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SOME EFFECTS OF ATROPINE ON SMOOTH MUSCLE

BY

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Contractions of the nerve-free smooth muscle of the chick amnion, either spontaneous or in response to electrical or mechanical stimuli, are potentiated by high concentrations (10^{-5} g/ml.) of atropine sulphate. In addition hyoscine, homatropine, lachesine, propantheline bromide and atropine *N*-methyl and atropine *N*-butyl salts potentiate spontaneous activity in the amnion. The effects of atropine do not appear to depend on inhibition of cholinesterase; on labilization, stabilization or depolarization of the muscle cell membrane; or on an increase in metabolism. It is suggested that the effects are the result of blockade of the muscarinic acetylcholine receptors. The way in which this may facilitate the conducted response is discussed.

This paper presents the results of an investigation following the chance observation that atropine sulphate potentiated the response of the chick amnion muscle to electrical stimulation. The way in which atropine potentiates the conducted response in this nerve-free smooth muscle preparation has been investigated and other atropine-like drugs have been tested for similar activity. A preliminary account of some of these results has been published (Cuthbert, 1963a).

METHODS

Isolated chick amnion preparation

Amniotic membranes were dissected from fertile hens' eggs and suspended in Hanks (1946) balanced salt solution at 37° C and gassed with air. Isotonic contractions were recorded on smoked paper or with an ink-writing lever as described previously (Cuthbert, 1962a). For electrical stimulation one end of the preparation was pulled through a pair of platinum electrodes of the type described by Burn & Rand (1960) and stimulated with rectangular cathodal shocks.

Amnion strips were also stimulated to contract by quickly stretching the tissue by means of a lever attached to the thread connecting the preparation with the recording lever. The stimulus artifact caused the recording lever only to fall. Contractions of the amnion were also caused by releasing the muscle from a stretch applied for 1 min by hanging a small weight onto the recording lever. In a few experiments isometric contractions of the amnion were registered on an oscilloscope using a mechano-electronic transducer which contained an RCA 5734 valve mounted as described by Bülbring (1955).

Cholinesterase determination

Cholinesterase activity in amnion tissue and hen plasma was determined by the method described previously (Blaber & Cuthbert, 1962).

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Oxygen uptake

The oxygen uptake of amnion tissue was determined using the Warburg constant volume respirometer. The tissue, together with 2.25 ml. of Hanks solution, was placed in the main compartment of the flask ; 0.25 ml. of 20% sodium hydroxide solution was placed in the centre well (to absorb carbon dioxide) and drugs, dissolved in distilled water, were placed in the side-arms. All flasks were gassed with air for 10 min at 37° C before reading was commenced. The manometers were read at 20 min intervals for 1 hr after which the drug solutions were tipped from the side-arms. The manometers were read again at 20 min intervals for a further 1 hr. The time between tipping and recommencing reading did not exceed 5 min.

Materials

The physiological liquid used throughout was Hanks solution which contained the following substances dissolved in distilled water (g/l.): NaCl, 8.00 ; KCl, 0.40 ; $CaCl_2$, 0.14 ; $MgSO_4\,7H_2O$, 0.10 ; $MgCl_2\,6H_2O$, 0.10 ; $Na_2HPO_4\,2H_2O$, 0.06 ; KH_2PO_4, 0.06 ; glucose, 1.00 ; and $NaHCO_3$, 0.35. The solution was gassed with air. The following drugs were used: acetylcholine chloride, methacholine chloride, carbachol, physostigmine salicylate, atropine sulphate, homatropine hydrobromide, hyoscine hydrobromide, lachesine chloride, atropine *N*-methyl nitrate, atropine *N*-butyl bromide, propantheline bromide, veratrum alkaloids, dinitrophenol and lignocaine hydrochloride. The amounts referred to in the text are the salts unless otherwise stated.

