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THE ROLE OF ELECTROGENIC SODIUM PUMPING IN THE RESPONSE OF SMOOTH MUSCLE TO ACETYLCHOLINE

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SUMMARY

1. Intracellular recording of membrane potential was made from the separated longitudinal muscle of the guinea-pig terminal ileum in physiological salt solution.

2. When acetylcholine was washed from the tissue following a brief application the membrane repolarized and then hyperpolarized ('after-hyperpolarization') beyond the level existing before the application of acetylcholine.

3. No after-hyperpolarization was observed following acetylcholine in potassium-free solution, in sodium-deficient (17 mM) solution, or in the presence of ouabain ($1{\cdot}7 \times 10^{-6}$ M). Repolarization under these conditions was delayed, especially after the membrane potential reached -20 to -30 mV, and was generally incomplete.

4. The after-hyperpolarization was significantly ($P < 0{\cdot}01$) greater when acetylcholine was applied in chloride-deficient (13 mM) solution.

5. It was incidentally observed that the membrane potential in the presence of acetylcholine was more positive in potassium-free solution (significance $P < 0{\cdot}025$), unchanged in chloride-deficient solution ($P > 0{\cdot}4$), and much more negative in sodium-deficient (17 mM) solution ($P \ll 0{\cdot}001$), confirming previous results using carbachol.

6. When a 2 min application of $1{\cdot}4 \times 10^{-6}$ M carbachol was made, the membrane potential 15–20 sec after beginning its application was not affected by ouabain (10^{-5} M), but showed a significantly ($P < 0{\cdot}005$) greater positive shift subsequently, so that the potential after 120 sec in carbachol was significantly ($P < 0{\cdot}025$) more positive in the presence of ouabain. After 45 sec in $5{\cdot}5 \times 10^{-5}$ M carbachol the membrane potential was also significantly ($P < 0{\cdot}005$) more positive in the presence of ouabain (10^{-5} M).

7. Calculations based on hypotheses concerning the movements of sodium and potassium showed that the positive shift of the membrane

potential in the presence of carbachol when sodium pumping was arrested, could be quantitatively explained by a decline in the sodium and potassium gradients across the membrane. It appeared that the electrogenic fraction of the sodium pumped was small in the presence of carbachol.

8. It was concluded that the application of acetylcholine or carbachol ($> 10^{-6}$ M) to this smooth muscle disturbs the sodium and potassium gradients across the membrane. These disturbances are in a direction which stimulates electrogenic sodium pumping. Some limitation of depolarization results, and the increased electrogenic extrusion of sodium is responsible for the after-hyperpolarization which follows the application of acetylcholine.

INTRODUCTION

It has been noticed that when acetylcholine is washed from smooth muscle after a brief application, the membrane repolarizes and then hyperpolarizes beyond the level of potential existing before its application (Burnstock, 1958; Bülbring & Burnstock, 1960; Bülbring & Kuriyama, 1963; Bolton, 1971*a*). This period of increased negativity of the membrane might be called an after-hyperpolarization.

The depolarizing action of acetylcholine on smooth muscle probably results from an increase in permeability mainly to sodium and potassium ions (Born & Bülbring, 1956; Lembeck & Strobach, 1956; Durbin & Jenkinson, 1961; Bülbring & Kuriyama, 1963; Bolton, 1972, 1973*a*; and others). As the smooth muscle cell is small and surrounded by narrow clefts of extracellular space (e.g. Gabella, 1973), it seems feasible that substances such as acetylcholine which increase the membrane conductance (Hidaka & Kuriyama, 1969; Bolton, 1972, 1973*a*) may produce a fall in the sodium and potassium gradients across the cell membrane, increasing both the internal sodium and the external potassium concentrations. Such changes would be expected to stimulate the activity of the sodium pump (Casteels, Droogmans & Hendrickx, 1971; Bolton, 1973*c*) resulting in an increased contribution of the electrogenic pump to the membrane potential. This might be responsible for the observed after-hyperpolarization and these experiments were done to test this. Some of them have been briefly communicated to the Physiological Society (Bolton, 1971*b*) and at a Discussion Meeting of the Royal Society (Bolton, 1973*b*).

METHODS

The methods used have been described (Bolton, 1972, 1973*c*). All results were obtained by recording intracellularly from small pieces of separated longitudinal muscle of guinea-pig terminal ileum kept in Krebs solution at 35° C for periods in excess of 1 hr before beginning experiments.

When it was required to measure the membrane potential this was done by withdrawing the electrode shortly after the point in the response at which it was desired to make measurement.

Potassium-free solution was made by omitting potassium chloride from the Krebs without compensating for the fall in tonicity. Sodium-deficient (17 mM) solution was made by replacing sodium chloride with Tris chloride at pH 7·4. To obtain chloride-deficient solution (13 mM), sodium chloride was replaced by sodium benzene-sulphonate.

RESULTS

Effects of agents which alter sodium pump activity on acetylcholine after-hyperpolarization

A large after-hyperpolarization following a 10 sec application of acetylcholine ($5{\cdot}5 \times 10^{-5}$ M) is shown in Fig. 1. The subjective impression was gained that after-hyperpolarization was greater in tissues when the flow of solution was high, presumably because acetylcholine was more quickly

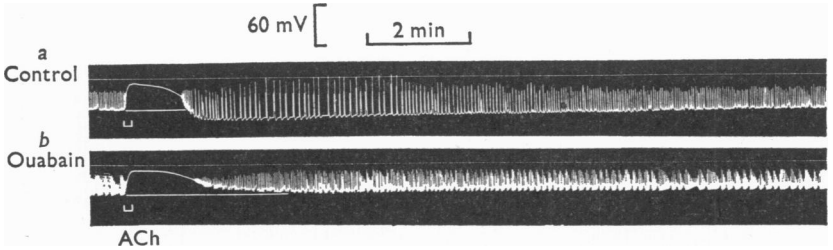

Fig. 1. Action of ouabain on the after-hyperpolarization which follows the application of acetylcholine (ACh). Acetylcholine ($5{\cdot}5 \times 10^{-5}$ M) was applied for the 10 sec period indicated by the bracket to the same tissue before (*a*) and in the presence of $1{\cdot}7 \times 10^{-6}$ M ouabain (*b*). The upper line in this and subsequent records is approximately at zero potential. The shorter horizontal lines indicate the level of membrane potential before the application of acetylcholine. In the presence of ouabain no after-hyperpolarization occurs and repolarization is delayed, especially after the membrane potential reaches -20 to -30 mV.

