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EFFECTS OF STIMULATING THE ACETYLCHOLINE RECEPTOR ON THE CURRENT–VOLTAGE RELATIONSHIPS OF THE SMOOTH MUSCLE MEMBRANE STUDIED BY VOLTAGE CLAMP OF POTENTIAL RECORDED BY MICRO-ELECTRODE

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SUMMARY

1. A double sucrose-gap voltage-clamp technique is described for use on smooth muscle strips longer than about 2 mm. It involves intracellular recording by microelectrode of the membrane potential of a narrow region of the strip ('node') sandwiched between two streams of deionized sucrose solution. Current was passed into the node across one or both sucrose streams.

2. Preliminary experiments in which potential was recorded intracellularly at two points during polarization of a 'short cable' preparation, formed by folding over a strip of smooth muscle, suggested that a node width of less than 0·15 mm was needed to achieve uniform potential during inward current flow. However, when node width between sucrose-gaps was reduced to 0·5 mm, spontaneous electrical activity was lost, and below 0·5 mm spike threshold was raised and the regenerative spike became graded. The currents flowing during the application of rectangular voltage-clamp command potentials were described.

3. Using taenia smooth muscle it was shown by recording with a second, independent micro-electrode that potential was not uniform for up to 200 ms or more following a step change in potential under voltage-clamp in nodes 0·4–0·5 mm wide where current was passed across both sucrose gaps. However, reasonably uniform nodal potentials were obtained using ramps with relatively slow rates of rise (25 mV/s).

4. Using such slow ramp commands under voltage clamp, the effects of carbachol on the current–voltage relationship of longitudinal muscle of ileum and taenia were studied in hypertonic solution.

5. In the presence of carbachol (10^{-6} to 10^{-5} g/ml.) additional inward current flowed across the membrane (in some experiments an equilibrium potential was observed at which this current reversed direction). The

magnitude of this additional current was linearly related to potential at potentials negative to the resting potential. At potentials positive to the resting membrane potential, this additional current increased with depolarization over the range -40 to -10 mV; in ileum the effect of this additional inward current on the current–voltage relationship was to produce a region of net inward current where before, in the absence of carbachol, a net outward current existed. In taenia the additional inward current flowing in the presence of carbachol was too small to produce a region of net inward current; thus carbachol produced regenerative slow oscillations of potential (slow waves) in ileum but not in taenia.

6. These results support a previous suggestion that activation of the acetylcholine receptor of ileal smooth muscle produces an additional inward current in the membrane which increases with depolarization and is responsible for the regenerative slow waves seen when muscarinic stimulants are applied. A similar effect apparently operates in taenia but the additional inward current is too small to produce regenerative slow waves.

INTRODUCTION

Stimulation of the acetylcholine receptor of smooth muscle of ileum produces large, relatively slow (~ 1 s) regenerative oscillations of the membrane potential. These may first appear as a prolonged delay of repolarization following a spike, and as the spike is reduced in size during the action of some agonist such as carbachol, they develop an almost sinusoidal form. The mechanism of these oscillations, or slow waves, is unknown, but they are sensitive to a reduction in the external sodium concentration and membrane conductance is increased at their peak. In sucrose hypertonic solution such waves do not occur in the *absence* of stimulation of the acetylcholine receptor, depolarizing current eliciting only repetitive spiking. However, if a small concentration of carbachol is present a similar depolarization (if suprathreshold) elicits slow waves of about 3 s duration upon which spikes are superimposed. Clearly the presence of slow waves depends upon stimulation of the acetylcholine receptor and their mechanism is a regenerative, voltage-dependent one (Bolton, 1971, 1972*a*).

Since these previous results were obtained using larger pieces of smooth muscle, it is conceivable that slow waves are a form of spike modified by the additional conductance introduced into the membrane by stimulating the acetylcholine receptor (Bolton, 1972*b*). Thus, a spike may arise in a remote or deep part of the muscle (Osa & Taga, 1973) but, upon propagating to the region of recording its duration is greatly increased and its size reduced by the increased conductance. An argument against this

hypothesis is that normal spikes can sometimes be recorded as taking off at the peak of the slow wave (fig. 7 of Bolton, 1971). Strong evidence against this hypothesis might be obtained if it was possible to record from pieces of smooth muscle which could be shown to be polarized sufficiently uniformly for asynchronous firing of spikes to be unlikely. This paper describes the results of experiments of this type.

An alternative slow wave mechanism which was suggested (Bolton, 1971) was that stimulation of the acetylcholine receptor somehow altered the current–voltage relationship of an inward current channel whose conductance increased with depolarization. Thus, without muscarinic stimulation this channel would carry insignificant current since slow waves do not occur, but, when the acetylcholine receptor is stimulated, this postulated channel would carry a significant inward current producing a slow wave.

Evidence to support such a hypothesis must come from some form of voltage-clamp technique. Early experiments (Bolton, 1974*a*) using the double sucrose-gap voltage-clamp apparatus of Rougier, Vassort & Stämpfli (1968) with extracellular potential recording, were somewhat disappointing, since muscarinic stimulants did not produce the oscillations of potential typical of their action on larger pieces of ileal smooth muscle. It was not, therefore, surprising that under voltage clamp, no important voltage-dependence of the additional conductance appearing in the presence of carbachol could be detected. A significant factor in the experiments was probably the small size of the region of active muscle ('node') between sucrose streams, which has the effect of increasing the relative importance of leakage current (McGuigan, 1974). To reduce the importance of leakage current, node width was increased in the present experiments, and an alternative voltage-clamp method developed (Bolton, 1974*b*).

There have been a number of voltage-clamp studies on smooth muscle in which the potential has been recorded extracellularly and in which the assumption of uniform nodal potential under voltage-clamp conditions has been made (e.g. Anderson, 1969; Kumamoto & Horn, 1970; Anderson, Ramon & Snyder, 1971; Kao, 1971; Mironneau & Lenfant, 1972; Mironneau, 1973; Kao & McCullough, 1975). Voltage-clamp techniques on multifibre preparations have been severely criticized on the grounds of lack of uniformity of nodal potential (Johnson & Lieberman, 1971). Experiments described in this paper substantially support the theoretical doubts which were raised. Because of this, and since the present voltage-clamp method has not been previously applied to smooth muscle, an important part of this paper is concerned with establishing conditions under which nodal potential is uniform, or nearly so.

The results of the present work support the previously suggested hypothesis that stimulation of the acetylcholine receptor of smooth muscle results in additional inward membrane current at the resting membrane potential, and this current increases with depolarization, as reported in preliminary communications (Bolton, 1975*a*, *b*).

METHODS

Double sucrose gap

Preparation

Strips of longitudinal smooth muscle were cut from taenia or terminal ileum of guinea-pigs weighing 200–400 g. Longitudinal ileal muscle was separated from the bulk of the circular muscle by a technique described previously (Bolton, 1972*b*). In cross-section taenia strips were about 0·1 mm thick and 0·3–0·5 mm wide when in position in the apparatus. Ileal strips were slightly thinner and about 0·8 mm wide.

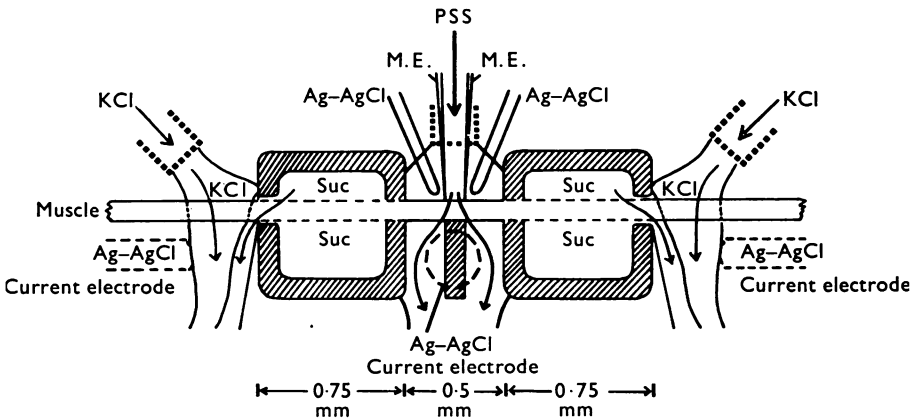

Fig. 1. Diagram of the sucrose-gap apparatus used. The muscle strip was drawn through holes in two chambers (hatched) through which deionized sucrose (Suc) flowed to escape, generally by the outer holes. The region between the chambers, the 'node', was perfused with physiological salt solution (PSS). Its width is shown as 0·5 mm here, but was varied in different experiments as described. The positions of silver–silver chloride (or calomel) electrodes are shown dotted, as are the positions of three inflow tubes. The solutions run to waste (arrows) under the influence of gravity. In the nodal solution is a support (also hatched), two micro-electrodes (M.E.) and two reference Ag–AgCl wires.

