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Action of Acetylcholine on the Longitudinal Muscle of Guinea-Pig Ileum: The Role of an Electrogenic Sodium Pump

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Action of acetylcholine on the longitudinal muscle of guinea-pig ileum: the role of an electrogenic sodium pump

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Intracellular recordings of membrane potential were made from the longitudinal muscle of guinea-pig terminal ileum. It was observed that ouabain or potassium-free solution depolarized the membrane. Upon readmitting potassium to potassium-free solution, the membrane potential rapidly increased. This response was blocked by ouabain and was potentiated in chloride-deficient solution, suggesting that it was due to the activity of an electrogenic sodium pump.

When acetylcholine was applied, and then washed from the tissue, there followed a period of increased negativity of the membrane potential, an after-hyperpolarization. This did not occur when responses to acetylcholine were obtained in the presence of ouabain, in potassium-free solution, or in sodium-deficient solution, but the after-hyperpolarization was increased in size in chloride-deficient solution. During a 2 min application of carbachol, the membrane potential fell more rapidly in the presence of ouabain (10^{-5} mol/l); this could be explained if sodium pump activity is important in retarding the decline of the sodium and potassium gradients that occurs at this time.

It was concluded that the application of acetylcholine or carbachol to ileal muscle increases internal sodium and probably external potassium concentrations. These increases stimulate the activity of the electrogenic sodium pump so that, when the membrane resistance recovers, there is an increased electrogenic contribution to the membrane potential. This produces the after-hyperpolarization which is a feature of the response to acetylcholine.

Because the smooth muscle cell is small and has a large surface area to volume ratio, substances such as acetylcholine which increases membrane conductance (Hidaka & Kuriyama 1969; Bolton 1972, 1973*a*) may alter the ionic gradients across the membrane. They may do this by changing both the internal and the external concentrations of ions adjacent to the cell membrane. As the increase in conductance produced by acetylcholine or carbachol is probably mainly to sodium and potassium ions (Bolton 1973*a*), the increased internal sodium and external potassium concentrations which might result would be expected to increase the activity of the sodium pump if one exists. This might exert effects in two ways: one by acting to restore the ionic gradients to potassium and sodium across the membrane, the other by generating an increased electrogenic potential. Recent papers (Bolton 1973*b*, *c*) present these results in full.

All the results which will be described were obtained on the longitudinal muscle of the guinea-pig terminal ileum, separated from the underlying circular muscle, and placed in physiological salt solution at 35 °C. All the records are of the membrane potential recorded intracellularly. Further details of the method are given elsewhere (Bolton 1972).

Evidence for the operation of an electrogenic sodium pump in longitudinal ileal muscle

Evidence that an electrogenic sodium pump operates in the taenia and rat uterus has been provided by Taylor, Paton & Daniel (1970, 1971), Casteels, Droogmans & Hendrickx (1971*a*, *b*), and Tomita & Yamamoto (1971). Experiments showed that a similar pump operates in the longitudinal muscle of guinea-pig ileum. For example, adding ouabain (1.7 μmol/l) to the

bathing solution produced a depolarization of several millivolts which was reversed upon reverting to ouabain-free solution. Potassium-free solution produced a similar effect to ouabain, and readmitting 5.9 mmol/l potassium to potassium-free solution produced a rapid increase in membrane potential beginning within a few seconds of readmitting potassium and reaching a maximum within 15 to 30 s (Bolton 1971*b*) (figure 1).

