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MUSCARINIC EXCITATION: A MICROELECTROPHORETIC STUDY ON CULTURED SMOOTH MUSCLE CELLS

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1 Acetylcholine was applied iontophoretically to smooth muscle cells cultured from taeniae coli of new-born guinea-pigs. Responses were recorded with intracellular microelectrodes.

2 Acetylcholine induced depolarization, spike generation and contraction. Large conductance increases could be measured during the action of acetylcholine.

3 Injection of depolarizing currents through the recording electrode reversed the sign of potential responses. The reversal potential was −5 to −25 mV.

4 Minimum latencies of responses to acetylcholine were 120-500 ms. These values were not attributable to diffusion time.

5 Attention is drawn to the long latencies of a variety of muscarinic responses, and the suggestion made that muscarinic mechanisms as a class may be characterized by a long activation time.

Introduction

The long latency of nerve-mediated responses that involve the interaction of acetylcholine with muscarinic receptors has been noted in a variety of autonomic neuro-effectors, including frog and cat heart (Brown & Eccles, 1934; del Castillo & Katz, 1955a), chick oesophagus (Ohashi, 1971), and cat salivary gland (Creed & Wilson, 1969). In mammalian intestinal muscle the latency of the excitory junction potential which follows a single transmural electrical stimulus has generally been reported to be greater than 100 ms (Burnstock, Campbell, Bennett & Holman, 1964; Gillespie, 1964; Bennett, 1966a; Beck & Osa, 1971). The reason for this long latency is unknown; estimates of the times required for conduction in the terminal and preterminal axons, relay in interposed synapses, and release and diffusion of transmitter, account for only a part of the observed latencies (Holman, 1970). It has been suggested that the long latencies may reflect a peculiarity in the response characteristics of the smooth muscle membrane to acetylcholine (Holman, 1970; Bennett, 1972).

In the present investigation, the response time of the smooth muscle membrane was examined using the precise microelectrophoretic method for application of acetylcholine (del Castillo & Katz, 1955b). The experiment requires a smooth muscle preparation in which acetylcholine can be applied with good spatial resolution close to the site of recording. This condition was fulfilled by the use of trypsin-dissociated cultures of the guinea-pig taenia coli. Some of the electrophysiological characteristics of the cultures have been reported (Purves, Mark & Burnstock, 1973).

Methods

Cultures were prepared from trypsin/collagenase-dissociated taeniae coli of new-born guinea-pigs. The culture methods were essentially those previously described (Mark, Chamley & Burnstock, 1973; Purves *et al.*, 1973) with the exception that cells were grown on plain glass coverslips. Cultures were used 4-21 days after plating. All experiments were performed on small (diameter 60-250 μm) irregular clumps of smooth muscle cells, formed by cellular division and aggregation. The number of cells in the clumps could not be determined accurately, but appeared to range from about 10 to several hundred.

Conventional intracellular recording techniques were used. The intracellular microelectrodes were filled with 3 M KCl or 2 M K-citrate and had resistances of 50-150 MΩ. Acetylcholine was applied iontophoretically from glass micropipettes of resistance 50-400 MΩ which required 'braking' currents of 2-8 nA. Cultures were maintained at 37°C by means of two feed-back devices, one of which controlled the temperature of the bathing culture medium; the other controlled the

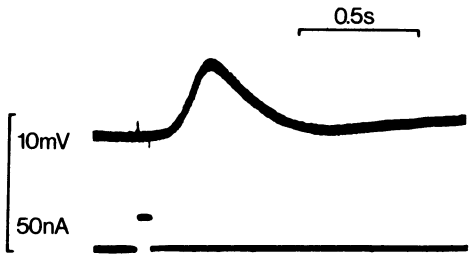

Fig. 1. Intracellular electrical record from small clump of taenia coli smooth muscle cells, showing the depolarization produced by acetylcholine. The lower trace in this and subsequent figures indicates current flow in the acetylcholine pipette. Latency 130 ms. 3-day culture.