washed from the tissue and the recovery of the membrane resistance more rapid. For this reason care was taken to ensure that the flow rates of the various solutions used was the same. Bülbring & Kuriyama (1963) remarked that in taenia after-hyperpolarization was greater the lower the membrane potential before the application of acetylcholine, and this also seemed to be true in longitudinal ileal muscle (Fig. 1).

Ouabain

If the after-hyperpolarization is produced by sodium pump activity then ouabain ought to reduce or abolish it. Ouabain itself depolarizes smooth muscle (Casteels, 1966; Matthews & Sutter, 1967; Tomita & Yamamoto,

1971; Bolton, 1973*c*) and in the presence of ouabain, smooth muscle continuously gains sodium and loses potassium (Casteels, 1966; Matthews & Sutter, 1967).

In a series of experiments acetylcholine ($5{\cdot}5 \times 10^{-5}$ M) was applied simultaneously with ouabain ($1{\cdot}7 \times 10^{-6}$ M), or after periods up to 15 min in ouabain-containing solution. After a brief application of acetylcholine (10–20 sec) no after-hyperpolarization occurred in the presence of ouabain ($1{\cdot}7 \times 10^{-6}$ M), although after-hyperpolarization averaged $6{\cdot}5 \pm 1{\cdot}2$ mV in twenty-nine responses on twelve preparations before ouabain. In the presence of ouabain the most negative potential attained in the 5 min period after acetylcholine on average fell $3{\cdot}3 \pm 1{\cdot}0$ mV short of the resting potential before its application, i.e. the tissues generally failed to repolarize completely (Fig. 1). This may reflect the progressive depolarization which occurs in ouabain. After reverting to ouabain-free solution, the after-hyperpolarization following acetylcholine reappeared, averaging $3{\cdot}7 \pm 0{\cdot}9$ mV (Fig. 4).

The depolarization produced by these brief applications of acetylcholine was slightly affected by the presence of ouabain ($1{\cdot}7 \times 10^{-6}$ M). In eleven preparations the most positive potential in the presence of acetylcholine ($5{\cdot}5 \times 10^{-5}$ M) was $-8{\cdot}4 \pm 0{\cdot}8$ mV before ouabain, $-6{\cdot}8 \pm 1{\cdot}2$ mV in the presence of ouabain, and $-8{\cdot}2 \pm 1{\cdot}1$ mV in ten of these preparations after washing out ouabain. Even brief exposure to acetylcholine reveals a tendency for the membrane potential to be less negative in the presence of acetylcholine when sodium pumping is reduced. Later, this phenomenon will be described in more detail.

Potassium-free solution

The activity of the sodium pump is reduced when potassium is absent from the bathing solution. Tissues continuously lose potassium (Axelsson & Holmberg, 1971; Casteels *et al.* 1971). As potassium leaves the cells some must be present outside the membrane but the concentration at this point is probably very low as detectable stimulation of the pump occurred with concentrations of potassium in the bathing solution of 0·1 mM or less (Bolton, 1973*c*). When acetylcholine is applied, the rate at which potassium leaves the cells must increase and hence also the concentration immediately outside the membrane. This will stimulate sodium pump activity but probably only so long as the effects of acetylcholine on membrane permeability last.

In seven tissues after-hyperpolarization following acetylcholine averaged $7{\cdot}0 \pm 1{\cdot}9$ mV in normal solution, while in potassium-free solution there was no after-hyperpolarization, repolarization was delayed especially beyond about -30 mV, and the most negative membrane potential attained in the

5 min period after acetylcholine fell short of the resting potential before application by $5{\cdot}4 \pm 2{\cdot}0$ mV, i.e. repolarization was incomplete (Figs. 2 and 4). In these experiments, acetylcholine ($5{\cdot}5 \times 10^{-5}$ M, applied for 10–20 sec) was applied 1–5 min after changing to potassium-free solution, i.e. the maximum membrane potential was measured in the period extending up to 10 min in potassium-free solution. The failure of such tissues to repolarize completely probably reflects the fall in the gradients of sodium and potassium.

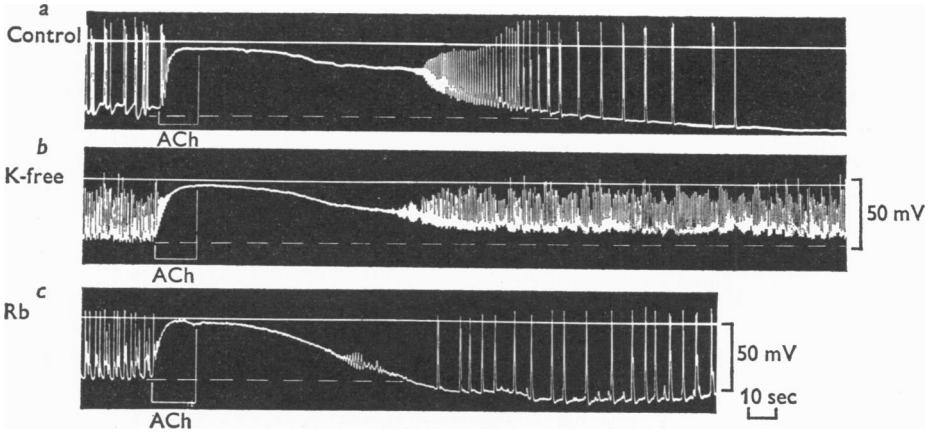

Fig. 2. *a–b*: action of potassium-free solution on acetylcholine after-hyperpolarization. Acetylcholine (ACh, $5{\cdot}5 \times 10^{-5}$ M) was applied for 15 sec as indicated by the bracket. After-hyperpolarization does not occur in potassium-free solution, and repolarization is delayed beyond about -30 mV. *c*: after-hyperpolarization following acetylcholine ($5{\cdot}5 \times 10^{-5}$ M) applied to a similar tissue in a solution where potassium had been replaced by rubidium (5·9 mM).