RESULTS

Isolated chick amnion muscle preparation

The isolated amnion responded equally to the three choline esters, acetylcholine, methacholine and carbachol. The response, in spontaneously active preparations, was an increase in tone and in frequency of contraction (Fig. 1). The responses to concentrations of these drugs which produced maximal effects were readily blocked

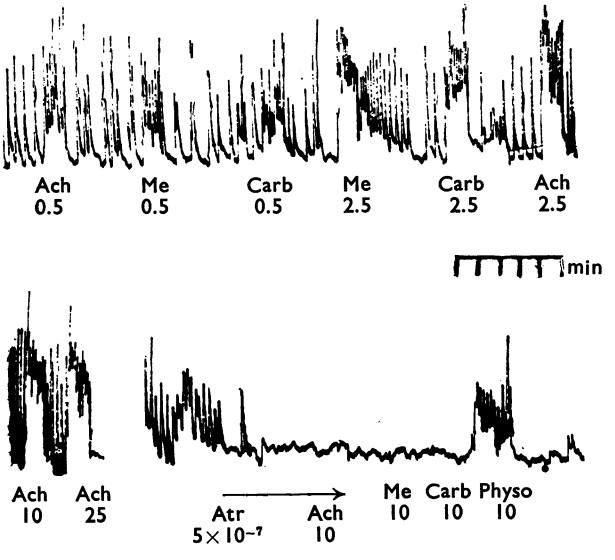

Fig. 1. 13 day amnion. Responses to acetylcholine (Ach), methacholine (Me), carbachol (Carb) and physostigmine (Physo). Concentrations are expressed as μg/ml. of the bases. Atropine (Atr, concentration in g/ml.) abolished the response to the choline esters.

by low concentrations (10^{-6} to 10^{-7} g/ml.) of atropine, but a response of the preparation could still be obtained to physostigmine, as described previously (Cuthbert, 1962a). Atropine (10^{-6} to 10^{-7} g/ml.) either did not affect spontaneous activity or abolished it. Concentrations of atropine far in excess of those required to block the effects of choline esters on the amnion increased the spontaneous activity; the concentration required to do this was usually in the region of 1 to 2×10^{-5} g/ml. In the presence of the drug spontaneous contractions were increased in amplitude and sometimes frequency and these changes were occasionally accompanied by an increase in tone. Some preparations which had previously been quiescent showed spontaneous activity in the presence of atropine, but some muscles were not stimulated by atropine.

Seven atropine-like drugs (10, 20 or 50 μg/ml.) were tested on spontaneous activity of amnion preparations after their resting activity had been recorded for approximately 30 min. The number of preparations which showed increased spontaneous activity is recorded in Table 1. The various forms of the responses

TABLE 1

CHICK AMNION PREPARATIONS SHOWING INCREASED SPONTANEOUS ACTIVITY IN RESPONSE TO DRUGS

Drug	No. of preparations showing: Increased activity	No change
Atropine sulphate	32	5
Hyoscine hydrobromide	5	3
Homatropine hydrobromide	7	3
Atropine *N*-methylnitrate	6	4
Atropine *N*-butylbromide	3	1
Lachesine chloride	7	1
Propantheline bromide	2	1

to atropine-like drugs are shown in Fig. 2. Addition of the drugs caused, in some cases, the appearance of spontaneous activity which was either continuous or intermittent (Fig. 2 *b, d*). In other cases addition of the drug caused an increase in the amplitude of the spontaneous contractions with little effect on frequency (Fig. 2 *a, c, f, g*). Some preparations showed intermittent activity in which the contractions were fused (Fig. 2 *h*); this type of response was also potentiated by atropine-like drugs.

The effect of other drugs on the response of the amnion to atropine was tested in order to elucidate the way in which atropine caused its effects. The local anaesthetic drug lignocaine (200 μg/ml.) inhibited spontaneous activity and inhibited the response to atropine. Also the stimulant effect of atropine was antagonized by the subsequent addition of lignocaine. Veratrum alkaloids (1 μg/ml.) produced a considerable increase in the frequency of contraction of the amnion but with a reduction in amplitude. Atropine, in the continued presence of the alkaloids, was able to increase the amplitude of the contractions (Fig. 3) and so appeared to act in a manner different to the alkaloids.