Apparatus

Strips were introduced into a double-sucrose gap apparatus (Fig. 1) which allowed one or two glass micro-electrodes (resistance 25–50 MΩ and filled with 3 M-KCl) to be inserted into the region between sucrose gaps (the 'node') which was perfused with either isotonic physiological salt solution or such a solution made hypertonic by the addition of sucrose (Tomita, 1966) or NaCl. The node is shown as 0·5 mm wide in

Fig. 1 but the effects of varying node width from 0·1 to 1 mm or more will be described.

The sucrose chambers were made of film base (Kodak 2495 RAR film) cemented together with Araldite epoxy resin (Ciba). Their width was about 0·8 mm and muscle strips were drawn through holes 0·3 mm diameter in these chambers from the nodal side. Usually both ends of the muscle strip were depolarized in 154 mM-KCl solution. A 292 mM sucrose solution was made by dissolving Analar grade sucrose and deionized by passing through an ion exchange resin (Elgastat type B102, Mk II). In later experiments 10^{-5} M-$CaCl_2$ was added to this solution (New & Trautwein, 1972; Kléber, 1974).

Current was passed between silver–silver chloride or calomel electrodes (Ives & Janz, 1961) connected downstream to the central node or K streams by 3 M-KCl 4% agar bridges about 1 cm long. Two chlorided silver wires (100 μm diam) dipped into the nodal solution and were positioned touching, or almost touching, the muscle at one side.

In the experiments where the effects of varying node width were examined, one sucrose gap was replaced by a short tube (i.d. about 0·5 mm) through which sucrose solution flowed and this side of the node was formed as an interface between physiological salt solution and sucrose solution without a physical partition. In this case that end of the muscle was not depolarized in high K and current was passed across only one sucrose gap. In all other experiments current was passed across both sucrose gaps.

Series resistance. This varied depending among other factors upon dimensions of the node. In the experiment illustrated in Fig. 8 the potential step observed upon withdrawing the electrode from the cell was less than 1 mV when about 3·5 μA were passed. Assuming all this current passes through r_s (Fig. 2) (i.e. $r_e \gg r_i$, which is almost certainly not true) then the series resistance is less than 300 Ω. This current, passing through r_m produced a steady-state hyperpolarizing electrotonic potential of 50 mV. Since the current–voltage relationship is linear, node resistance at the resting membrane potential is about 15 kΩ and the ratio of r_m/r_s is 50. This ratio is unaffected if the assumption that $r_e \gg r_i$ is not true. In the experiment of Fig. 9 where the node width was 0·5 mm, r_m/r_s is about 25.

Electronics

The essentials of the circuit used are shown in Fig. 2. The muscle strip in the node is represented by a single battery, resistor, and capacitor in parallel but under some conditions this representation is insufficient and a second membrane unit is introduced connected by interrupted lines. The internal (core) resistance of the muscle through both gaps (in parallel) is r_i, and the external resistance across the gaps (in parallel) is r_e. The resistance in series with the membrane is r_s(ohms).

One chlorided silver wire was used as an independent electrode for the input which was differential for both micro-electrodes. The other chlorided silver wire was connected to the negative input of an operational amplifier and nodal solution was clamped at virtual ground potential by the appropriate position of switch *C*. The current required to do this during voltage-clamp, or when passing rectangular current pulses was measured as the potential across a 1 kΩ resistor. This potential was fed into the voltage-clamp amplifier, when rectangular current pulses were required, by the appropriate position of switch *B*; R_S was then reduced and R_F increased, clamping the current between nodal and KCl streams. Rectangular or ramp currents could then be applied from the wave-form generator. The membrane was always held at the resting membrane potential (zero current) when in the current clamp mode.

For voltage clamp the output from one electrode was chosen by the position of

switch A and fed into the voltage-clamp amplifier by means of switch B. The resistance R_S was reduced and R_F increased. The holding potential was chosen by adjusting the offset, and voltage-clamp commands were produced by the wave form generator. The resistance of both sucrose gaps in parallel ($= (r_i r_e)/(r_i + r_e)$) was measured as the potential across the 1 kΩ resistor when a 100 mV, 10 Hz, sine wave was applied between the node and the KCl streams by the appropriate positions of switch C. Typical resistances lay in the range 40–200 kΩ.

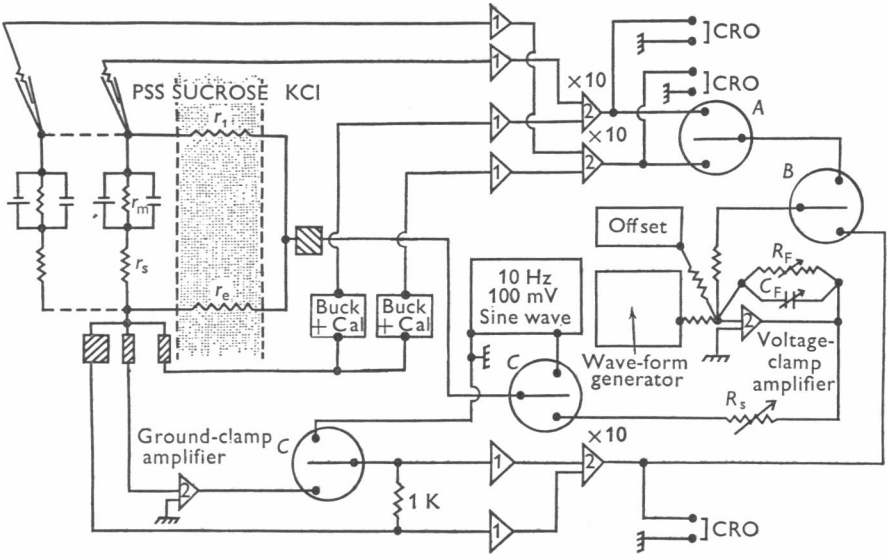

Fig. 2. Simplified diagram of the circuit used. The double sucrose gap is represented by a single gap here (gaps were connected in parallel). Thus r_i (Ω) is the internal (core) resistance of the preparation across both gaps in parallel; r_e (Ω) is the external resistance across both gaps in parallel. Operational amplifiers labelled 1 had high input impedance, those labelled 2, low input impedance. CRO are the inputs to the oscilloscope. The hatched boxes are Ag–AgCl chloride or calomel electrodes connected to the nodal solution or KCl streams. By appropriate positions of switch B either the current flowing between the node, the KCl streams, or the potential recorded by one or other of the micro-electrodes, can be clamped. For further details see text.

To prevent oscillation during voltage or current clamp a suitable value of C_F was chosen. As can be seen from the records, control of recorded potential after a step potential change under voltage clamp could be achieved within a few milliseconds which was adequate for present purposes. In some ramp experiments the current output was filtered by a RC circuit with variable time constant immediately before the input to the oscilloscope. A routine check was made to ensure that this did not affect the shape of the ramp obtained.

Physiological salt solutions and drugs

The isotonic Krebs solution used to bathe the node had the following composition (mM): NaCl 120; KCl 5·9, $CaCl_2$ 2·5, $NaHCO_3$ 15, NaH_2PO_4 1·2, $MgCl_2$ 1·2, glucose 11. Hypertonic solution was made by adding 320 mM sucrose or 234 mM (extra) sodium chloride to isotonic Krebs solution. All solutions were equilibrated with 3 % CO_2 and 97 % O_2 before use. The temperature of the nodal solution was 32–34° C. Carbachol chloride was introduced to the muscle by changing the physiological salt solution to one that contained it.

Short-cable folded preparations

A few experiments were done on ileal muscle strips about 2 mm wide. These were folded over as shown diagrammatically in Fig. 3 to create a short cable in which the decline of potential is expected to be less than in the usual 'infinite cable' preparation. Current was injected close to the folded end of this preparation by means of a silver–silver chloride plate electrode (electrode 3) insulated on the side facing two recording micro electrodes (electrodes 1 and 2) as depicted. To reduce short-circuiting a 2 mm sucrose gap was formed between the current passing electrode and its partner (electrode 4) in a KCl pool. The folded end of the muscle was secured by micropins (T. Gerrard & Co.) and bathed in sucrose hypertonic physiological salt solution.