The dashed line indicates the level of membrane potential existing before the application of acetylcholine.

It was incidentally observed in these experiments that the membrane potential in the presence of acetylcholine was $-10{\cdot}3 \pm 1{\cdot}9$ mV in normal solution (5·9 mM potassium) compared with $-3{\cdot}1 \pm 1{\cdot}5$ mV in potassium-free solution. The difference is significant ($P < 0{\cdot}025$). A similar positive shift was seen in experiments using carbachol (Bolton, 1972, 1973*a*) and is in the opposite direction to the one expected if acetylcholine increases the potassium permeability. This discrepancy has been discussed (Bolton, 1973*a*).

Rubidium

As rubidium is known to be able to substitute for potassium in maintaining the activity of the sodium pump in smooth muscle (Paton, 1971; Taylor, Paton & Daniel, 1971; Tomita & Yamamoto, 1971) as in other tissues, three experiments were done in which acetylcholine was applied in potassium-free solution containing 5·9 mM rubidium. After-hyperpolarization occurred and averaged $7{\cdot}7 \pm 1{\cdot}7$ mV, similar to that observed in normal, potassium-containing solution (Figs. 2*c* and 4).

Sodium deficiency

Reducing the sodium concentration of the bathing solution from 137 to 17 mM would be expected to shift E_{Na} from about 50 mV to near zero (Casteels & Kuriyama, 1965, 1966; Casteels, 1966, 1969, 1971; Bülbring, Casteels & Kuriyama, 1968) and reduce the influx of sodium during the action of carbachol. In normal solution the after-hyperpolarization averaged $3{\cdot}8 \pm 1{\cdot}3$ mV ($n = 7$) while in sodium-deficient (17 mM) solution no after-hyperpolarization occurred and the maximum negative potential attained in the 5 min following acetylcholine fell short of the potential existing before its application by $1{\cdot}5 \pm 1{\cdot}1$ mV (Figs. 3 and 4). It was incidentally observed that the membrane potential in the presence of acetylcholine was $-9{\cdot}2 \pm 1{\cdot}5$ mV in normal solution and $-29{\cdot}3 \pm 0{\cdot}9$ mV in sodium-deficient solution. The difference is extremely significant ($P \ll 0{\cdot}001$).

The delay in repolarization was conspicuous (Fig. 3) and larger than might be expected from the calculated reduction in sodium influx in sodium-deficient solution. If the sodium conductance, ΔG_{Na}, opened by acetylcholine is independent of the external sodium concentration and given by $\Delta G_{Na} = i_{Na}/(V - E_{Na})$ (eqn. (2), p. 724) then the shifts in E_{Na} and V would be expected to reduce sodium influx by about half. The pronounced delay in repolarization and the complete absence of any after-hyperpolarization are therefore surprising (Fig. 3).

Chloride deficiency

On applying chloride-deficient solution the membrane of smooth muscle first depolarizes and then repolarizes or hyperpolarizes (Burnstock & Straub, 1958; Holman, 1958; Kuriyama, 1963). It was observed that in chloride-deficient (13 mM) solution the response to readmitting potassium to potassium-free solution was increased about $2\frac{1}{2}$ times, supporting the suggestion that the sodium pump operates electrogenically (Bolton, 1973*c*). Control responses to acetylcholine showed an average after-hyperpolarization of $3{\cdot}8 \pm 2{\cdot}0$ mV but in these same preparations after

changing to chloride-deficient solution (13 mM) the after-hyperpolarization was significantly ($P < 0{\cdot}01$) increased to $13{\cdot}8 \pm 3{\cdot}8$ mV ($n = 7$). Upon reverting to normal solution the after-hyperpolarization averaged $5{\cdot}2 \pm 2{\cdot}3$ mV (Fig. 4).

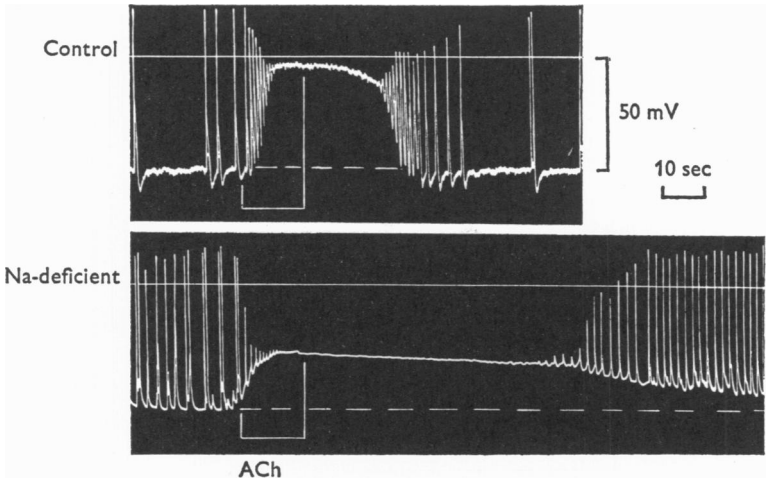

Fig. 3. Effect of sodium-deficient solution on repolarization and the after-hyperpolarization following acetylcholine. In the control response in normal Krebs solution (137 mM sodium), when acetylcholine (ACh, $5{\cdot}5 \times 10^{-5}$ M) was washed from the tissue repolarization was rapid, although the after-hyperpolarization was small. In sodium-deficient (17 mM, Tris substitution) solution repolarization was delayed and no after-hyperpolarization was observed. Also in sodium-deficient solution depolarization is less. The dotted line indicates the level of membrane potential existing before the application of acetylcholine.