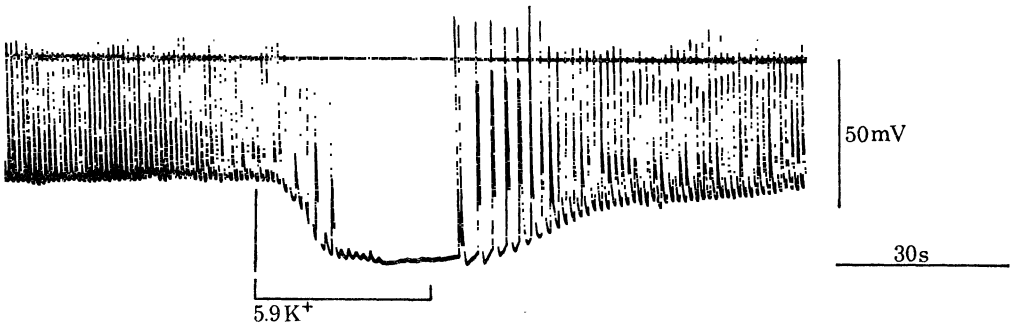

FIGURE 1. Effect of readmitting potassium to potassium-free solution. Potassium (5.9 mmol/l) was readmitted for a 30 s period indicated by the bracket and produced a rapid increase in membrane potential. Intracellular record of membrane potential.

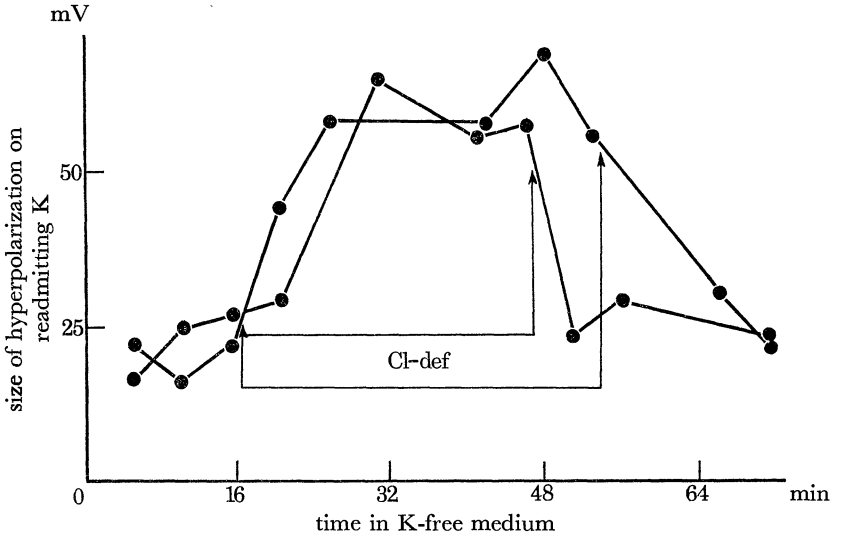

FIGURE 2. Effect of chloride-deficient solution on the response to readmitting potassium to potassium-free solution (cf. figure 1). Potassium (5.9 mmol/l) was readmitted to potassium-free solution at intervals and the sizes of the resulting hyperpolarizations are plotted against time for two preparations. During the periods indicated by the arrows the potassium-free solution was changed to one which was also chloride-deficient (13 mmol/l, benzene sulphonate replacement).

The response to readmitting potassium (5.9 mmol/l) to potassium-free solution was blocked by ouabain (1.7 μmol/l) showing that it resulted from the activity of a sodium pump. Experiments in which chloride-deficient solution was used showed that the response to readmitting potassium to potassium-free solution resulted largely, if not exclusively, from the electrogenic property of the sodium pump. In chloride-deficient solution (13 mmol/l) in which chloride was replaced by benzene sulphonate, the membrane resistance was approximately doubled. The response to readmitting potassium to potassium-free solution was also increased two fold or more when the potassium-free solution was changed to one which was also chloride-deficient (figure 2). The potentiation of the response produced by pump activity in chloride-deficient or

-free solutions is similar to that observed on other tissues and indicates that the extrusion of sodium is electrogenic (see, for example, Rang & Ritchie 1968; Thomas 1972). In these experiments, readmitting potassium to potassium-free, chloride-deficient (13 mmol/l) solution sometimes produced a membrane potential which exceeded -100 mV; this is probably more negative than the potassium equilibrium potential under these conditions (Bolton 1971*b*).

The size of the hyperpolarization observed upon readmitting potassium to potassium-free solution was increased by increasing the concentration of potassium over the range 0.1 to 20 mmol/l. The response to 0.6 mmol/l was increased less than twofold by increasing the potassium concentration ten times. Increasing the concentration above 6 mmol/l produced only a small increase in the size of the response. It is possible that the small effect of increasing the concentration of potassium above 6 mmol/l was due to a fall in membrane resistance and a positive shift in E_K upon readmitting higher concentrations of potassium.