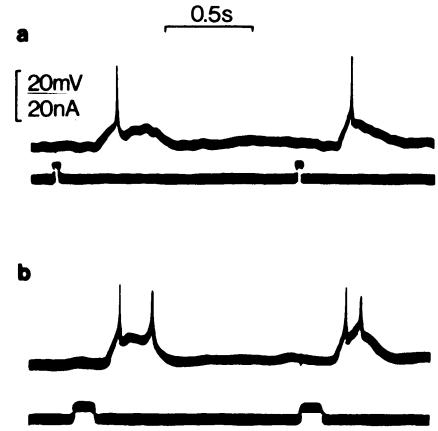

Fig. 2. Single and double spikes produced by application of acetylcholine. (a) Pulse length 50 ms. Latency 250 ms. (b) Pulse length 130 ms. Latency 200 ms. 9-day culture.

temperature of a diffuse air stream directed at the under-surface of the culture chamber. Accurate positioning of micropipettes was facilitated by the use of an inverted microscope (Zeiss Standard UPL) equipped with phase contrast optics (total magnification 200 or 500). Successful impalements generally lasted 1-5 min, being terminated by a more or less abrupt decline of resting potential. Occasionally the recording time was in excess of 10 minutes.

Results

Intracellular recordings from small clumps of muscle cells showed resting potentials up to −45 mV. Many cells, especially those in young cultures, were spontaneously active, discharging spikes at a frequency of 0.5-1.5 Hz (Purves *et al.*, 1973). The discharge frequency could be altered by the passage of small (0.2-1.0 nA) currents in either direction through the recording electrode. To allow easier visualization of the responses to acetylcholine, spontaneously active cells were usually hyperpolarized to a membrane potential of −40 to −50 mV, at which level spontaneous spiking was abolished. Most of the results described below were obtained from the relatively small proportion of quiescent cells. The responses to acetylcholine seen in quiescent cells were similar in every way to those seen in spontaneously active cells.

Acetylcholine responses

The responses to acetylcholine were tested by placing the iontophoretic electrode within 20 μm of the recording electrode, and then lowering it until a faint dimpling of the cell surface indicated contact. It was then withdrawn 2-5 μm before current pulses were applied. A typical response to acetylcholine is shown in Figure 1. Small pulses evoked subthreshold depolarizing potentials with a latency of 120-500 ms, an amplitude of 2-10 mV, a rise time of 40-150 ms, and a total time course of about 1 second. In any given cell, larger pulses caused greater depolarization which could lead to spike generation (Figure 2). A vigorous contraction lasting 5-15 s and involving the entire clump of cells could be seen through the microscope when these large pulses were applied. The contraction was frequently strong enough to dislodge the recording electrode. An interesting feature of Fig. 2 is the shortened latency obtained with a longer acetylcholine pulse. This result was observed in only three cells, and has not received specific study. Latencies were not dependent on the intensity of the acetylcholine pulse.

The minimum charge required to elicit a recordable response was 0.2-0.5 nC. For technical reasons it has not yet been possible to investigate quantitatively the dose-response relationship. Some impalements gave fluctuating responses to successive pulses of acetylcholine, especially when application was repeated at intervals of 5 s or less. In these cases the first response of a series was generally the largest, suggesting that some form of desensitization was occurring. Desensitization was not seen when the interval between pulses was 10 s or greater.

Both the contractile and the electrical response to acetylcholine were blocked by the addition of hyoscine to the chamber (final concentration 2 x