It was incidentally observed that the membrane potential in the presence of acetylcholine ($5{\cdot}5 \times 10^{-5}$ M) was $-10{\cdot}8 \pm 2{\cdot}0$ mV in normal solution (134 mM chloride), $-9{\cdot}8 \pm 1{\cdot}0$ mV in chloride-deficient solution, and $-6{\cdot}9 \pm 1{\cdot}3$ mV upon reverting to normal solution. The pair differences were not significant ($P > 0{\cdot}5$, $n = 7$ and $P > 0{\cdot}4$, $n = 5$ respectively). These results confirm those of similar experiments using carbachol (Bolton, 1972, 1973*c*).

Effects of ouabain during the action of carbachol

The foregoing results imply that the brief application of larger concentrations of acetylcholine disturb the sodium and potassium gradients, increasing internal sodium and possibly external potassium concentrations. These increases could stimulate sodium pumping directly and it is not necessary to postulate any direct action of acetylcholine on the sodium

pump. Thus the sodium pump might exert its effects on the membrane potential in two ways: one by generating an electrogenic potential, the other by counteracting the decline of the sodium and potassium gradients during the action of acetylcholine or carbachol.

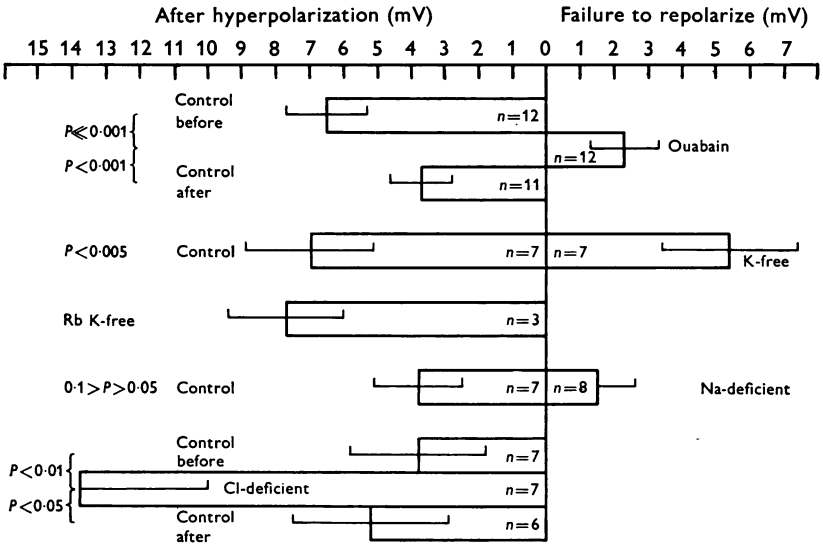

Fig. 4. Summary of the effects on acetylcholine after-hyperpolarization of procedures known to affect the operation of the sodium pump. From above downwards are shown the effects of ouabain ($1{\cdot}7 \times 10^{-6}$ M), potassium-free solution, rubidium-containing (5·9 mM) potassium-free solution, sodium-deficient (17 mM, Tris replacement) solution, and chloride-deficient (13 mM, benzene sulphonate replacement) solution. The means ± S.E. of the indicated number of preparations (n) are given. In the experiments with ouabain and chloride-deficient solution, control responses to acetylcholine ($5{\cdot}5 \times 10^{-5}$ M) were obtained both before and after responses in the test solution. In experiments with potassium-free and sodium-deficient solutions, all control responses were averaged; some of these preceded, others followed, responses in the test solution. Controls on the same tissues were not done in the case of rubidium.

In all cases the maximum negative potential observed following the application of acetylcholine was subtracted from the membrane potential existing immediately before its application. The results show that in normal solution after-hyperpolarization averaged 3–8 mV but was generally not seen in ouabain, potassium-free or sodium-deficient solutions. Here the most negative potential attained, on average, fell short of the membrane potential existing before the application of acetylcholine by a few millivolts. After-hyperpolarization was unchanged in rubidium-containing potassium-free solution, and potentiated in chloride-deficient solution. Also given are the results of a t test applied to pair differences.

Short periods of carbachol application

In order to obtain further evidence on this point, applications of carbachol were made in the presence of sufficient ouabain to at least severely impair sodium pumping. Carbachol was used in preference to acetylcholine because of its greater stability, and because the increase in conductance produced by the two concentrations used ($1{\cdot}4 \times 10^{-6}$ M and $5{\cdot}5 \times 10^{-5}$ M) had been estimated previously under the same conditions (Bolton, 1972).

It had been noticed that the membrane potential in the presence of acetylcholine was slightly more positive in the presence of $1{\cdot}7 \times 10^{-6}$ M ouabain (above) although the shift did not prove statistically significant. Experiments were now done using 10^{-5} M ouabain and carbachol ($1{\cdot}4 \times 10^{-6}$ M) applied for periods generally between 15 and 30 sec, but occasionally for up to 60 sec. In the presence of ouabain the most positive potential attained was $-3{\cdot}3 \pm 2{\cdot}0$ mV ($n = 6$) while in controls it was $-11{\cdot}2 \pm 1{\cdot}5$ mV ($n = 7$). The difference is significant ($P < 0{\cdot}005$). When $5{\cdot}5 \times 10^{-5}$ M carbachol was used the corresponding values were $-0{\cdot}8 \pm 1{\cdot}1$ mV ($n = 4$) and $-6{\cdot}3 \pm 3{\cdot}0$ mV ($n = 3$). Here the period of carbachol application was 10–15 sec and 10^{-4} M ouabain was used. Most of the responses to carbachol were obtained after several minutes in ouabain and it was noticed that with increasing time in ouabain the membrane potential in the presence of carbachol shifted positively.