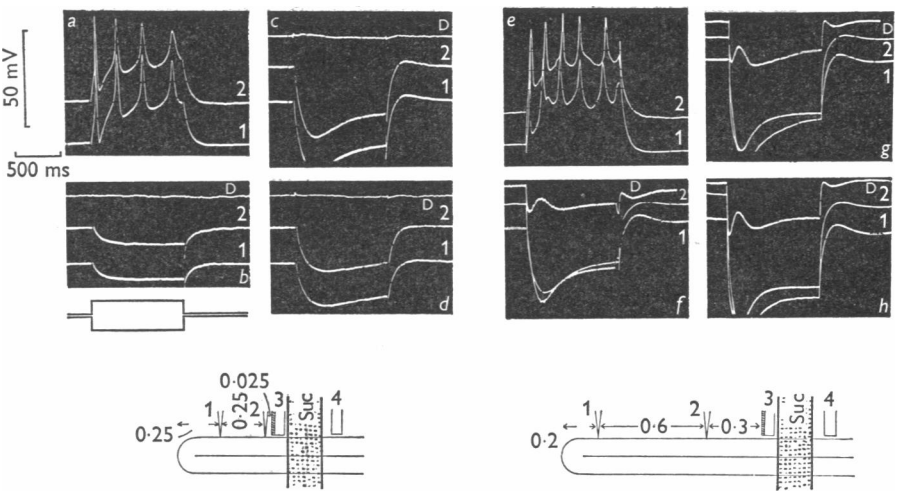

Fig. 3. Decline of potential in a 'short-cable' preparation of ileal smooth muscle. A closed end of the cable was formed by folding over the muscle strip as shown in the diagrams. Current was passed into the cable by means of electrode 3 (an external Ag–AgCl electrode) and sucrose gap (Suc). Electrode 4 is the indifferent electrode in the KCl pool. Potential was recorded at the indicated points (dimensions in mm) in two preparations by means of micro-electrodes 1 and 2. The records above are labelled accordingly. The inset shows the form of the current pulses applied. As shown by the differential records (D) when the preparation was short, $X \approx 0{\cdot}5$ (a–d, and left-hand diagram) the decline in the electrotonic potential was very small, but, when the preparation was longer, $X > \lambda$ (e–h, and right-hand diagram) then there was an appreciable decline in the electrotonic potential.

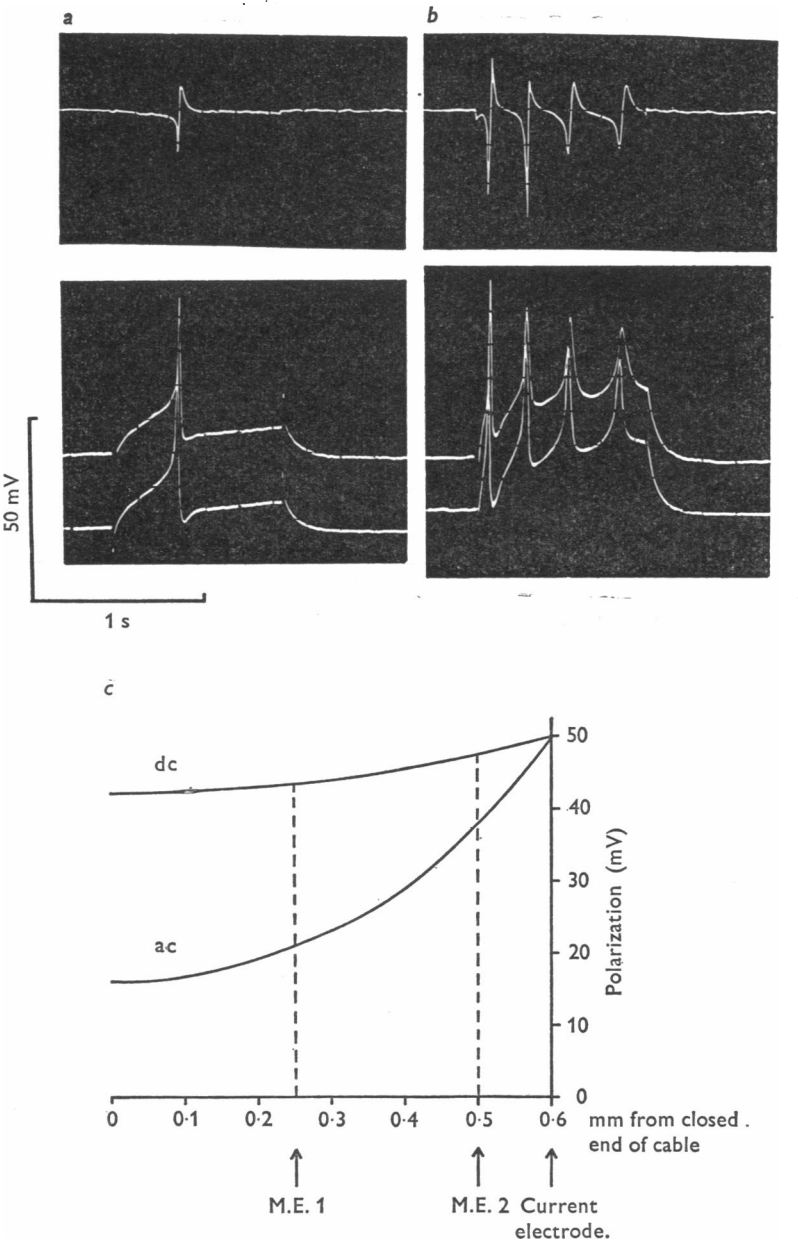

Fig. 4. For legend see facing page.

RESULTS

Potential decline in 'short-cable' preparations

Despite their multifibre nature, the passive electrical properties of strips of intestinal smooth muscle are approximately those of an electrical cable, the decline of extrapolar potential with distance from a current-passing electrode being exponential (Shuba, 1961; Tomita, 1966; Abe & Tomita, 1968). Potential decline in a cable is reduced near a closed end (e.g. see Adrian, Chandler & Hodgkin, 1970). To test this, a 'short cable' preparation of smooth muscle was created by folding over strips of longitudinal ileal muscle, as indicated diagrammatically in the insets to Fig. 3. The folded ends of the strips were polarized as described by an external current passing electrode (and sucrose gap). In this arrangement the upper and lower parts of the folded strip can be considered to be two cables both of which are 'closed' at the fold, since no axial current can flow at this point. (Alternatively the portion of the muscle between the external electrode and the fold can be considered to represent the end of a single cable, terminating at the fold, with a longitudinal internal septum which does not affect current distribution.) Thus the folded end of the smooth muscle strip is theoretically analogous to the end of a skeletal muscle fibre as used by Adrian *et al.* (1970). Two micro-electrodes were used to record the potential at different points in hypertonic physiological salt solution while hyperpolarizing or depolarizing rectangular current pulses were passed. The difference in potential between these two points was also displayed on the oscilloscope (Figs. 3*b–d*, *f–h* and Fig. 4, upper line).

When the length of muscle, x (mm), beyond the current electrode was short compared to the space constant, λ (mm), of this muscle (about 1·3 mm according to Hidaka & Kuriyama, 1969), the decline in potential observed in response to constant hyperpolarizing currents was very small:

Fig. 4. Variation in potential in a 'short-cable' preparation during spike discharge. The preparation is the same one as in Fig. 3*a–d*. The upper lines in *a* and *b* show the recorded potential difference between the two electrodes. Providing membrane resistance does not fall there is little decline of direct current polarization along this preparation, but considerable potential variation occurs during a spike. In *c* is shown the theoretical decline of potential in a short cable in which current injection is made 0·6 mm from a closed end. Line d.c. shows the decline of polarization produced by a constant current when $\lambda = 1{\cdot}0$ mm. Line a.c. shows the expected decline if membrane resistance falls to a tenth of its normal value ($\lambda = 0{\cdot}32$), which is equivalent to its capacitive reactance to a 50 Hz sine wave assuming the membrane capacitance is 1 μF. cm^{-2}. The positions of the two recording micro-electrodes (M.E.) are indicated. For further details see text.

for example, in Fig. 3*b–d* the largest electrotonic potential elicited was 37 mV recorded at electrode 2 while the difference in potential recorded between the two electrodes (top line) was 1–2 mV. The short cable equation, $V = V_0 \cosh(x/\lambda)$, V (volts) being the potential recorded at a distance x from the closed end of a cable ($x = 0$) where the potential is V_0, predicts a 3 mV difference between electrodes in these positions for 50 mV polarization (at $x = 0{\cdot}6$ mm) of a cable of space constant 1·0 mm (Fig. 4*c*, line d.c.). Thus, providing there is no appreciable decrease in the resting membrane resistance, a closed cable length of about $\frac{1}{2}\lambda$ achieves reasonable constancy of potential. However, when the length of muscle beyond the

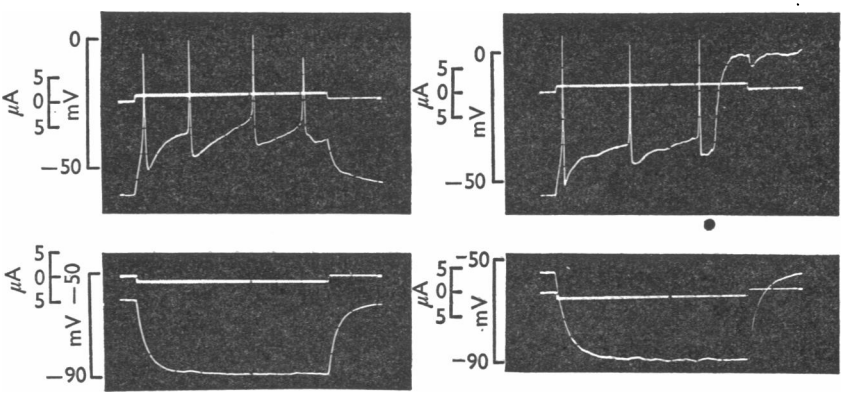

Fig. 5. Responses recorded from two cells of an 'infinite cable' preparation of taenia when 1·5 s rectangular current pulses were applied in isotonic physiological salt solution. The sucrose chambers were about 1 mm apart and one sucrose stream was not switched on. At ● the electrode was dislodged by the contraction.

current electrode exceeded one space constant, a significant decline in potential was observed as recorded by two electrodes (Fig. 3*f–h*, top lines). These experiments suggest that, while the passive electrical properties of a smooth muscle strip can normally be described by equations for an infinite cable (Tomita, 1970) they can, under appropriate conditions, also be described by equations for a short cable.