The metabolic inhibitor dinitrophenol, in a concentration of 10^{-4} M, caused a slight increase in the amplitude of contraction, but dinitrophenol (10^{-3} M) caused

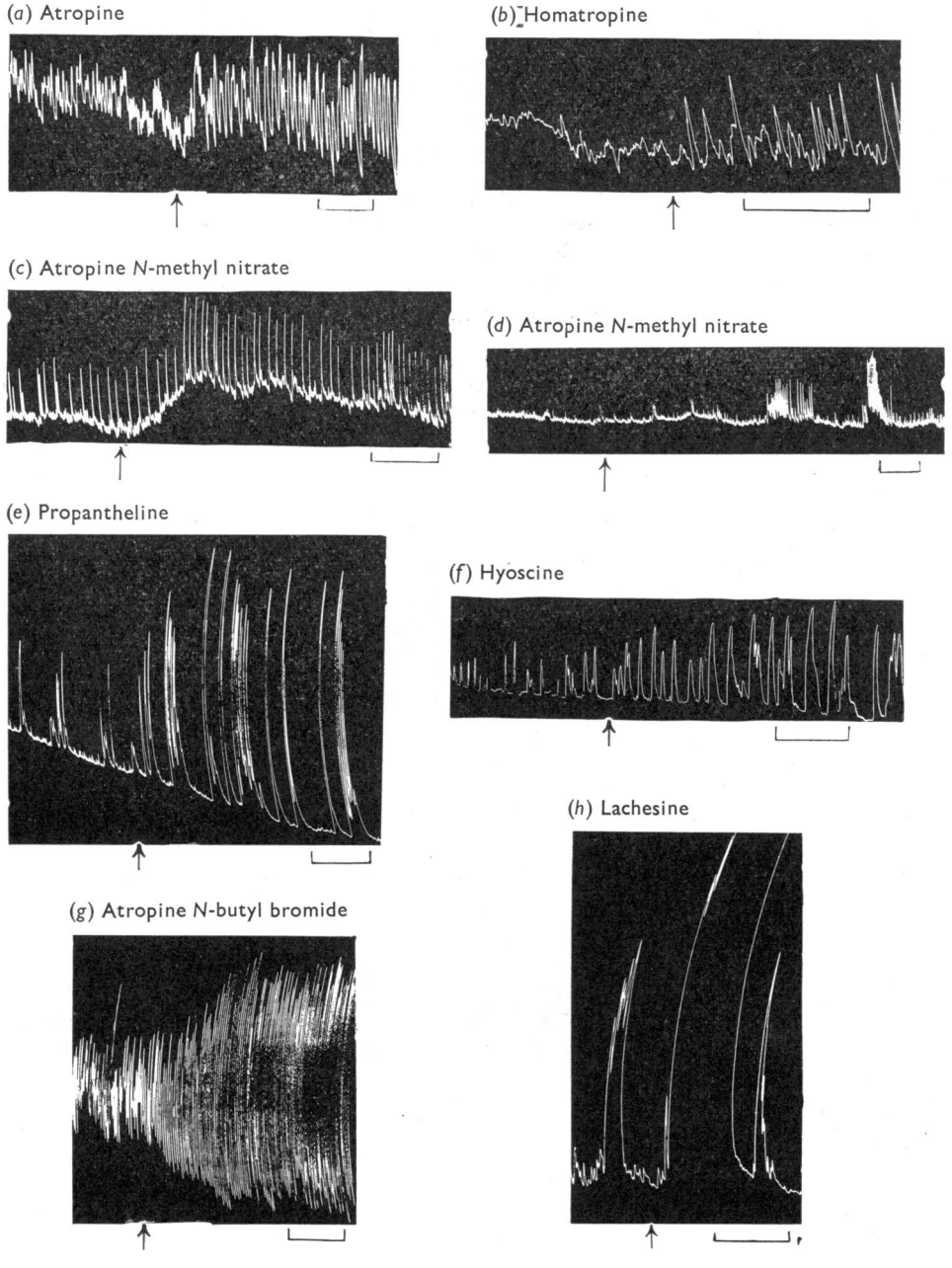

Fig. 2. The effects of atropine-like drugs on spontaneous activity of amnion muscle preparations. Time marks in 5 min. (*a*) 10 day, atropine (50 μg/ml.) added at arrow. (*b*) 14 day, homatropine (20 μg/ml.) added at arrow. (*c*) 16 day, atropine *N*-methyl nitrate (20 μg/ml.) added at arrow. (*d*) 17 day, atropine *N*-methyl nitrate (20 μg/ml.) added at arrow. (*e*) 16 day, propantheline (20 μg/ml.) added at arrow. (*f*) 13 day, hyoscine (20 μg/ml.) added at arrow. (*g*) 16 day, atropine *N*-butyl bromide (20 μg/ml.) added at arrow. (*h*) 16 day, lachesine (20 μg/ml.) added at arrow.