Long periods of carbachol application

It seemed likely that E_{Na} and E_K might change when the tissue was in 10^{-5} or 10^{-4} M ouabain for several minutes, and that changes in these equilibrium potentials might be the cause of the change in the response to carbachol. For this reason experiments were done in which ouabain was applied for only 90–120 sec before introducing carbachol so that the changes in E_{Na} and E_K would be minimal. Two concentrations of carbachol were used. Using the lower concentration ($1{\cdot}4 \times 10^{-6}$ M) of carbachol the period of application was 2 min. Under the conditions of these experiments it had been observed previously that the effects of carbachol on membrane conductance were constant about 15 sec after carbachol began to exert its effects (Bolton, 1972).

The membrane potential in these experiments, where $1{\cdot}4 \times 10^{-6}$ M carbachol was applied, was measured at two points, one 15–20 sec after introducing carbachol, the other after 120 sec, just before switching back to carbachol-free solution. The first point might represent a time when the disturbance of the ionic gradients is small and the second when some degree of perturbation of these may have occurred. The membrane

potential at 15–20 sec was unaffected by ouabain (10^{-5} M) being $-10{\cdot}0 \pm 1{\cdot}5$ mV ($n = 6$) in its absence and $-10{\cdot}0 \pm 2{\cdot}2$ mV ($n = 6$) in its presence. After 120 sec the membrane potentials were $-7{\cdot}6 \pm 1{\cdot}6$ mV ($n = 5$, no ouabain) and $-1{\cdot}9 \pm 2{\cdot}3$ mV ($n = 5$, ouabain present) (Table 1). The pair differences were significant ($P < 0{\cdot}025$). The decay in the membrane potential in the presence of carbachol was noticeably faster (Fig. 5) and significantly ($P < 0{\cdot}005$, pair differences) greater when ouabain was present.

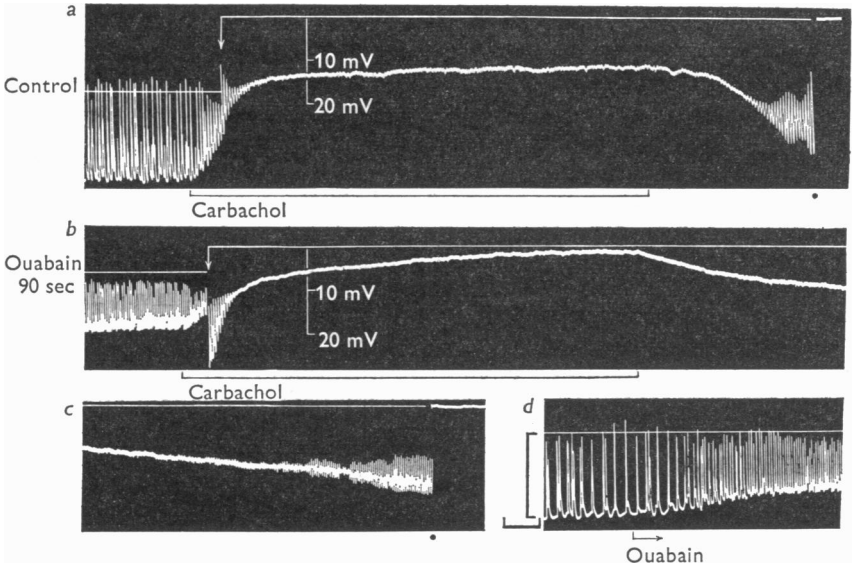

Fig. 5*a–c*: effect of sodium pumping on the membrane potential during prolonged application of carbachol. During the period (2 min) indicated by the bracket the solution bathing the tissue was changed to one containing $1{\cdot}4 \times 10^{-6}$ M carbachol. In the control (*a*), carbachol was applied in normal Krebs solution and in *b*, 90 sec after changing to a solution which contained 10^{-5} M ouabain. Shortly after switching to carbachol-containing solution the gain of the recording system was increased (at ↓) and a new zero potential applies. After returning to carbachol-free solution the electrode was withdrawn (at ●) to check the zero potential. During the period of increased gain the calibration near *d* represents 20 mV, at other times 50 mV. The horizontal calibration is 10 sec. Records *b* and *c* are continuous. Notice the more rapid decay of the membrane potential in the presence of carbachol when sodium pumping is impaired by ouabain, and the subsequently delayed repolarization. *d*: this shows the effect of changing the solution to one containing 10^{-5} M ouabain. This record and *b* are continuous. Calibrations 50 mV and 10 sec.

The presence of ouabain for 90–120 sec before introducing carbachol, depolarized the membrane (Fig. 5*d*) presumably mainly by abolishing the electrogenic contribution of the sodium pump to the membrane potential.

Upon returning to carbachol-free solution repolarization occurred, more slowly in the presence of ouabain. When it was clear that the electrode had not been dislodged during the contraction produced by carbachol, it was withdrawn to check the level of the zero potential (Fig. 5).

A similar series of experiments were done using $5{\cdot}5 \times 10^{-5}$ M carbachol which was applied for 45 sec. As appreciable disturbance of the ionic gradients probably takes place even during the onset of action of carbachol, measurements of membrane potential were made only at 45 sec. Ouabain (10^{-5} M) shifted the membrane potential from $-6{\cdot}8 \pm 0{\cdot}8$ mV to $-2{\cdot}7 \pm 0{\cdot}8$ mV at this time. The effect is significant ($P < 0{\cdot}005$) (Table 1).