With time in potassium-free solution, the response to readmitting a fixed concentration of potassium increased. A likely explanation for this increase is that with time in potassium-free solution the internal sodium concentration rises (Axelsson & Holmberg 1971; Casteels *et al.* 1971*a*, *b*). It is possible that this rise causes an increase in the amount of sodium extruded in response to a given potassium stimulus applied to the pump.

Thus the longitudinal muscle of the guinea-pig terminal ileum possesses an electrogenic sodium pump. Results supported the view that the activity of this pump was increased either by increasing the external potassium concentration, or by increasing the internal sodium concentration (or both), a situation which might very well exist during the application of acetylcholine.

Effects of conditions known to modify sodium pump activity on the after-hyperpolarization produced by acetylcholine

When acetylcholine is applied to smooth muscle the membrane depolarizes (Bülbring 1954, 1955, 1957; Burnstock 1958; Bülbring & Burnstock 1960; Bülbring & Kuriyama 1963; Hidaka & Kuriyama 1969; Bolton 1972, 1973*a*). Upon returning to acetylcholine-free solution the membrane repolarizes and then hyperpolarizes beyond the level existing before the application of acetylcholine (Bülbring & Kuriyama 1963; Bolton 1971*a*, *b*). This latter part of the response might be called an after-hyperpolarization.

The hyperpolarization which follows activity in nerve has been shown to be due to an electrogenic sodium pump (Ritchie & Straub 1957; Connelly 1959; Straub 1961; Hurlbut 1963; Rang & Ritchie 1968). It seemed likely that the after-hyperpolarization produced by acetylcholine in smooth muscle also resulted from the electrogenic extrusion of the additional sodium which enters during the increase in membrane permeability produced by acetylcholine.

Experiments supported this. The after-hyperpolarization did not occur after the application of acetylcholine (5.5×10^{-5} mol/l) in the presence of ouabain (1.7 μmol/l) or in potassium-free solution (Bolton 1971*b*). Under these conditions repolarization was delayed, especially after the membrane potential reached 20 or 30 mV, and was generally incomplete, i.e. did not return to the level existing before the application of acetylcholine (figure 3). The presence of ouabain, or the removal of potassium, both impair sodium pump activity (Harris & Maizels 1951; Schatzmann 1953). If an equimolar amount of rubidium was used to replace potassium in making potassium-free solution, the after-hyperpolarization occurred normally and was not

noticeably different from that which occurred in normal, potassium-containing solution. It is known that rubidium can substitute for potassium in maintaining the activity of the sodium pump in smooth muscle (Paton 1971; Taylor *et al.* 1971; Tomita & Yamamoto 1971) as in other tissues.

In sodium-deficient (17 mmol/l) solution in which sodium is replaced by tris, the influx of sodium during the action of acetylcholine might be expected to be reduced. If acetylcholine produces an appreciable increase in permeability only to sodium and potassium ions, then presumably any increase in the external potassium concentration which occurs during the action of acetylcholine, will also be less. These changes would be expected to reduce the after-hyperpolarization if it was due to sodium pumping. Sodium-deficient solution not only abolished the after-hyperpolarization but very greatly delayed repolarization (figure 4).