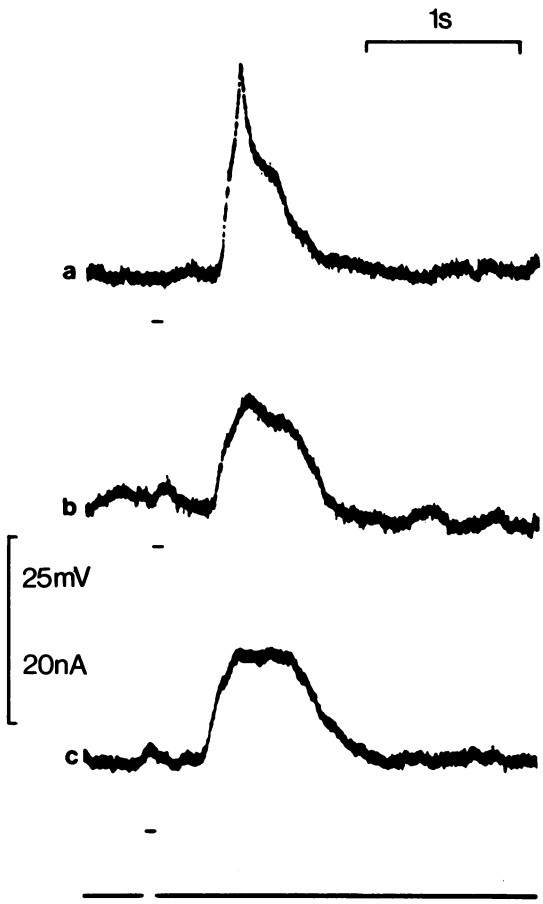

Fig. 3. Responses produced by equal pulses of acetylcholine applied at various distances vertically above the impaled cell. The separations were: (c) <5 μm; (b) 8 μm; (a) 15 μm. Note the spike formation and very small increase in latency at the larger separations. 7-day culture.

10^{-6}M). Hexamethonium (5×10^{-4}M) and (+)–tubocurarine (8×10^{-5}M) had no effect.

In six experiments, the effect of withdrawing the acetylcholine pipette to various vertical distances from the cells was examined. Withdrawal from the closest position (2-4 μm) to 15-20 μm separation increased the latency by less than 10% (Figure 3). The response amplitude generally decreased as the separation was increased, although not uncommonly, as in Fig. 3, a paradoxical increase was seen, due to spike generation. The conductance increase (see below) following close application in the latter instances was apparently great enough to quench the spike mechanism. Exploration of a clump in the

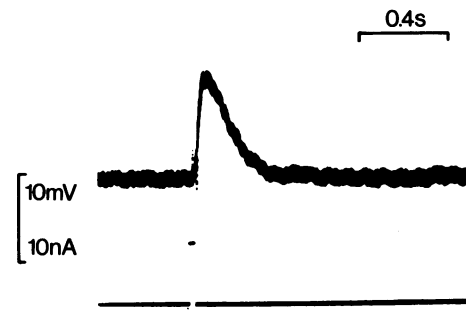

Fig. 4. Intracellular electrical record from chick myotube in culture. The latency of the depolarization produced by a pulse of acetylcholine was less than 5 ms. 12-day culture.

horizontal plane, with the acetylcholine pipette 2-5 μm above the surface never gave responses of latency less than 120 milliseconds.

The extraordinarily long latencies might have resulted from anomalous release of acetylcholine from the electrophoretic pipettes. However, adjustments of the reverse 'braking' current in the range 0-10 nA never altered the latency. Control experiments were performed with cultured myotubes derived from explants of chick thigh muscle (cultures kindly supplied by Mrs C. Hill) in place of smooth muscle. Minimum latencies of responses to acetylcholine pulses of 1-50 ms duration from the same iontophoretic pipettes used in the smooth muscle experiments were less than 5 ms (Figure 4). No evidence for delayed release was seen.

Localization of receptors

Attempts to map out the chemosensitivity of the surface membrane of the muscle cells did not reveal any striking regional differences in responsiveness. Similar depolarizations could be obtained from close application of acetylcholine to any part of the cell, or even to neighbouring cells within 100 μm of the recording site.

Accidental impalement by the iontophoretic pipette was not uncommon. When this occurred, there was no slow depolarizing response to current pulses. Instead, an electronic potential of zero latency was observed (Figure 5). Deterioration of the cell was common following such accidental impalement; rarely, normal acetylcholine potentials could again be evoked after withdrawal of the iontophoretic electrode. Long-latency excitatory responses could not be evoked when the acetylcholine pipette was intracellular.

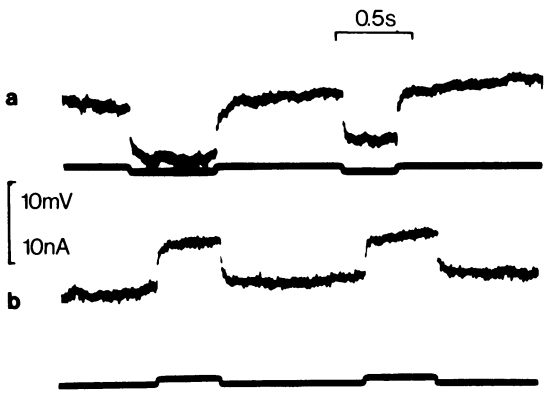

Fig. 5 Following impalement of the smooth muscle clump by the acetylcholine pipette, electrotonic potentials of zero latency were obtained in response to (a) inward and (b) outward current pulses. 14-day culture.