TABLE 1. Effect of ouabain on the membrane potential in the presence of carbachol. Carbachol was applied for 120 sec ($1{\cdot}4 \times 10^{-6}$ M) or 45 sec ($5{\cdot}5 \times 10^{-5}$ M) before, and 90–120 sec after adding 10^{-5} M ouabain to the bathing solution. The membrane potential was measured at the indicated times after switching to carbachol-containing solution. The number of preparations (n) is also given

	Concentration of carbachol			
	$1{\cdot}4 \times 10^{-6}$ M		$5{\cdot}5 \times 10^{-5}$ M	
Time in carbachol	Control	Ouabain	Control	Ouabain
15–20 sec	$10{\cdot}0 \pm 1{\cdot}5$ mV ($n = 6$)	$10{\cdot}0 \pm 2{\cdot}2$ mV	—	—
45 sec	—	—	$6{\cdot}8 \pm 0{\cdot}8$ mV ($n = 7$)*	$2{\cdot}7 \pm 0{\cdot}8$ mV
120 sec	$7{\cdot}6 \pm 1{\cdot}6$ mV ($n = 5$)†	$1{\cdot}9 \pm 2{\cdot}3$ mV	—	—

* Pair differences significant $P < 0{\cdot}005$.
† Pair differences significant $P < 0{\cdot}025$.

THEORY

Decline of sodium and potassium gradients when sodium pumping is arrested in the presence of carbachol

A plausible explanation for the more rapid decline of the membrane potential when carbachol is applied in the presence of ouabain is that, when sodium pumping is prevented or reduced, the sodium and potassium gradients decline. The feasibility of such a proposal is difficult to test mainly because the rate of exchange of ions between the bathing solution and the extracellular space is not known, but the following is one way in which this problem might be approached.

A number of simplifying assumptions can be made initially. Previous evidence suggests that carbachol at 10^{-6} M or greater causes the membrane potential to approach the equilibrium potential for the carbachol-operated

ion channels (Bolton, 1972, 1973*a*). This ignores any electrogenic contribution from the sodium pump. We may, therefore, write as a first approximation for the membrane potential

$$V = \frac{\Delta G_K}{\Delta G} E_K + \frac{\Delta G_{Na}}{\Delta G} E_{Na}, \quad (1)$$

where ΔG_{Na} and ΔG_K are defined as

$$\Delta G_{Na} = i_{Na}/(V - E_{Na}) \quad \text{and} \quad \Delta G_K = i_K/(V - E_K). \quad (2)$$

It will be assumed that ΔG_{Na} and ΔG_K are independent of internal and external ion concentrations. At present there seems little justification for using a more complicated model particularly in view of the results obtained on receptor-operated channels at the frog end-plate (Takeuchi & Takeuchi, 1960; Takeuchi, 1963; Ginsborg, 1967).

Thus the contributions of ions other than sodium and potassium to the increase in conductance produced by carbachol are ignored. The weight of evidence suggests that any chloride contribution is small (Bolton, 1972, 1973*a*). If then sodium and potassium ions exchange one for one across the membrane, the rate of change of the internal sodium concentration will be equal in size but opposite in sign to the rate of change of the internal potassium concentration. Hence

$$\frac{d[Na^+]_i}{dt} = \frac{-d[K^+]_i}{dt} = \frac{\Delta G_{Na} \cdot (V - E_{Na})}{Fv}, \quad (3)$$

where F is Faraday's constant and v is the volume (l) in which the sodium crossing 1 cm^2 of cell membrane is distributed.

Eqn. (3) describes how the internal concentrations of sodium and potassium might vary with time. To obtain numerical solutions to eqn. (1), it is necessary to know also how their external concentrations vary with time. This will depend not only upon their exchange across the cell membrane, but also on their exchange with the bathing solution. Two models of ion movements were considered; both may be regarded as unrealistic extremes between which the true situation might lie.

Model I. In this model exchange of sodium and potassium ions across the cell membrane was assumed to be negligibly small compared with their rates of exchange between the extracellular space and the bathing solution. Under such conditions the external potassium and sodium concentrations will not vary appreciably from their values in the bathing solution (5·9 and 137 mM respectively).

Model II. This is essentially the converse of model I. The extracellular concentrations of sodium and potassium were assumed to be determined by their rates of exchange across the cell membrane and to be negligibly affected by their rates of exchange with the bathing solution; thus, in such

a case, the total amounts of sodium and potassium in the tissue (= extracellular + intracellular space) will remain constant during the application of carbachol. If the extracellular space is assumed to be half the intracellular space and therefore equal to 33 % of the tissue volume then

$$\frac{d[Na^+]_o}{dt} = -\frac{2d[Na^+]_i}{dt}. \quad (4)$$

Assuming that potassium ions move in a reciprocal manner to sodium, a similar equation will apply.

Numerical solutions for the value of V, at different times, t, after the application of carbachol (when sodium pumping was arrested) were then obtained by computing the time, Δt, taken for small changes in internal sodium concentration, $\Delta[Na^+]_i$, to occur. When the change in $[Na^+]_i$ was rapid, $\Delta[Na^+]_i$ was taken as 0·0005 M, at other times 0·005 M. Potassium was treated similarly so that the time courses of $[Na^+]_i$ and $[K^+]_i$, $[Na^+]_o$ and $[K^+]_o$ could be obtained by summing the values of Δt. The time course of V was then obtained from eqn. (1). The following values were used at $t = 0$:

$\Delta G_{Na} = 1{\cdot}3 \times 10^{-4}$ mho.cm^{-2}, $\Delta G/G = 10$ ($1{\cdot}4 \times 10^{-6}$ M carbachol), or $\Delta G_{Na} = 1{\cdot}3 \times 10^{-3}$ mho.cm^{-2}, $\Delta G/G = 100$ ($5{\cdot}5 \times 10^{-5}$ M carbachol) (Bolton, 1972), $G = 0{\cdot}25 \times 10^{-4}$ mho.cm^{-2} (Tomita, 1970), $E_{Na} = 0{\cdot}050$ volt; $E_K = -0{\cdot}081$ volt (Casteels & Kuriyama, 1965, 1966; Casteels, 1966, 1969, 1971; Bülbring, Casteels & Kuriyama, 1968), $v = 1{\cdot}5 \times 10^{-7}$ l (corresponding to a cell diameter of 6 μm).