When spikes were elicited by a rectangular depolarizing current pulse significant potential differences were recorded between the two electrodes even at cable lengths $< \frac{1}{2}\lambda$ (Fig. 4*a*, *b*). The spike duration at half maximal size was about 20 msec. Assuming the peak of the spike is roughly equivalent to a 50 Hz sine wave, the capacitative reactance, $Z\,(= 1/(2\pi fc))$ of the membrane would be about 3 kΩ.cm², if a capacitance of 1 μF.cm^{-2} is assumed. A d.c. space constant of 1·0 mm gives a membrane resistance of about 30 kΩ.cm² if cable core specific resistivity is 300 Ω.cm (Tomita, 1969,

1970). Thus during the spike the impedance of the membrane is reduced to a tenth, and thus λ to approximately a third, of its d.c. value. The decline in potential is greatly increased (Fig. 4*c*, line a.c.) and the calculated potential difference between the two micro-electrodes expected in this experiment would be 17 mV for a 50 mV polarization applied at the current passing electrode, compared with a 3 mV difference during d.c. polarization. The actual difference observed was 14 mV for a 50 mV spike (Fig. 4*b*). These results suggested that, to obtain reasonably uniform potential during inward current flow in this muscle in hypertonic solution, a short cable length of 0·15 mm or less is needed.

Double-sucrose gap experiments

Effects of node width on electrophysiological properties of muscle

Constant currents. To obtain short cable preparations of smooth muscle strips down to 0·1 mm in length, a double-sucrose gap apparatus was used. Taenia strips showed normal electrophysiological activity in isotonic PSS providing the node width was 1 mm or more. Such strips were often spontaneously active after a period in the apparatus. The membrane potential was 55–60 mV and inward current elicited the usual electrotonic potential with a time to 63 % steady-state size of 70–110 msec. Depolarizing current elicited repetitive spiking, some of the spikes showing overshoot (Fig. 5).

When node was reduced to 0·3–0·5 mm, spontaneous activity was seldom seen. Depolarizing current usually produced only a single spike followed by a hump in the electrotonic potential (Fig. 6*a–e*). It can be seen that the threshold for spiking was rather high. Further reduction of node width, to 0·1–0·3 mm, produced a further increase in threshold and the regenerative spike seen in wider nodes was often converted to a graded phenomenon (Fig. 7*a–e*). Thus, reducing node width had several effects on electrophysiological activity, namely abolition of spontaneous activity, elevation of spike threshold, and conversion of all-or-none to graded spike.

Voltage clamp. When responses to rectangular current pulses (such as are shown in Figs. 6 and 7) had been obtained following a penetration, then attempts were made to voltage-clamp the membrane potential at its resting level and apply rectangular command potentials under voltage-clamp. Since these experiments were done in isotonic physiological salt solution, the contraction of the muscle sooner or later dislodged the micro-electrode. It was found easier to remain within a cell in narrow nodes (0·1–0·3 mm) than in wider nodes ($>$ 0·3 mm) probably because the total amount of movement possible during contraction was less in the former. Fig. 6*f* shows the currents flowing in response to three rectangular depolarizing

command potentials, applied while recording from the same cell as that from which records *a–e* were obtained, after clamping at the resting membrane potential. The three records obtained were stored on the oscilloscope and then photographed.

The smallest depolarization (by about 11 mV) elicited a roughly constant outward current after the initial capacitative surge. Stronger depolarization elicited a net inward current which reached a peak after about 45 ms (23 mV depolarization). The peak of this current occurred earlier in time

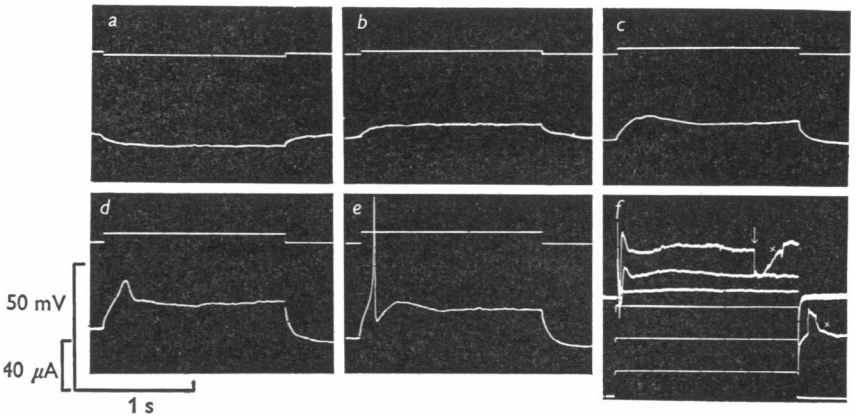

Fig. 6. Responses recorded from a node 0·5 mm wide using taenia smooth muscle in isotonic physiological salt solution. *a–e*, the responses to rectangular current pulses. After clamping at the resting potential the records shown in *f* were obtained. This shows three responses (superimposed using a storage oscilloscope) to rectangular depolarizing command potentials of increasing size (lower three lines). The upper three lines are the resulting currents which flowed. At ↓ the electrode was partially dislodged and the record which follows (X) should be disregarded. Notice that in this and in subsequent records the actual potential recorded by micro-electrode under voltage-clamp is shown and this attained its new value within a few milliseconds after potential stepping.

(at about 30 ms) with stronger depolarization (by about 36 mV). Following this, current became outward and reached an early peak, declined, and reached a second more gradual peak at about 500–700 ms. The form of these current records is very similar to those described by Vassort (1974) using extracellular potential recording. However, the results described earlier in this paper on short cable folded preparations make it very unlikely that all parts of the preparation at a node width of 0·5 mm (and using current injection across only one sucrose gap) were held at the potential recorded by the micro-electrode and displayed in Fig. 6*f*.