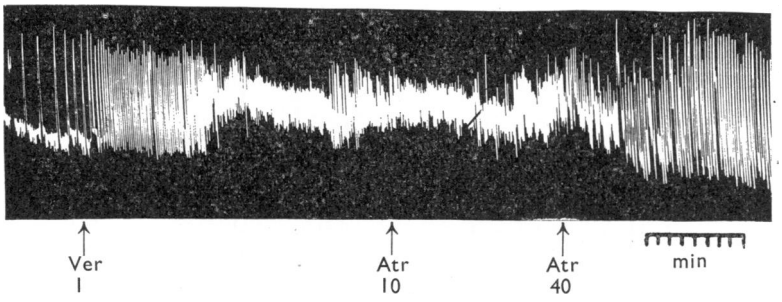

Fig. 3. 10 day amnion. Ver, veratrum alkaloids, and Atr, atropine, were added to the bath when indicated by the arrows. Concentrations are in μg/ml. and time marks in min.

an easily reversible inhibition of activity. In the presence of 10^{-4} M-dinitrophenol, atropine caused a further increase in the contraction amplitude (Fig. 4).

When the potassium ion concentration of Hanks solution was increased, by stages, spontaneous movements of isolated amnion preparations were at first increased and then depressed. The increases in potassium ion concentration required