The predicted time courses of V (in the absence of sodium pumping) are shown in Fig. 6 for model I (exchange) and model II (no exchange between bathing solution and extracellular space). The calculated lines were obtained using $\Delta G = 2{\cdot}5 \times 10^{-4}$ mho.cm^{-2} ($1{\cdot}4 \times 10^{-6}$ M carbachol) and $\Delta G = 2{\cdot}5 \times 10^{-3}$ mho.cm^{-2} ($5{\cdot}5 \times 10^{-5}$ M carbachol). Also shown are the actual time courses observed using these concentrations of carbachol in two experiments and the means of all experiments done (from Table 1). The filled symbols are in the presence of ouabain (10^{-5} M) and the open symbols in its absence.

The observed time courses lie between the time courses predicted by the two models, the one assuming that exchange between bathing solution and extracellular space is very rapid, the other very slow, compared with exchange across the cell membrane. If it is more realistically assumed that these two rates of exchange are of a similar order, then a better, or even perfect fit could obviously be obtained. The very poor fit given by model I suggests that the extracellular concentrations of sodium and potassium deviate considerably from their concentrations in the bathing solution. A combination of a raised external potassium concentration and

raised internal sodium concentration would be expected to provide a powerful stimulus to electrogenic sodium pumping (Bolton, 1973*c*).

Contribution of electrogenic sodium pumping to the membrane potential in the presence of carbachol

So far, however, no account has been taken of any electrogenic contribution the sodium pump might make to the membrane potential during the action of acetylcholine or carbachol. Experiments described in the previous paper (Bolton, 1973*c*) suggested that the sodium pump might

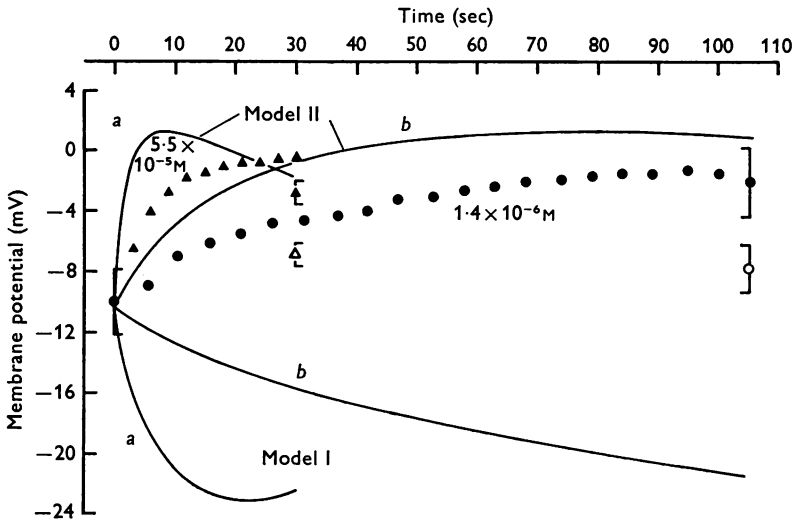

Fig. 6. Comparison of actual time courses of membrane potential in presence of carbachol with those predicted by Model I and Model II. The actual time courses of the membrane potential in the presence of 10^{-5} M ouabain are shown for two experiments where $1{\cdot}4 \times 10^{-6}$ M carbachol (●) and $5{\cdot}5 \times 10^{-5}$ M carbachol (▲) were applied. Time zero was taken as 15–20 sec after switching to carbachol-containing solution, i.e. after the period when carbachol concentration is changing. Also shown are the means ± S.E. (measured at the indicated points in time) of all such experiments in the absence (open symbols) and in the presence (closed symbols) of ouabain (10^{-5} M) (data from Table 1). The continuous lines are the time courses according to Model I and Model II using $\Delta G = 2{\cdot}5 \times 10^{-3}$ mho. cm^{-2} (*a*, corresponding to $5{\cdot}5 \times 10^{-5}$ M carbachol) and $\Delta G = 2{\cdot}5 \times 10^{-4}$ mho. cm^{-2} (*b*, corresponding to $1{\cdot}4 \times 10^{-6}$ M carbachol).

contribute something like 10 mV to the resting membrane potential. It is therefore conceivable that electrogenic sodium pumping could contribute to the membrane potential even during the action of carbachol, particularly if the internal sodium and external potassium concentrations rise.

If during the action of carbachol, the rises in internal sodium and

external potassium concentration stimulate pump activity, a new equilibrium could be reached where the inward passive current created by sodium flowing down its electrochemical gradient is equal in size and opposite in direction to the outward pump current, i.e. from eqn. (2)

$$i_{\text{pump}} = \Delta G_{\text{Na}} (V - E_{\text{Na}}). \tag{5}$$

If the fraction of the sodium current which is extruded electrogenically is ρ then

$$\rho i_{\text{pump}} = \Delta G . E, \tag{6}$$

where ΔG is the conductance of the membrane (mho.cm^{-2}) in the presence of carbachol and E is the electrogenic potential (volts) generated by the pump. By substitution from (5) and (6)

$$E = (V - E_{\text{Na}}) \rho b, \tag{7}$$

where $b = \Delta G_{\text{Na}}/\Delta G$. Thus, if the inward passive and outward active currents of sodium are equal in size and opposite in direction during the action of carbachol, the electrogenic potential generated will be independent of the membrane conductance providing the relative contribution of sodium to the total membrane conductance remains constant (i.e. b is constant).

The electrogenic contribution to the membrane potential in the presence of carbachol, E, will be about 10 mV if $b = 0{\cdot}5$ (Bolton, 1972), $E_{\text{Na}} = 0{\cdot}050$ volts (Casteels & Kuriyama, 1965, 1966; Casteels, 1966, 1969, 1971), $V = -0{\cdot}010$ (during the action of carbachol) and $\rho = 0{\cdot}33$ (assuming that smooth muscle resembles other tissues in that one third of the sodium pumped is extruded electrogenically, Thomas, 1972). It is conceivable, therefore, that the membrane potential in the presence of carbachol differs from zero potential only because of the electrogenic activity of the sodium pump.