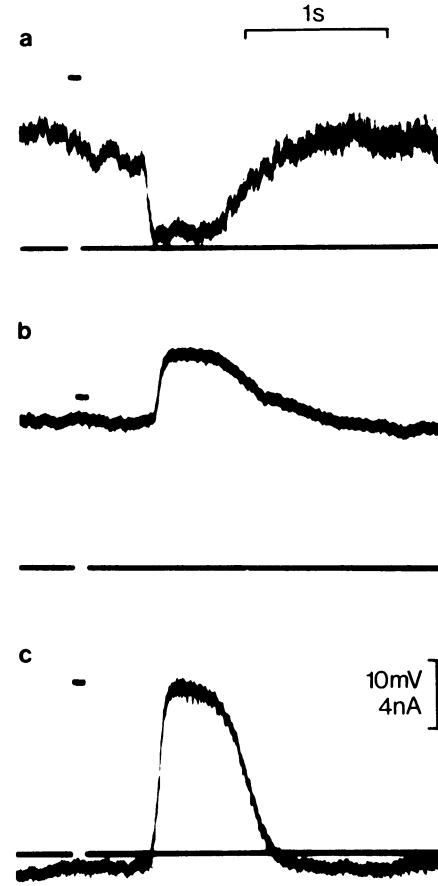

Fig. 6. Effect of passage of polarizing current through the recording electrode on the responses to equal pulses of acetylcholine. Acetylcholine was applied every 10 seconds. In record (a) an outward polarizing current of 1.7 nA was passed. Record (b) was obtained without polarizing current. In record (c) an inward current of 1.6 nA was passed. The resting membrane potential was approximately −35 mV. 5-day culture.

The reversal potential

In preliminary experiments it was noted that the application of acetylcholine to cells which had been damaged by impalement and which consequently had low resting potentials sometimes gave rise to hyperpolarizing potential responses. Attempts were made to demonstrate a reversal of the response in 15 relatively undamaged cells. Unfortunately the delayed rectification which developed during the injection of depolarizing current usually prevented an actual reversal from appearing, as insufficient depolarizing current could be passed through the high-resistance recording electrode. In addition, the generation of spikes and mechanical movement of the cells often caused the impalement to fail. The results of one of four successful experiments are shown in Figures 6 and 7. Accurate assessment of the membrane potential during current injection requires careful compensation for the electrode resistance, which must be constant over the range of current employed. Although this was checked on several occasions during the experiment, using a fast sweep speed and full capacitance neutralization of the input circuit, balance may have been imperfect. Furthermore, resting potentials could not be measured accurately, because of microelectrode tip potentials. The membrane potential values given are therefore very approximate. Reversal occurred at a point some 10-20 mV more positive than the resting potential i.e. at about −5 to −25 mV. The reversal potential was also estimated for five cells by extrapolation of the responses during injection of hyperpolarizing and small depolarizing currents (Figure 8). Values obtained in this way were also in the range −5 to −25 mV.

Conductance changes

The results described in the previous section imply a conductance increase during the action of acetylcholine. A more direct indication is given in Figure 9. An acetylcholine pulse of 1.5 nC here caused a conductance increase of at least ten-fold, lasting for over 1 second. The resting input

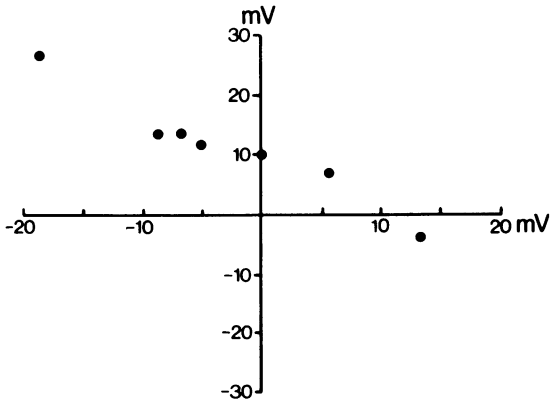

Fig. 7. Relationship between membrane potential and response to acetylcholine. Horizontal axis: alteration of intracellular potential produced by the passage of polarizing current through the recording electrode. Vertical axis: voltage response to equal pulses of acetylcholine applied every 10 seconds. Depolarizations are shown as positive quantities. Reversal occurred at about 12 mV positive to the resting potential. 5-day culture; same cell as in Figure 6.