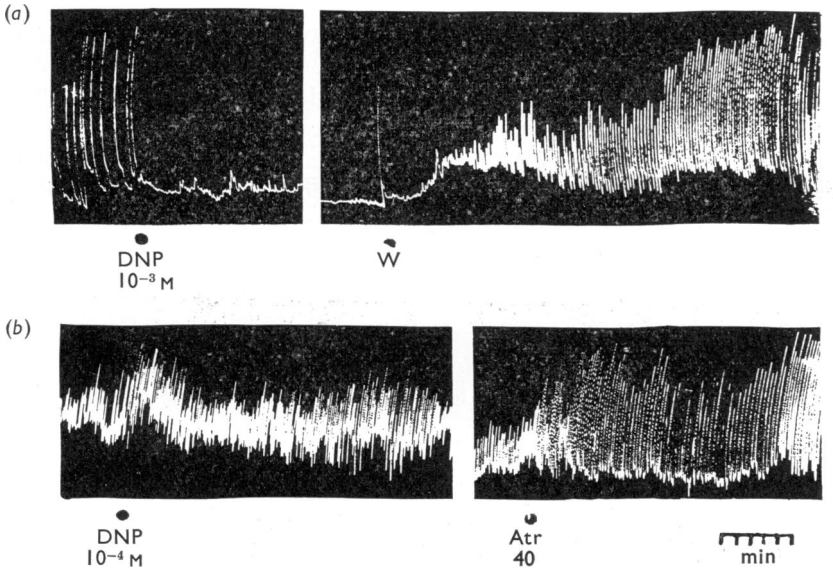

Fig. 4. (*a*) 13 day amnion showing reversible inhibition of spontaneous activity caused by dinitrophenol (DNP, 10^{-3} M). The drug was removed at W, 60 min after adding it to the organ bath. (*b*) 14 day amnion showing stimulant effect of dinitrophenol (10^{-4} M). Atropine (Atr, 40 μg/ml.) was added 45 min after dinitrophenol. Time marks in min.

to suppress spontaneous activity varied between two- and nine-times the potassium concentration of normal Hanks solution, that is between 11 and 48 mM. When activity was depressed by excess potassium, atropine was able to restore spontaneous activity (Fig. 5).

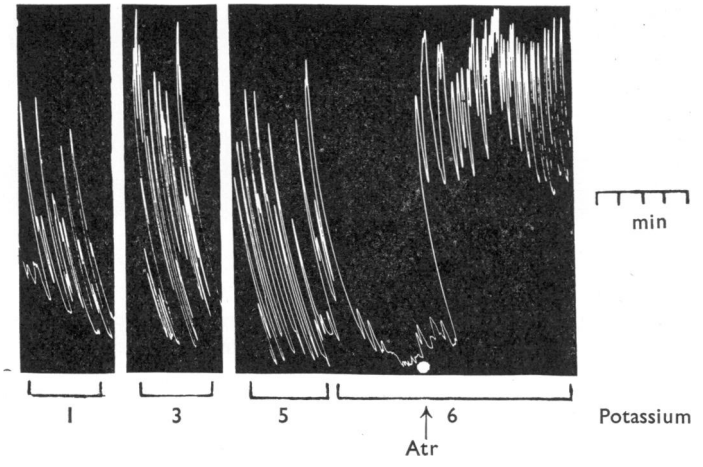

Fig. 5. 13 day amnion showing spontaneous activity in potassium-rich solutions. The potassium concentrations are shown below and are expressed as multiples of the potassium concentration of normal Hanks solution, which contains 5.5 mM potassium ions. Atropine (Atr, 20 μg/ml.) was added at the arrow. Time marks in min.

The effect of atropine-like drugs on contractions elicited by electrical and mechanical stimuli has also been investigated. Fig. 6 shows isometric contractions of an amnion muscle stimulated by single cathodal shocks at 2 min intervals. Addition of atropine (10 μg/ml.) to the organ-bath caused an immediate, but slight, increase in the tension developed in response to a stimulus. After 15 min the response to a single shock was considerably increased and, in many cases, this gave rise to several contraction waves. Several-fold increases in the height of isotonic

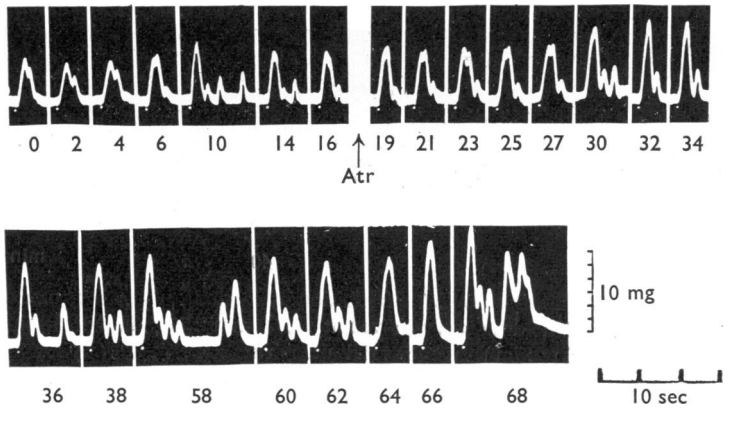

Fig. 6. Isometric contractions of a 10 day amnion strip in response to single cathodal shocks of 95 msec duration given every 2 min (at white dots). At the arrow, 10 μg/ml. of atropine (Atr) was added to the organ-bath. The numbers below each panel give the times of recording in minutes relative to the first panel. The preparation was not stimulated between the 38th and 58th minutes. Time marks are 10 sec, and tension graduations are 10 mg.

contractions in response to single electric shocks were also obtained in the presence of atropine (10 μg/ml.).