A greater possibility of voltage homogeneity exists in narrow nodes

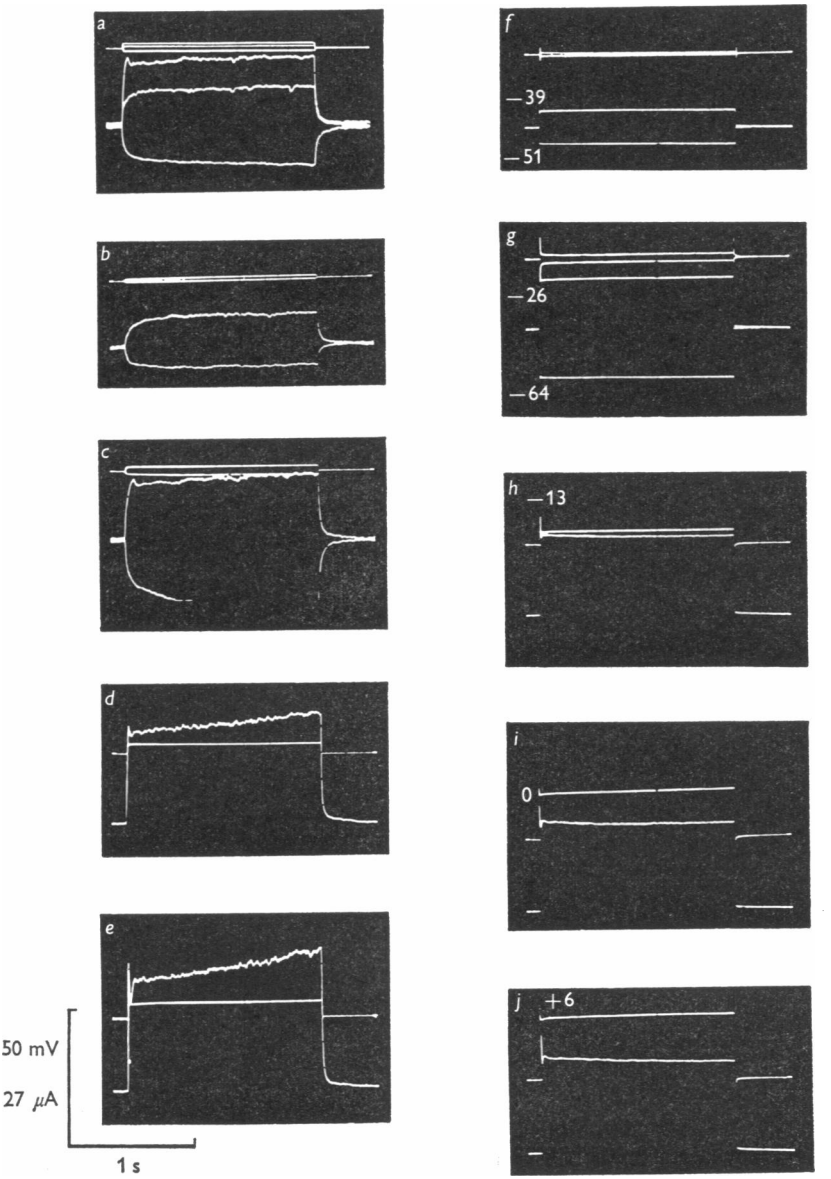

Fig. 7. Records from a node 0·15 mm wide of taenia in isotonic physiological salt solution. *a–e* are the responses to rectangular current pulses. Notice that the 'spike' is graded. Records *f–j* were obtained from the same cell, after clamping at the resting potential (−45 mV). They show the currents flowing into the node in response to rectangular command potentials. The numbers are the potential (mV) existing during the clamp pulse.

(0·1–0·3 mm). Fig. 7*f–j* illustrates responses obtained under voltage-clamp while in the same cell from which the records *a–e* were obtained. The membrane was clamped at the resting potential (−45 mV). Rectangular command potential steps under voltage-clamp did not produce any net inward current. This is to be expected since regenerative spikes did not occur. Outward current reached an early peak as in wider nodes

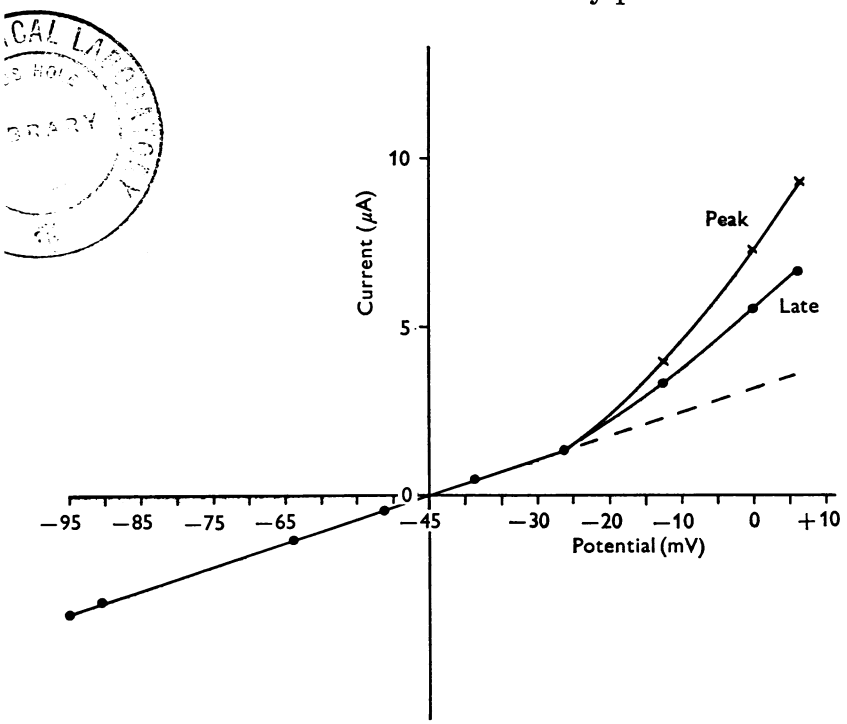

Fig. 8. Current–voltage relationship obtained from the records shown in Fig. 7 (node 0·15 mm wide). Outward current is upwards on the ordinate. Absolute potential is plotted on the abscissa. The two continuous lines show the current flowing at the peak of outward current, and 1·5 s after the beginning of a rectangular pulse (late).

and then declined steadily. A later peak in outward current was seen in one such preparation with strong depolarizing command steps. Since contraction occurs in isotonic solution, some features of the recorded current may be affected. Measured either at the time of maximum outward current (Fig. 8, labelled 'peak') or at the end of the pulse ('late') the current–voltage relationships of the membrane revealed outward rectification, but were linear in a hyperpolarizing direction.

These results show that as the node width was reduced in an attempt to achieve potential homogeneity, the electrophysiological properties of the

muscle were changed. It seems likely that most of the effects arose due to the fact that a sucrose gap is an imperfect approximation to the 'short cable' and that as the node width (x) becomes small compared to the d.c. space constant of the tissue, λ, then leakage current becomes relatively important. According to McGuigan & Tsein (McGuigan, 1974) when $X = x/\lambda = 1{\cdot}0$ then the effects of leakage current are minimized. In these experiments, where $X < 0{\cdot}1$, the leakage current would be expected to be relatively large.

Two micro-electrode experiments

Constancy of nodal potential. Since it was clear that the electrophysiological properties of the muscle were changed as $X \rightarrow 0{\cdot}1$, it was decided to use slightly wider nodes (0·4–0·5 mm) and to pass current into the node across both sucrose gaps (both ends of the muscle depolarized in high K) instead of across only one sucrose gap as in the experiments described so far. This ought to double the node width at which a given percentage potential variation across the node occurs.

Additionally, the node was bathed in a hypertonic physiological salt solution in order to prevent or minimize artifacts due to contraction. This solution also allowed penetrations to be maintained for a much longer period of time but in taenia converted the regenerative spike usually seen at this node width into a graded spike.

The usual procedure in these experiments was to penetrate cells at two different (usually well separated) points in the node. Responses to rectangular current pulses were first obtained (Fig. 9*a–b*). Following this, if the form of these responses was similar, the potential recorded by one micro-electrode was clamped at the resting membrane potential and rectangular voltage-clamp command pulses applied.

Although the clamped potential could be efficiently stepped to a new potential within a few milliseconds, the potential recorded by the second, independent, electrode always lagged. The difference between the potentials recorded by the two micro-electrodes was serious for 50–100 ms when the potential stepping did not activate inward current (Fig. 9*d–h*). When inward current was activated a potential difference persisted for longer periods and strong depolarization produced a spike-like response recorded at the monitoring electrode (Fig. 9*i–k*). However, in most experiments, after about 200 ms the potentials recorded at two points within a node were similar.

In some experiments the current–voltage relationships were measured by plotting the potential at the end of a 1·5 sec pulse at each electrode against current. Fig. 11 shows that these relationships were very similar for moderate depolarization but some deviation occurred in this particular

experiment (part of which is illustrated in Fig. 9) either with hyperpolarization or strong depolarization. Presumably this situation may arise due to the imperfect cable properties of the smooth muscle strip.