DISCUSSION

It is pertinent to enquire whether the rate of sodium pumping can be great enough during the action of carbachol for an equilibrium between inward and outward sodium fluxes to be achieved. If $\Delta G_{\text{Na}} = 1{\cdot}3 \times 10^{-4}$ mho.cm^{-2} ($1{\cdot}4 \times 10^{-6}$ M carbachol) or $\Delta G_{\text{Na}} = 1{\cdot}3 \times 10^{-3}$ mho.cm^{-2} ($5{\cdot}5 \times 10^{-5}$ M carbachol) (Bolton, 1972) then the sodium current would be 8×10^{-6} or 8×10^{-5} A.cm^{-2} respectively (eqn. (2)). The maximum pumping rate in sepia axon at 15–20° C is about 5×10^{-6} A.cm^{-2} (Hodgkin & Keynes, 1955) while in unmyelinated mammalian nerve Landowne & Ritchie (1970) estimated a maximum rate of 4×10^{-7} A.cm^{-2} (at 20° C). The value obtained for ileal muscle depends upon measurements of resting conductance (Tomita, 1970) and of the increase in conductance produced by carbachol (Bolton, 1972). A combination of errors in these might make the

estimate for ileal muscle too large by a factor of ten but probably not by a factor of a hundred. Allowing for this and the difference in temperature, the maximum pumping capability in ileal muscle and sepia axon (Hodgkin & Keynes, 1955) could be of the same order.

However, the evidence was that the electrogenic sodium pump contributed at most 1–2 mV to the membrane potential during the action of carbachol, as 10^{-5} M ouabain had no effect 15–20 sec after the application of $1{\cdot}4 \times 10^{-6}$ M carbachol (Table 1). At this time, 10^{-5} M ouabain had severely impaired pump activity as evidenced by the fall in resting membrane potential (Fig. 5*d*). The electrogenic contribution is much less than the 10 mV calculated from eqn. (7) which assumes equilibrium conditions and one third of the sodium extruded electrogenically. As the membrane potential subsequently moved positively, there is no argument for the electrogenic contribution becoming more substantial at later times.

If we assume, nevertheless, that the electrogenic pump does contribute a small amount, e.g. 1–2 mV, to the membrane potential in the presence of $1{\cdot}4 \times 10^{-6}$ M carbachol, eqn. (6) would indicate a total sodium pump current of $8\text{–}16 \times 10^{-7}$ A.cm^{-2} (one third of sodium extruded electrogenically). This is a fifth or a tenth of the sodium current, 8×10^{-6} A.cm^{-2} (eqn. (2), $\Delta G_{Na} = 1{\cdot}3 \times 10^{-4}$ mho.cm^{-2}, $V - E_{Na} = -0{\cdot}060$ V) which would appear to be flowing into the cells at this time. If the sodium pump is maximally activated at this time due to the rises in internal sodium and external potassium concentrations, then the maximum pump current in ileal muscle would be about 10^{-6} A.cm^{-2}, in which case the discrepancy between pump and the inward current when $5{\cdot}5 \times 10^{-5}$ M carbachol is applied would be even greater; eqn. (2) indicates an inward current of 8×10^{-5} A.cm^{-2} so that the pump could do very little towards altering the distribution of sodium and potassium ions. Nevertheless, ouabain had a clear effect on the membrane potential in the presence of $5{\cdot}5 \times 10^{-5}$ M carbachol: this implies that pump current, if not equal to inward sodium current, is at least sufficiently substantial to affect the distribution of sodium and potassium ions at this time. An alternative explanation for the apparent small electrogenic contribution of the pump to the membrane potential in the presence of carbachol could be that the fraction of the sodium extruded electrogenically in the presence of carbachol (when internal sodium and external potassium concentrations are raised) is much smaller than the one third which was assumed.

Repolarization occurred even in the presence of 10^{-5} M ouabain, although the rate was slowed. If pumping was arrested by ouabain, and the sodium and potassium gradients decline severely in the presence of carbachol, then repolarization would presumably not occur. According to model II, E_{Na} need only fall by 10 mV and E_K shift by about 30 mV to

explain the observed value of the membrane potential after 2 min in $1{\cdot}4 \times 10^{-6}$ M carbachol with ouabain present. Thus, repolarization would be possible but delayed compared with the normal tissue. In the latter, as membrane conductance decreases, *electrogenic* activity apparently comes to exert an effect on the membrane potential, accelerating repolarization, particularly beyond -20 or -30 mV, and causing after-hyperpolarization.

After-hyperpolarization following the application of acetylcholine suggested that electrogenic sodium pumping was increased at this time. If a direct action of acetylcholine on pump activity is discounted, an increase in the internal sodium concentration, an increase in the external potassium concentration, or a combination of these is implied (Bolton, 1973*c*). In support of this it was shown that a fall in the sodium and potassium gradients was a feasible explanation for the change in membrane potential occurring with time in carbachol when sodium pumping was impaired (Fig. 6). Paton & Rothschild (1965) also found that the sodium and potassium content of isolated longitudinal muscle of guinea-pig ileum was changed by $1{\cdot}1 \times 10^{-5}$ M or $5{\cdot}5 \times 10^{-4}$ M acetylcholine during a 5 min application. There seems little doubt that the application of acetylcholine or carbachol to these small cells produces a fall in the sodium and potassium gradients across the membrane. This increases sodium pumping which retards the decline of the sodium and potassium gradients and reduces the depolarization produced, although the electrogenic contribution of the pump to the membrane potential at this time is apparently small. The increased electrogenic extrusion of sodium subsequently is responsible for the after-hyperpolarization that follows an application of acetylcholine.

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