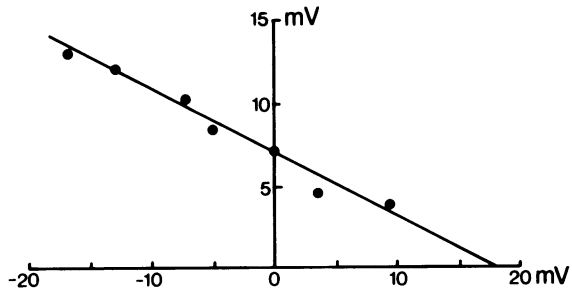

Fig. 8. Estimation of reversal potential by extrapolation. Straight line fitted by eye. The estimated value for this cell is 18 mV more positive than the resting potential. Axes as in Figure 7. 5-day culture.

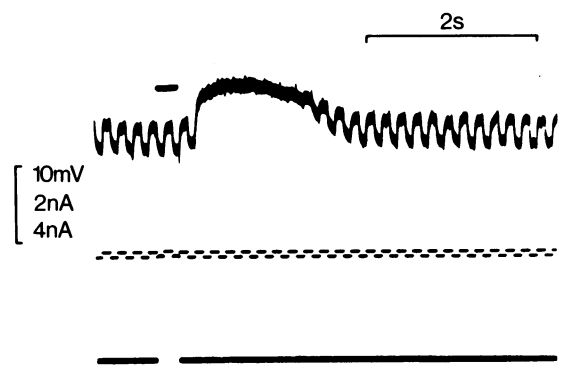

Fig. 9. Conductance change during the action of acetylcholine. Small repetitive current pulses of rectangular waveform (5.5 Hz; middle trace) were passed through the recording electrode. The potential drop across the electrode resistance was compensated during display of the voltage trace at high sweep speed. 5-day culture.

conductance of this cell was 0.05 μS. Conductance increases were demonstrable much more readily than was reversal of the voltage response, since only small currents had to be passed through the recording electrode.

The observation of such large conductance increases suggests another means of estimating of the reversal potential. If the conductance increase produced by acetylcholine were sufficiently large to swamp all other conductances, the membrane potential would tend to a value very close to the reversal potential. With the largest doses of acetylcholine this condition was probably met, as indicated by the flattened peaks of the voltage responses in Figure 6. Close application of large doses quenched the spike mechanism (Fig. 3), presumably by virtue of the increased shunt conductance. Thus the plateau potential of responses to large doses of acetylcholine is likely to be a good approximation to the reversal potential (see Bolton, 1972), the only significant source of error being the difficulty of measuring resting potentials. Values of reversal potential obtained in this way from 22 cells ranged from -12 to -22 mV, with a mean of -17 mV.

Discussion

Cultured smooth muscle cells from the guinea-pig taenia coli respond to the application of acetylcholine with transmembrane conductance increases, depolarization, spike generation and contraction. These responses are qualitatively similar to those seen in adult intestinal smooth muscle differentiated *in vivo* (Bülbring, 1954, 1955; Burnstock, 1958; Hidaka and Kuriyama, 1969; Kuriyama, 1970; Bolton, 1972). In addition, the spontaneous electrogenic behaviour of cultured muscle is very similar to that of the adult taenia coli (Purves *et al.*, 1973). The above observations suggest that other electrical properties of cultured smooth muscle, in particular the latency of responses to acetylcholine, may be representative of the adult condition.