A total of six experiments were done in which it was possible to monitor

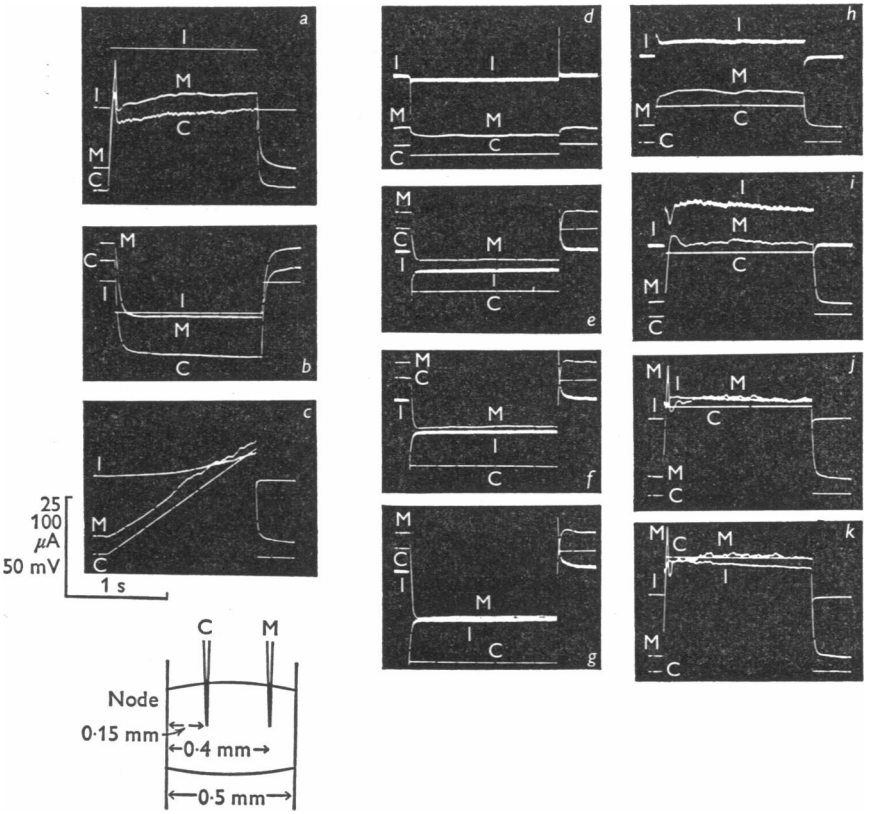

Fig. 9. Test for uniformity of nodal potential during rectangular voltage clamp commands. The membrane potential was recorded at two points in a taenia node at the positions indicated in the diagram. Node width was 0·5 mm and current was passed across both sucrose gaps. Responses to rectangular current pulses were first obtained (*a–b*). The potential recorded by electrode C was then clamped at the resting membrane potential and subjected to hyperpolarizing (*d–g*) or depolarizing (*h–k*) rectangular command potentials. Notice that when the inward current channel is not activated, potential at the monitoring electrode (M) lags the clamped potential (C) and differences exist for 50–100 ms following a step change in potential. When inward current channels are activated then differences last longer and even spiking may occur (*j–k*). Where ramp commands are used under voltage-clamp (*c*) then potential varied little between the two points of recording. I, the current record. Sucrose hypertonic PSS. Calibration 25 μA in *a–i* and 100 μA in *j–k*.

the potential at some other point in the node while voltage-clamping the potential recorded by a second electrode. In some experiments, potential variations were greater than observed in Fig. 9, although the current trace gave no indication of this. Fig. 10*a–g* shows an experiment in which the responses to rectangular current pulses recorded at two points were very similar, although not identical. However, when the potential recorded by

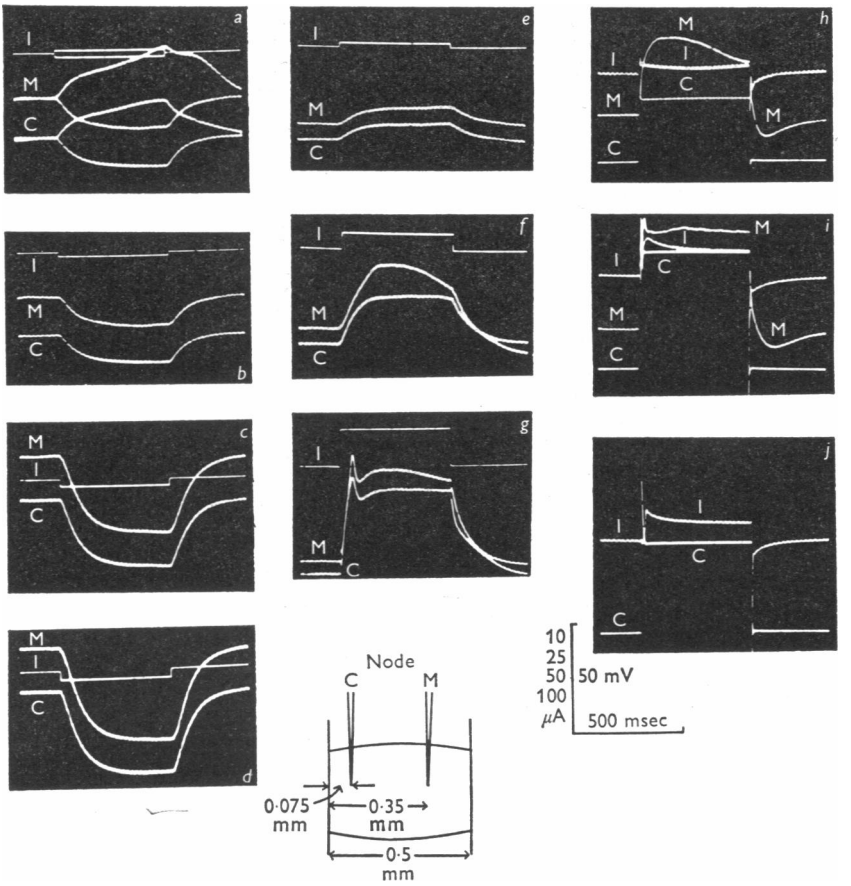

Fig. 10. Nodal potential variation under voltage clamp. Recording was made at two points within a 0·5 mm node of taenia at the positions shown in the diagram. The responses to rectangular current pulses were similar (*a–g*), but under voltage clamp, the potential recorded by electrode M differed considerably from clamped potential, and this difference persisted for up to 500 ms. Upon repolarization the potential recorded by electrode M was hyperpolarized for a similar period. The monitoring electrode was dislodged before record *j* was obtained. Sucrose hypertonic physiological salt solution. Calibrations 10 μA (*e*, *f*), 25 μA (*a–d*, *g*), 50 μA (*h*), 100 μA (*i–j*).

electrode *C* was clamped at the resting membrane potential and then subjected to rectangular command pulses, the potential recorded independently by electrode *M* showed considerable variation from the hoped-for rectangular form (Fig. 10*h–j*). Variation persisted for up to 500 ms upon a depolarizing step and the early peak in outward current was associated with a varying potential at electrode *M*. Upon repolarization to the resting membrane potential, potential recorded at electrode *M* was considerably hyperpolarized while potential recorded at electrode *C* was clamped at the resting (= holding) potential. Such experiments indicate that under the conditions of these experiments, the assumption of constant nodal potential following a step depolarization or a step repolarization would not be justified except possibly at times greater than 100–200 ms or more following such step changes. The assumption of constant nodal potentials, made by others using voltage-clamp of extracellular potential of smooth muscle strip (e.g. Anderson, 1969; Kumamoto & Horn, 1970; Anderson *et al.* 1971; Kao, 1971; Mironneau & Lenfant, 1972; Mironneau, 1973; Kao & McCullough, 1975) would, therefore, seem to require re-examination, particularly since Tarr & Trank (1974) and McGuigan (1974) have obtained very similar results to these described here, on multi-fibre preparations of cardiac muscle.

Current–voltage relationship. As a check on the voltage-clamp technique a comparison was made between the current–voltage relationship obtained using rectangular current pulses and rectangular voltage-clamp command potentials, while the electrodes were within the same two cells. Current was measured at the end of a 1·5 s pulse in each case. The two methods gave good agreement although current pulses were not tested over so wide a range (Fig. 11).

Also tested were ramp command potentials under voltage-clamp, and ramp currents. It was found that at a rate of ramp rise (voltage-clamp) of 20–30 mV/s, then the potentials recorded at two different points showed insignificant differences (Fig. 9*c*). The current–voltage relationship obtained was virtually identical to that found using rectangular pulses in a depolarizing direction (Fig. 11). However, in a hyperpolarizing direction this relationship was similar for only moderate hyperpolarizations (50 mV in Fig. 11) and rectangular voltage-clamp pulses (○) activated more current than ramps at more negative potentials. Hyperpolarizing ramp currents also produced a similar current–voltage relationship for moderate hyperpolarizations (their rate of rise was sufficiently slow for capacitive current to exert a negligible influence).

