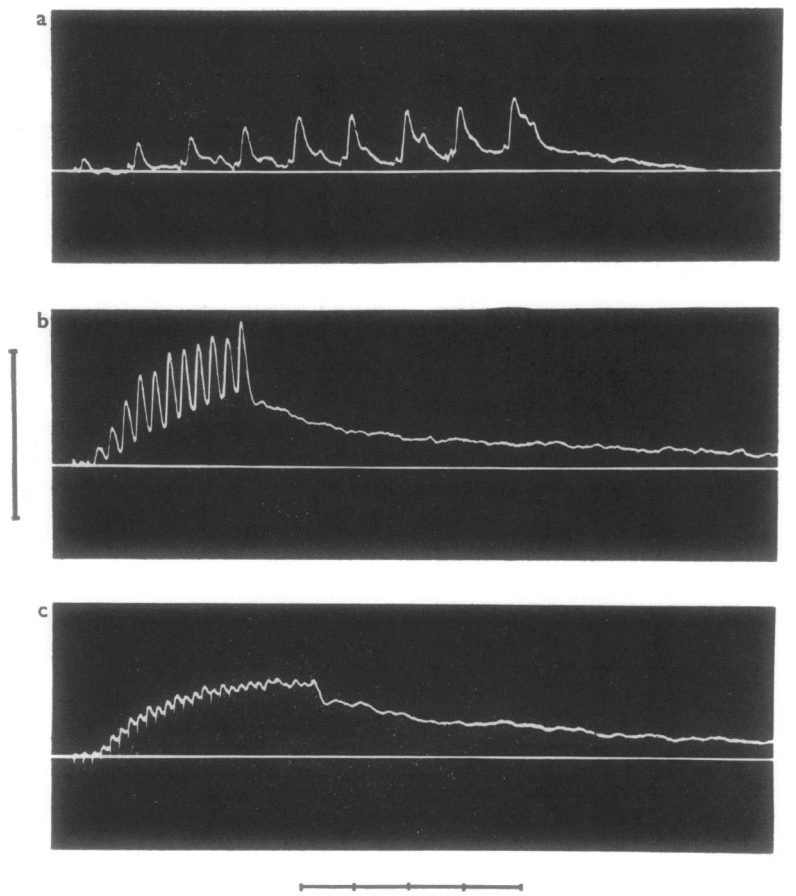

FIG. 6. Intracellular records from another smooth muscle cell of the same preparation as in Fig. 5d, e, f, after the concentration of physostigmine had been reduced by flushing the bath. Recording electrode about 2 cm from the stimulating electrode. Transmural stimulation (pulse duration 0·2 msec; pulse strength 90 V (maximal)) at frequencies of (a) 1·0 pulse/sec, (b) 3·7 pulses/sec, (c) 5·0 pulses/sec. Stimulation at low frequencies (a) evoked complex E.J.P.s but with higher stimulation frequencies only simple E.J.P.s were seen (b, c). Note slow membrane repolarization after stimulation was stopped. Figure retouched. Vertical calibration 20 mV; horizontal calibration 4 × 1 sec.

with the myenteric plexus intact, and only when the recording electrodes were distant (1–2 cm) from the stimulating electrodes. Cells which responded with multiple E.J.P.s in response to low frequency stimulation, showed only simple E.J.P.s when the stimulation frequency was increased (Fig. 6b, c).

Discussion

It was previously reported (Bennett, 1969b) that the E.J.P.s recorded from smooth muscle cells of the avian gizzard were due to stimulation of cholinergic nerves since the E.J.P.s were blocked by hyoscine. In the present study, hyoscine (10^{-6} g/ml.) initially reduced the amplitudes and durations of the E.J.P.s. The observation of Bell (1967) that atropine decreases the durations of the E.J.P.s recorded from the guinea-pig vas deferens is not directly comparable with the observations on the avian gizzard since only a certain component of the E.J.P. recorded from the guinea-pig vas deferens is due to stimulation of cholinergic nerve fibres. However, it may be pertinent to the present study that atropine causes a reduction in the amplitude and duration of the e.p.p. recorded from the frog sartorius neuromuscular junction. (Beránek & Vyskočil, 1968 ; Kordaš, 1968). Beránek & Vyskočil suggested that the shortening of the e.p.p. by atropine might be explained by an enhanced removal of acetylcholine, or by a spatial gradient of effectiveness of the blocking drug, or by both these mechanisms. The mode of action of hyoscine at the autonomic cholinergic neuromuscular junction is not clear from the present observations, and further investigation of this point is required.

Bell (1967) showed that physostigmine caused an increase in the duration of the falling phase of some E.J.P.s recorded from smooth muscle cells of the guinea-pig vas deferens. In the present study, E.J.P.s were recorded, where possible, from the same cell before and after the application of anticholinesterases. This was considered desirable because it has been shown earlier that the time course of E.J.P.s recorded from different cells, even in the same piece of tissue, may differ markedly (Bennett, 1969b). It has been suggested that the long time course of autonomic neuromuscular transmission may be due to a long time constant of the muscle cell syncytium (Tomita, 1967 ; Bennett & Burnstock, 1968), thus the duration of the junction potential would be determined by the time taken for the membrane capacitance to discharge. The duration of the junction potential could therefore be much longer than the duration of the permeability changes caused by the action of transmitter. In these conditions an increase in effectiveness and duration of transmitter action, caused by anticholinesterases, would produce no detectable change in the duration of the E.J.P. until the duration of transmitter action exceeded the time constant of the muscle cell syncytium. Thus, in low concentrations anticholinesterases would produce an increase in E.J.P. amplitude with no concurrent increase in E.J.P. duration and, in high concentrations, they would produce an increase in E.J.P. duration, as observed in the present study.

An alternative explanation of the long time course of autonomic junctional transmission is that, due to the absence of an enzyme to inactivate the transmitter, the duration of the permeability changes caused by the action of transmitter dictate the time course of transmission (Bennett & Burnstock, 1968). From the evidence of the present results, and from histochemical observations (Bennett, 1969a), it would appear that, in the smooth muscle preparation of the avian gizzard, there is con-

siderable true cholinesterase activity. If, however, inactivation of transmitter were slow relative to the time taken for the membrane capacitance to discharge, then inhibition of the enzyme would increase both amplitude and duration of the E.J.P. This effect occurs with high, but not with low concentrations of anticholinesterase and requires further analysis.

In some experiments anticholinesterases transformed the response to a single stimulating pulse from a single E.J.P. to multiple E.J.P.s, which were recorded only from preparations with an intact myenteric plexus and only when cells distant from the stimulating electrodes were impaled. In these conditions, it is feasible that stimulation of preganglionic nerve fibres contributed to the responses (Bennett, 1969b). Recently, Riker & Guerrero (1968) found that low concentrations of neostigmine facilitated repetitive action potential firing in postganglionic neurones. High concentrations of neostigmine, which caused depolarization of the neuronal membrane, depressed this repetitive activity, as did high frequency orthodromic stimulation. In the present study, multiple E.J.P.s were not evoked by a single stimulating pulse when the cells were depolarized by high concentrations of anticholinesterases. When the tissue concentration of anticholinesterase was reduced by washing, the muscle membrane potential repolarized, and stimulation at low, but not at high, frequencies occasionally evoked multiple E.J.P.s. If the assumption is correct that preganglionic nerve fibres are stimulated, then the multiple E.J.P.s recorded from smooth muscle cells in the presence of low concentrations of anticholinesterases may be due to repetitive activity of ganglion cells in the myenteric plexus.

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