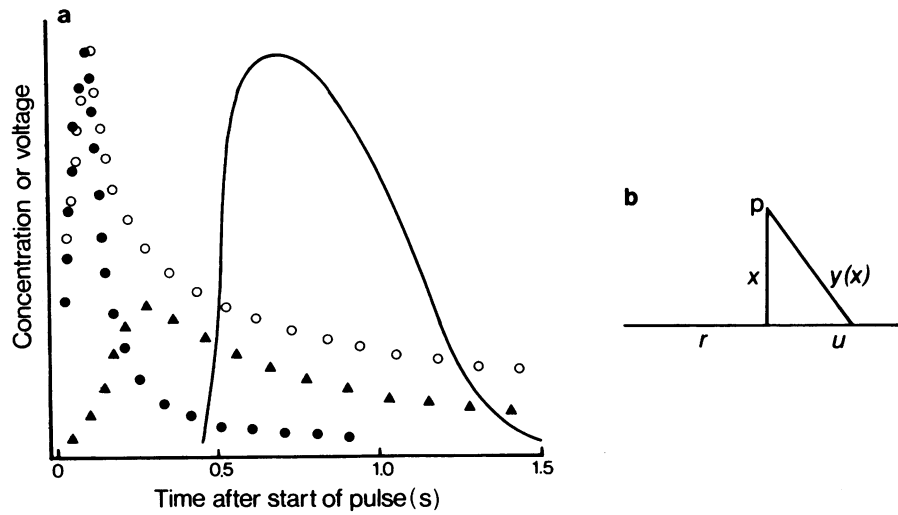

Fig. 10 (a) Comparison of a typical voltage response with the calculated time course of concentration change of acetylcholine 8 μm (circles) and 32 μm (triangles) from a point source. The concentration curves were calculated numerically from the diffusion equations (Jaeger, 1965), for a continuous point source of 100 ms duration, the diffusion constant being taken as 8.10^{-6} $cm^2 s^{-1}$. The vertical scale is arbitrary, though linear. Concentrations for the 32 μm curve were multiplied by 10 for display purposes. The voltage curve was redrawn from a trace obtained from an 8-day culture when a 100 ms pulse of acetylcholine was applied 4 μm from the surface of the clump. Open circles represent average concentrations at the surface of the whole clump, scaled to the same peak height as the 8 μm curve.
(b) Schematic drawing of geometry assumed for calculation of average concentrations. Acetylcholine is released at point P, distance x above centre of circular clump of radius r. $y(x) = \sqrt{(x^2 + u^2)}$ where u is a dummy radius variable. See text for further details.

Latency of the acetylcholine response

The long latencies of the electrical responses to iontophoretically applied acetylcholine are of particular interest, for they indicate that a large part of the latency of nerve-mediated cholinergic responses in smooth muscle may be due to a delay in the reaction of the muscle to the neurotransmitter. Before this conclusion is accepted it is necessary to show that in the present study the time course of responses (particularly of their onset) was not access-limited. Figure 10a compares a typical voltage response with the concentration changes of acetylcholine 8 μm (filled circles) and 32 μm (triangles) from a point source of 100 ms duration. The concentration curves were computed from the diffusion equations (Jaeger, 1965), assuming no chemical reaction of the transmitter. In view of the electrical coupling between cells (see below) and the large size of the clumps, it is unrealistic to treat the system as a 'point receptor'. The average concentration of acetylcholine presented to the entire clump may be a more valid basis of comparison with the voltage response. If it is assumed that the acetylcholine pipette is situated above the centre of a circular clump (Fig. 10b), then a measure of the average concentration at any time can be obtained by integrating the concentration-area product over the whole surface of the clump. The open circles of Fig. 10a represent average concentrations calculated as:

$$\frac{m}{2D}\int_0^r \frac{u}{y(x)}\left(\operatorname{erfc}\frac{y(x)}{2(Dt)^{1/2}} - z\right) du$$

where $z = 0$ for $t \leqslant t_1$

$$= \operatorname{erfc}\frac{y(x)}{2[D(t-t_1)]^{1/2}} \text{ for } t > t_1 \text{ (Jaeger, 1965)}$$

D is the diffusion constant, u, r and $y(x)$ have the meanings indicated in Fig. 10b, and m is the rate of supply of acetylcholine during a pulse of duration t_1. For evaluation of the integral, x was taken as 4 μm, r as 100 μm, and t_1 as 100 milliseconds.

A marked discrepancy is evident between the time courses of the average concentration and the observed voltage response. The computer

programme which generated the average concentration curve was modified so as to derive the times at which maximum average concentrations would be obtained for various values of x. The predicted value of x corresponding to the observed peak time of the voltage response (680 ms) was found to be 30 μm. The actual electrode separation during the experiment was only 4 μm. If the geometrical model of Fig. 10b is an appropriate one, these calculations would suggest that diffusion time cannot account for the delayed onset of the responses.