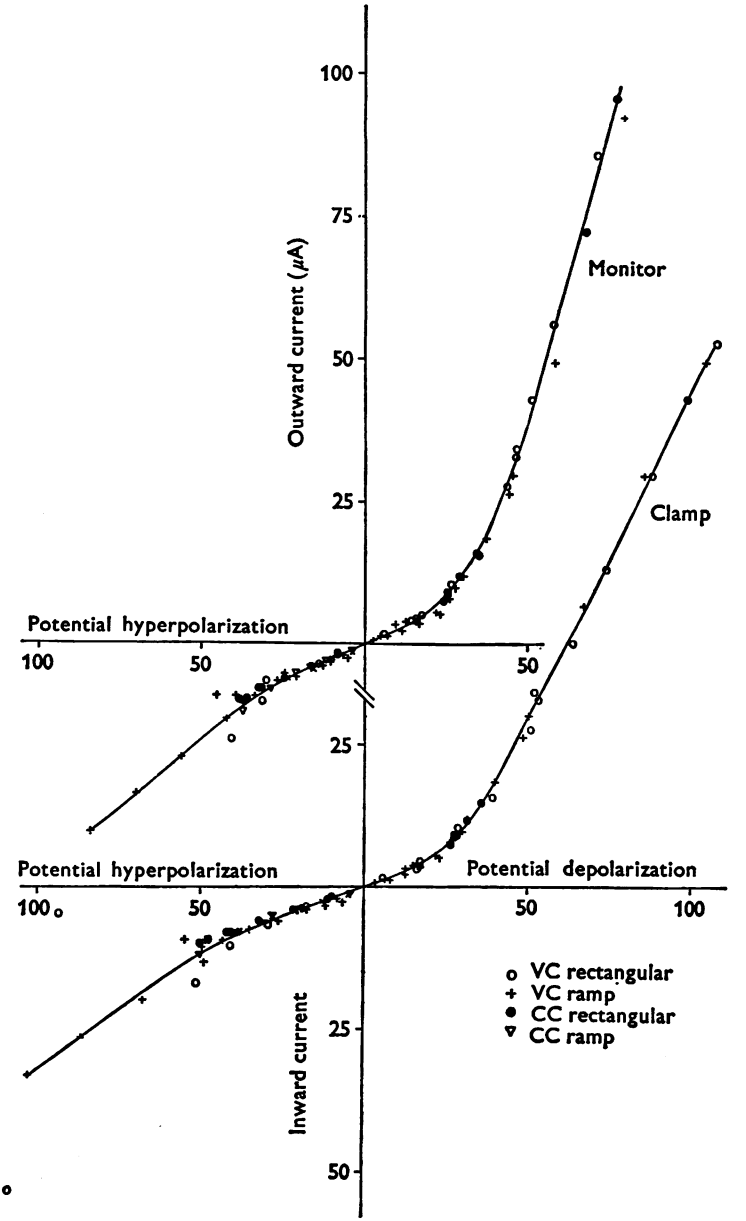

Fig. 11. Comparison of the current–voltage relationships obtained by two electrodes in the experiment of Fig. 9. The current–voltage relation was obtained using rectangular voltage-clamp command potentials (○), ramp voltage clamp commands (+), rectangular current pulses (●) and ramp currents (▽). This relationship in the case of rectangular pulses (current or potential) was measured at the end of a 1·5 s pulse. The rate of ramp rise was about 30 mV/s. The potential shown is the deviation from the resting potential and has been corrected for the potential across the series resistance (r_s).

Effects of stimulating the acetylcholine receptor on the current–voltage relationship

An important fact to emerge from the experiments described so far was that a fairly slowly rising ramp (25 mV/s) under voltage clamp could provide an estimate of the current–voltage relationship over a wide range and that at any instant during the ramp the potential across the node did not vary appreciably.

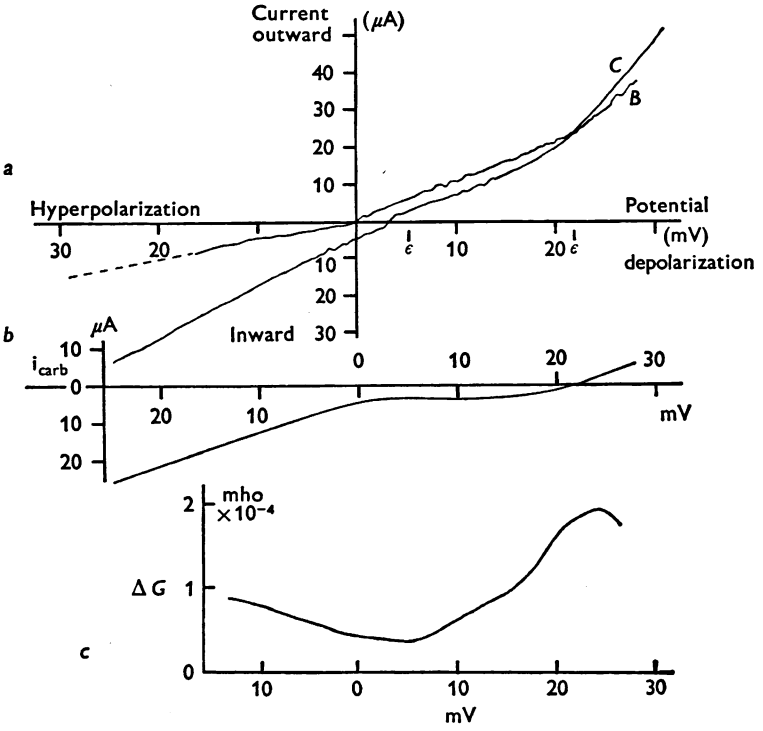

Fig. 12. Effect of carbachol on the current–voltage relationship of taenia smooth muscle in NaCl hypertonic solution. *a*, the current–voltage relationship before (*B*) and in the presence of carbachol (*C*, 10^{-6} g/ml.) were traced from current records obtained by applying ramps under voltage clamp, and fitted to appropriate axes. The abscissa shows the displacement of the membrane potential from the resting potential existing before carbachol application. Where the current–voltage relationships intersect there is an equilibrium potential, ϵ. During voltage clamp the holding potential was the existing resting potential, i.e. in the presence of carbachol it was 3 mV more positive. *b*, the relationship between the additional current flowing in the presence of carbachol (i_{carb}) and potential. The curve was fitted by eye to specimen points obtained by subtracting line *B* from line *C*. *c*, shows the variation in the conductance, ΔG ($=i_{carb}/(V-\epsilon)$) with potential. Notice the increase in ΔG upon depolarization.

In hypertonic physiological salt solution, the oscillations of the potential (slow waves) produced by stimulating the acetylcholine receptor have a duration of 1–5 s (Bolton, 1971, 1972*a*). Rates of ramp rise of about 25 mV/s should therefore be suitable for detecting any effects of stimulating the acetylcholine receptor on the current–voltage relationship. Experiments were done in which this relation was determined by applying

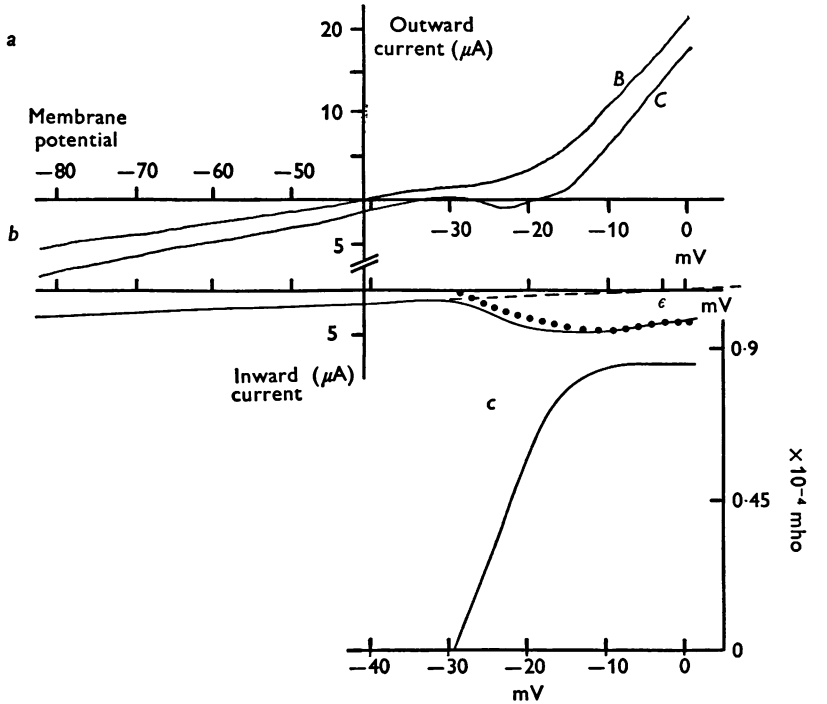

Fig. 13. Effect of carbachol on the current–voltage relationship of ileal smooth muscle in sucrose hypertonic solution. Similar experiment to that on taenia shown in Fig. 12 but the holding potential in the presence of carbachol was the same as before its application and was equal to the resting potential. Here the absolute potential is plotted. *a*, the current–voltage relation before (*B*) and in the presence of carbachol (*C*, 10^{-6} g/ml.). Notice that in ileum there is no equilibrium potential in the voltage range which could be studied. *b* the behaviour of i_{carb} with potential (continuous line) obtained as in Fig. 12. The dotted line was obtained by subtracting an extrapolated current (dashed line) from i_{carb}. *c*, this shows the behaviour of the conductance through which the current (shown as a dotted line in *b*) flows. For further details see text.

two ramps (one hyperpolarizing, one depolarizing) before, and in the presence of carbachol ($1–5 \times 10^{-6}$ g/ml.) (at the peak of its depolarizing effect) while recording from the same cell. Sometimes it was possible to

obtain a third current–voltage relationship while in the same cell 5–10 min after returning to carbachol-free solution.

Initial experiments were done on strips of taenia since these had been most studied. Carbachol had the effect of producing an additional inward current at all potentials negative to a point, an equilibrium potential (ϵ (volts)). At potentials positive to ϵ, the additional current flowing in the presence of carbachol was outward (Fig. 12). In some experiments no equilibrium potential was obtained because it was found that strong depolarization of the membrane damaged the preparation presumably by increasing the ratio r_i/r_e (see also New & Trautwein, 1972).

The additional current flowing in the presence of carbachol, i_{carb} (Amps) was not linearly related to the displacement of potential from ϵ (Fig. 12*b*). (In this experiment the membrane potential was held at a more depolarized potential in the presence of carbachol, but the non-linearity is in the wrong direction to be explained by inactivation of inward current channels.) This finding was confirmed in several experiments in which the leakage current across the gap was relatively low. When leakage current was high, the non-linearity was insignificant, as observed previously (Bolton, 1974*a*).