The amnion smooth muscle contracts in response to a quick stretch (Evans & Schild, 1956) or to a release from a prolonged stretch. The responses to these stimuli were also potentiated by addition of atropine (10 μg/ml.) to the organ-bath.

Amnion cholinesterase determinations

In concentrations up to 10^{-3} g/ml. atropine had no inhibitory activity on the hydrolysis of acetylcholine by amnion cholinesterase. The results are summarized in Table 2. No explanation can be offered for the increased rate of hydrolysis of

TABLE 2

LACK OF ACTION OF ATROPINE ON THE HYDROLYSIS OF ACETYLCHOLINE BY AMNION CHOLINESTERASE

Atropine concentration (μg/ml.)	Cholinesterase activity (% of control)	
	3×10^{-2} M acetylcholine	3×10^{-3} M acetylcholine
10	102·9	102·2
100	104·0	—
1,000	120·8	100·5

acetylcholine (3×10^{-2} M) in the presence of 10^{-3} g/ml. of atropine. A similar potentiation of hydrolysis was seen with hen plasma cholinesterase, an enzyme like that found in the amnion (Blaber & Cuthbert, 1962).

Oxygen consumption of amnion tissue

The oxygen consumption of whole amniotic membranes was measured as described in Methods. The oxygen consumption of 13 day amniotic membranes varied between 35 and 50 μl. of oxygen/20 min at the beginning of an experiment and these values fell to a slightly lower and steady level after 1 hr. Addition of Hanks solution, atropine (10 or 50 μg/ml.) or dinitrophenol (10^{-5} M) after 1 hr did not alter the oxygen consumption of the tissue. Dinitrophenol (10^{-4} M), however, approximately doubled the oxygen consumption of 13 day amnions and this increase was maintained for at least 1 hr. It was also this concentration of dinitrophenol which produced a moderate increase in the activity of the isolated amnion. These results suggest that atropine and dinitrophenol (10^{-4} M) stimulate activity in the amnion by different means.

DISCUSSION

Prosser & Rafferty (1956) showed that the nerve-free smooth muscle of the chick amnion showed spontaneous conducted electrical activity. Subsequently, Cuthbert (1962b) showed that the conducted electrical activity was associated with contraction waves. The present results show that conducted contraction waves in the amnion, either spontaneous or elicited in response to electrical and mechanical stimulation, are potentiated by atropine. Similar results on spontaneous activity were obtained with six other atropine-like drugs. It was considered that these drugs may have direct excitant effects either on the cell membrane or on intracellular processes, or that they facilitated cell-to-cell conduction causing each contraction wave to spread further in the presence of the drugs.

Secondly, it was considered that the effects may be unrelated to blockade of muscarinic acetylcholine receptors. Atropine, for instance, has weak anticholinesterase activity in some species (Edge, 1955), but in this work no such activity was found even with concentrations far in excess of those which potentiated the response. Drugs acting extracellularly on the cell membrane may have caused stabilization, labilization or depolarization of the cell membranes. Membrane labilizers did increase the excitability of the tissue, as shown by the considerable increase in contraction frequency in the presence of veratrum alkaloids. However, conduction was not facilitated as the amplitude was reduced by veratrum alkaloids. Depolarization with potassium salts also, at first, increased excitability, presumably by lowering the resting membrane potential. However when spontaneous activity was depressed in potassium-rich solutions atropine restored this activity. Evans, Schild & Thesleff (1958) found that in potassium sulphate-Ringer solution (in which the membrane potential fell to zero) the amnion still responded to acetylcholine and it might be argued that atropine had some similiar direct effect on the depolarized membrane. However the response of the amnion to acetylcholine in potassium sulphate-Ringer was a contracture, whereas in these results atropine restored a rhythmical response. It is doubtful that stabilization of the cell membranes could account for the results with atropine on the amnion. Experiments with local anaesthetics confirmed this and suggested that this was not the way in which atropine produced its effects.