It is conceivable, however, that the effective diffusion pathway could be greater than that assumed in Figure 10b. For instance, there may be no receptors on the exposed surface of the cells; only a few cells may be sensitive; the electrotonic properties of the clump may be markedly anisotropic; and so on. Although the existence of these complications cannot be ruled out, consideration of the time course of the voltage response shows that they are unlikely to have given rise to significant access delays. For if the onset of the response reflects the rise of acetylcholine concentration at the receptors, the initial delay due to diffusion should be only one-fifth to one-quarter of the time to peak (Katz & Miledi, 1965). In fact, the initial delay was usually greater than the time to peak by a factor of 2-5. The time course of the underlying conductance change is not known, but it cannot have been less abrupt in onset than the voltage response; this abruptness constitutes the strongest argument against simple diffusional access-limitation from any cause. More complicated forms of access-limitation can be considered, such as a saturable pool of high-affinity sites of loss which might be imagined to intervene between the acetylcholine pipette and the receptors, thus delaying the onset of transmitter action. Cholinesterase is an obvious candidate for this role, but its presence in cultures of skeletal muscle is not associated with long latency cholinergic responses.

The binding of acetylcholine to receptors will also affect the time course of concentration change. A precise computation is not possible but the following rough calculation will indicate the magnitude of the effect. With an upper limit of 2×10^6 receptors per cell (Tschöpe & Ziegler, 1969) there may be 10^8 receptors on the exposed upper surface of a clump of average size. A 1 nC electrophoretic pulse should liberate $1\text{-}2 \times 10^9$ acetylcholine molecules, of which less than half would be available for combination with receptors, owing to the geometry of the release situation. It is clear that appreciable distortion of the theoretical concentration time course could occur, although it is difficult to see how this effect could produce a long latency.

A further possibility is that the iontophoretic pipettes had anomalous properties which prevented the release of acetylcholine until some time after the end of the current pulse. Control experiments (Fig. 4) on cultured chick skeletal muscle did not support this possibility. Minimum latencies obtained by activation of the nicotinic receptors of these cells, using the same acetylcholine pipettes as in the smooth muscle experiments, were less than 5 milliseconds.

A reasonable conclusion is that the electrical response of the smooth muscle cells was delayed by 120-500 ms from the time at which acetylcholine reached the cell surface. The range of latencies observed was wide; unfortunately no correlation could be made between latency and such factors as culture age, clump size and so on. The delay is three orders of magnitude greater than that shown by the end plate of skeletal muscle (Katz & Miledi, 1965; Nastuk, Manthey & Gissen, 1966). Such a long delay could conceivably be due to a membrane abnormality in consequence of the *in vitro* conditions or the trypsin treatment during preparation of the cultures. For the reasons given earlier this is thought unlikely. Furthermore, the muscarinic receptor has been reported to be resistant to trypsin (Cuthbert, 1966). It is, however, highly desirable that the results be confirmed with tissues differentiated *in vivo*. Iontophoretic application of acetylcholine to smooth muscle of the guinea-pig jejunum (Hidaka & Kuriyama, 1969) evoked excitatory electrical changes whose latency (0.5 s) was comparable to the largest values observed in the current investigation. However, the experimental conditions used by Hidaka & Kuriyama (1969) did not allow application at accurately-known small distances from the muscle cells. The present results are in accord with, or exceed slightly, most published values of cholinergic (muscarinic) junction potential latency. Some authors, however, have reported an 'early' junction potential in various intestinal preparations, whose latency is considerably less than 100 ms (Kuriyama, Osa & Toida, 1967; Hidaka & Kuriyama, 1969; Furness, 1969; Ito & Kuriyama, 1973). The present experiments provide no evidence for rapid cholinergic mechanisms in the taenia.