Since it is in longitudinal ileal muscle that activation of the acetylcholine receptor produces the striking oscillations of potential, similar experiments were done on this muscle. The current–voltage relationship in the absence of carbachol was similar to taenia, except that conductance often increased with increasing hyperpolarization. However, in the presence of carbachol a region of net inward current was observed (Fig. 13*a*). (The holding potential was the same before, during and following carbachol application.)

The increase in i_{carb} in the region of potential up to some 40 mV positive to the resting membrane potential, was relatively greater than in experiments on taenia, and no equilibrium potential was observed in the potential range studied (Fig. 13*b*). The net inward current in the presence of carbachol observed in ileum, but not in taenia, is significant, since regenerative slow waves upon application of carbachol were observed in these experiments only in ileal muscle.

DISCUSSION

An interesting result which emerges from this voltage-clamp study is that, when the acetylcholine receptor of ileal muscle is activated, a net inward current appeared to flow across the membrane over a certain range of potential where before there was a net outward current. This inward current has a threshold some 10 mV positive to the resting membrane potential and is presumably responsible for the regenerative, slow

oscillations of the membrane potential (slow waves) which occur in this muscle if depolarization produced by stimulating the acetylcholine receptor is sufficient (Bolton, 1971, 1972*a*).

It is a moot point to what extent voltage variations within the node affect the interpretation of voltage-clamp results (Johnson & Lieberman, 1971). It was possible in several experiments, using an independent micro-electrode, to record from another region of the node during slow voltage-clamp ramps and to show that the potential there was not greatly different from the voltage-clamped one (step changes initially produced large variations in potential across the node which lasted for up to several hundred milliseconds, as others have found in cardiac muscle (McGuigan, 1974; Tarr & Trank, 1974)). Nevertheless there may be regions possibly inaccessible to micro-electrode penetration, where the potential is considerably different from the voltage-clamped one. It seems unlikely that these are sufficient to affect the *direction* of the recorded currents, although their *size* may be altered. The increase in membrane conductance in the presence of carbachol at potentials negative to the resting potential was very modest, and it seems most unlikely that an additional *voltage-independent* conductance alone could bring about sufficient undetected nodal voltage variation to explain the observed change in the current–voltage relationship. A likely and simple explanation for these results and others (Bolton, 1971, 1972*a*) is that there is an opening of a population of ion channels whose conductance increases sharply with depolarization beyond about -35 mV.

While regenerative slow waves occurred in ileum when carbachol was applied in these experiments, they did not occur in taenia. Although i_{carb} increased slightly with depolarization in taenia, it is significant that its size was insufficient to produce a region of inward current in the current–voltage relationship.

The interpretation of these results in terms of the properties of ion channels opened in the membrane during stimulation of the muscarinic receptor by carbachol must be somewhat speculative. One interpretation might be that there is a single set of ion channels with fixed ionic selectivity such that they have an equilibrium potential, ϵ (V). Their conductance might then be defined as $\Delta G = i_{carb}/(V-\epsilon)$ where V (V) is the membrane potential at some instant. The way ΔG so defined varies with potential in taenia is shown in Fig. 12*c*. An alternative scheme would be to suppose that the ionic selectivity of these channels, e.g. for Na and K, varied with potential.

A more attractive hypothesis is suggested by the results on ileum (Fig. 13). Here no equilibrium potential was encountered in the potential range that could be studied. However, the linear relationship of i_{carb} in a

hyperpolarizing direction might indicate a population of ion channels, operated by the activated acetylcholine receptor, whose conductance is independent of potential and which have an equilibrium potential (obtained by extrapolation) around -4 mV. Most of the additional current flowing in the presence of carbachol in the range between -32 mV and zero potential might then be through a second population of channels with a different ionic selectivity and equilibrium potential. We may tentatively call this ϵ_{Na}, since this current is presumably sensitive to a reduction in the external sodium concentration (Bolton, 1971, 1972*a*). Linear extrapolation of the current–voltage relationships in Fig. 13*a* (and subtracting the part of i_{carb} which is linearly related to potential) give ϵ_{Na} a value of $+55$ mV. Using this value of ϵ_{Na} and defining the conductance of these channels as $\Delta G_{Na} = i_{carb,\,Na}/(V-\epsilon_{Na})$ the activation curve for these channels may be obtained and is shown in Fig. 13*c*. This ignores the possibility that at the rate of ramp rise used in this experiment $i_{carb,\,Na}$ may not be fully activated.

The postulated presence of two populations of ion channels operated by activated acetylcholine receptors in ileum (and possibly in taenia) should be compared with the pharmacological evidence for the existence of two populations of acetylcholine receptors in this muscle (Burgen & Spero, 1968, 1970; Burgen & Hiley, 1974).

It is necessary also to reconcile the present results with those of previous studies (Bolton, 1972*b*) in which it was found that the maximum depolarization produced by acetylcholine (or other strong agonists at the muscarinic receptor) in ileum was about -8 mV. This value was suggested as being limiting upon depolarization because it was close to the equilibrium potential of the channels responsible for depolarization. These results on ileum might suggest an equilibrium potential which ought to be much nearer to the Na equilibrium potential. However, recent experiments (unpublished) indicate that much of i_{carb} activated over the potential range -40 to -10 mV can be inactivated by a few seconds depolarization to around -10 mV. If this is the case, then the equilibrium potential would be determined by those channels remaining open when the potential had moved to around -10 mV under the influence of a fairly high concentration of acetylcholine or carbachol, and no serious discrepancy would exist. Additionally, it is clear that the larger concentrations of cholinergic agonists which are needed to depolarize to this level, will also disturb the ionic gradients across the membrane of these small cells, probably shifting the equilibrium potential (Bolton, 1973).

Many of the features of the action of acetylcholine on ileal muscle are seen when a variety of other stimulant substances act on the membranes of other smooth muscles. Noradrenaline has an action on the membrane potential of anococcygeus muscle which, superficially at least, closely

resembles the action of acetylcholine on ileum in hypertonic solution (Gillespie, Creed & Muir, 1973). Without producing appreciable changes in the level of the resting potential, pentagastrin acts to increase slow wave frequency in canine antrum muscle (Szurszewski, 1975), noradrenaline and histamine prolong the plateau of the guinea-pig ureter (Shuba, 1975; Shuba, Taranenko, Kochemasova, Gurkovskaya, & Klevetz, 1975) and a number of stimulants (prostaglandin, oxytocin, acetylcholine) in low concentrations increase the frequency of occurrence and duration of spike bursts in uterine muscle (Kuriyama, Osa & Suzuki, 1975). It is conceivable that an effect on a voltage-sensitive inward channel is a mechanism of general importance, underlying the actions of a variety of stimulant substances on the membranes of rhythmically contracting visceral smooth muscles.

The action of acetylcholine on ileum resembles its action on cortical neurones (Krnjević, Pumain & Renaud, 1971; Krnjević, 1974) and on spinal motoneurones (Zieglgänsberger & Reiter, 1974) where it produces depolarization and a delay in repolarization following the spike. This can result in a plateau-like component appearing. Krnjević (1974) has suggested a unifying hypothesis, applicable to all these cells, of a reduction in outward K current which has the dual effect of delaying repolarization following a spike and reducing the potential in the interspike interval. Certainly the actions of acetylcholine on ileum and on neurones are superficially very similar, but acetylcholine produces a large *increase* in conductance in smooth muscle (Bolton, 1972*b*; Magaribuchi, Ito & Kuriyama, 1973; Osa & Taga, 1973; Bülbring & Szurszewski, 1974) while it *reduces* the conductance of neuronal membrane (*loc. cit.*). The *increase* in conductance in smooth muscle is detectable when point injection of current is made by micro-electrode (Hidaka & Kuriyama, 1969; Purves, 1974). It has been pointed out to me by Purves that this latter observation would seem to exclude any primary action of acetylcholine to uncouple smooth muscle cells as suggested by Krnjević (1974), since an increase in coupling resistance would be expected to *increase*, rather than *decrease*, the electrotonic potential elicited by intracellular injection of current. While the suggestion of a generalized reduction of smooth-muscle membrane conductance by acetylcholine is clearly untenable, the idea of a reduction by acetylcholine of the outward potassium current responsible for repolarizing following a spike, is worthy of consideration. The experiments described in this paper alone do not exclude a *reduction* in outward current rather than an *increase* in inward current, being responsible for the effects of carbachol on the current–voltage relationship. However since a reduction in $[Na^+]_o$ abolished regenerative slow waves elicited by carbachol (Bolton, 1971, 1972*a*) while variations in $[K^+]_o$ had little effect

(Bolton, 1972*a*) it would seem that, at least in this muscle, activation of the acetylcholine receptor increases an inward Na current rather than decreases an outward K current.

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