An intracellular site of action for atropine seemed unlikely as quaternary atropine-like compounds, which are thought to be unable to penetrate the cell membrane, also produced similar effects. Such compounds were atropine *N*-methyl nitrate, atropine N-butyl bromide and lachesine. However, dinitrophenol (10^{-4} M) did produce an effect similar to atropine on the spontaneous activity of the amnion presumably by an intracellular action. Uncoupling agents of this type increase oxygen consumption at low concentrations and inhibit respiration at higher concentrations (Brody, 1955). Atropine failed to increase the oxygen consumption of amnion tissue in concentrations of 50 μg/ml. and its action does not therefore appear to depend on the uncoupling of oxidative phosphorylation in this tissue.

In view of the foregoing negative findings and the fact that similar actions were found with seven different atropine-like drugs it is suggested that the effects may be the result of blockade of muscarinic acetylcholine receptors in the cell membrane. An acetylcholine-like material can be found in the amnion (Cuthbert, 1963b) and evidence has also been obtained that such a substance is released when the amnion contracts (Cuthbert, unpublished). Electron micrographs of the amnion show that the adjacent muscle cell membranes show regions of close apposition (D. H. L. Evans, personal communication). If intercellular conduction takes place in these regions it may be mediated or moderated by the release of acetylcholine from the cells. The receptors involved in these junctional sites are presumed to be unavailable to, or not blocked by, atropine. Muscarinic acetylcholine receptors are believed to be situated in the cell membrane and those receptors adjacent to the junctional regions may act as sinks for some of the liberated acetylcholine. If, however, these sites are occupied by atropine, more acetylcholine may be available for activation of the junctional regions. It is suggested that in this way atropine increases the safety factor for conduction in the amnion and so potentiates the contractions. One

objection to this idea is the high concentrations of atropine required to produce this response compared with those required to inhibit responses to applied acetylcholine. However atropine is equally ineffective in blocking the effects of physiologically released acetylcholine as compared with applied acetylcholine at other synapses (Dale & Gaddum, 1930 ; Dale, 1938 ; Ambache, 1955 ; Ursillo & Clarke, 1956).

A second objection to the role of acetylcholine as an intercellular transmitter in this tissue is that electrical activity can be recorded using the sucrose-gap electrode (Cuthbert, 1962b). This implies there must be some low resistance pathway between adjacent cells for electrical conduction through the sucrose solution, as favoured by protagonists of ephaptic conduction in smooth muscle (Prosser, 1962). However, the mechanism of conduction in isotonic sucrose solution is not necessarily the same as in saline solutions in which the external resistance is low.

The failure of some preparations to show increased spontaneous activity in the presence of atropine-like drugs may be because the conducted responses are already maximal. This would be the case if each contraction wave spread throughout the whole of the tissue or if the conduction mechanism had broken down. Prosser & Rafferty (1956) suggested that the failure of conduction, with increasing age, in the amnion was a result of increased separation of cells.

It seems relevant to mention that in other situations occupation of receptors by an antagonist leads to an increase in the amount of active substance released. The output of acetylcholine from the cerebral cortex is increased by atropine (MacIntosh & Oborin, 1953 ; Mitchell, 1963). Similarly the output of noradrenaline from the spleen is increased by sympathetic blocking drugs (Brown & Gillespie, 1957). How far the results with the amnion are applicable to other unitary smooth muscles is being investigated.

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