The phenomenon of long-latency cholinergic effects is not confined to autonomic neuro-effectors. Latency differences corresponding to activation of different pharmacological receptors have been described for central and peripheral neurones. Renshaw cells, whose predominant cholinoceptive behaviour is nicotinic (Curtis &

Ryall, 1966) give excitatory responses to iontophoretically applied acetylcholine whose onset is much more rapid than responses of cortical or thalamic cells, the receptors of which are mainly muscarinic (Krnjević & Phillis, 1963; Andersen & Curtis, 1964). Some mammalian autonomic ganglion cells possess both nicotinic and muscarinic excitatory receptors. Slow synaptic excitatory responses in the rabbit superior cervical ganglion have been attributed to activation of the muscarinic receptors (Libet, 1967) latencies being of the order of 100-200 milliseconds. Although it was originally postulated (Eccles & Libet, 1961) that the slow responses occurred following diffusion of acetylcholine to sites remote from the synapses, it is more likely that the long latency is a consequence of the receptor type rather than of spatial factors.

A tentative hypothesis is advanced, according to which the differential pharmacological properties of cholinergic receptors can be correlated with differential kinetic properties: nicotinic receptors give fast-onset responses, whereas muscarinic receptors give responses only after a relatively long activation time. This correlation was pointed out many years ago by Dale (1938); it is surprising that so little experimental investigation of it has since been attempted.

At present it is not clear whether the delayed onset of muscarinic responses represents a receptor or a post-receptor phenomenon. Recent investigations of the time course of the end-plate current at the skeletal neuromuscular junction provide evidence that conformational changes in the receptors may be rate-limiting for the decay phase (Kordaš, 1972; Magleby & Stevens, 1972). The onset of muscarinic responses is perhaps similarly rate-limited by a conformational change of appropriate kinetic characteristics. The time constants for conformational change in various enzymes range from less than 1 ms to several minutes (Massey & Curti, 1966; Hammes, 1968).

Receptor localization

Attempts to demonstrate localized regions of high sensitivity to acetylcholine in these denervated cultured smooth muscle cells by the method of del Castillo & Katz (1955b) were unsuccessful. The membrane thus appears to be uniformly sensitive, although it must be recognized that the long response latencies would have allowed diffusion of acetylcholine over a wide area. Real differences in sensitivity might have been masked.

Intracellular application of acetylcholine never gave rise to delayed conductance increases. Thus, the receptors in smooth muscle, as in skeletal muscle (del Castillo & Katz, 1955b), are probably located on the external surface of the membrane.

The conductance increase

Point polarization with an intracellular microelectrode has generally been found inadequate to demonstrate changes of smooth muscle membrane properties during the action of neurotransmitters (Bennett, 1967; Bennett & Rogers, 1967; Holman, 1970; but see Hidaka & Kuriyama, 1969). The reason for this is that smooth muscle cells are electrically coupled to form a three-dimensional syncytium (Tomita, 1970), whose driving-point conductance may theoretically be almost independent of membrane conductance (George, 1961). The ready demonstration of large conductance changes in the present study therefore requires explanation. One explanation might be that cultured smooth muscle cells, even when in tightly-packed clumps, are not electrically coupled. This possibility has been refuted by the recording of electronic potentials in cells 50-200 μm from a current-passing intracellular microelectrode (Purves *et al.*, 1973; Purves, unpublished observations). It is considered more likely that neighbouring cells are coupled, but that the dimensions of the small clumps used in the present experiments (60-250 μm diameter; 1-3 cells thick) are comparable with, or less than, the electrical space constant. The dependence of driving-point conductance on membrane conductance would in this case be greatly increased, compared with an infinite syncytium.

Accurate assessment of the acetylcholine reversal potential was impossible in these experiments, largely owing to vagaries of microelectrode resistance and tip potential. A further source of error is the horizontal diffusion of acetylcholine across the clumps, producing transmitter action at sites remote from the recording electrode. Polarizing currents conducted electrotonically through the syncytium would be relatively ineffective in altering the membrane potential at these remote sites, especially during transmitter action, when the electrical space constant would be much smaller than its resting value (Woodbury & Crill, 1961). The rough estimates made are compatible with the previously reported values for intestinal smooth muscle, namely -9 mV (Bolton, 1972) and -20 to -26 mV (Bennett, 1966b).

In the present studies, individual ionic components of the conductance increase produced by acetylcholine were not resolved, although it may be possible to attempt this by manipulating extracellular ion concentrations. It should also be possible to examine the mode of action of other

known or putative transmitter chemicals, including noradrenaline and adenosine triphosphate.

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