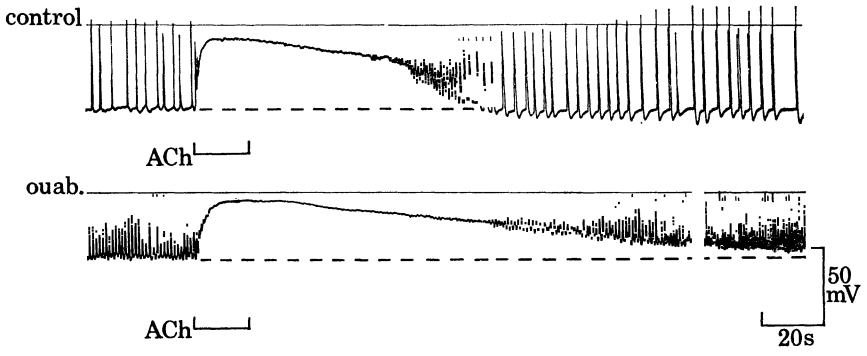

FIGURE 3. Effect of ouabain on the after-hyperpolarization which follows acetylcholine application. Acetylcholine (ACh) (5.5×10^{-5} mol/l) was applied for the period indicated by the bracket in ouabain-free solution (control) and to the same preparation in the presence of ouabain (ouab) (10^{-5} mol/l). Repolarization is delayed in the presence of ouabain, particularly after the membrane potential reaches -20 to -30 mV, and is incomplete. No after-hyperpolarization occurs. The gap in the lower record represents a period of 1 min. Intracellular records of membrane potential.

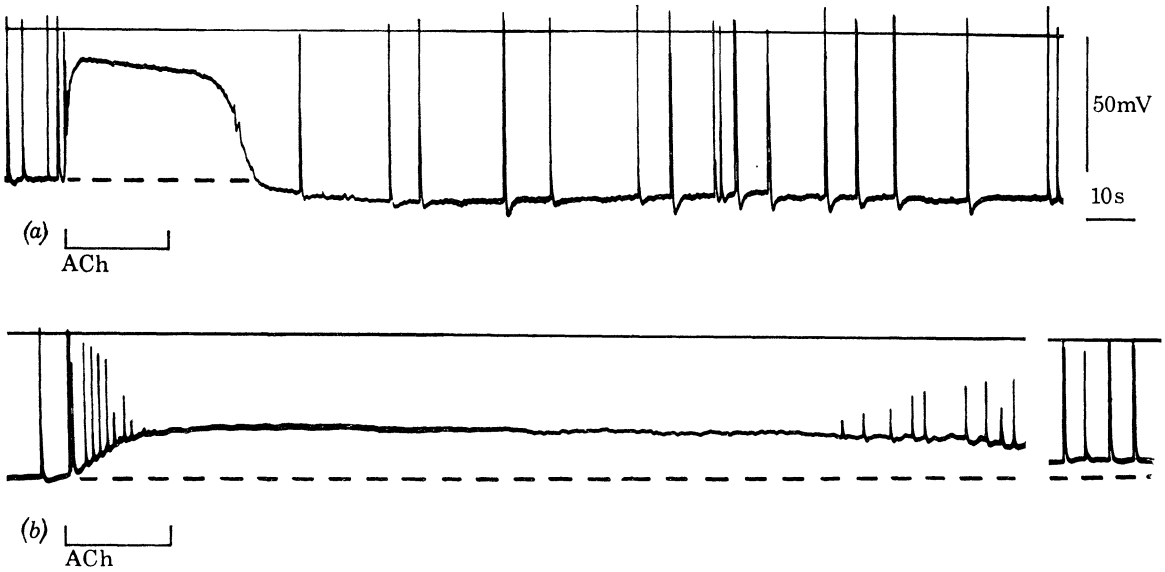

FIGURE 4. Effect of sodium-deficient solution on the after-hyperpolarization which follows acetylcholine application. Acetylcholine (ACh) (5.5×10^{-5} mol/l) was applied for the period indicated by the bracket in (*a*) normal solution (137 mmol/l sodium) and in (*b*) sodium-deficient solution (17 mmol/l, tris substitution). Notice that repolarization is very much delayed in the latter and no after-hyperpolarization occurs. The gap in the lower record represents a period of 3 min. Intracellular records of membrane potential.

The delay in repolarization observed upon reducing the external sodium concentration from 137 to 17 mmol/l by replacing sodium with tris was much greater than might be expected from the calculated reduction of sodium influx in sodium-deficient solution. If the sodium equilibrium potential is +50 mV in normal solution (Casteels & Kuriyama 1965, 1966; Casteels 1966, 1969, 1971; Bülbring, Casteels & Kuriyama 1968) then in the presence of acetylcholine, when the membrane potential is about −10 mV, the driving force on the sodium ion will be about 60 mV. In 17 mmol/l sodium the sodium equilibrium potential will be about 0 mV, if the internal sodium does not change very much. As in the presence of acetylcholine the membrane potential is now about −30 mV, the driving force on the sodium ion is about 30 mV, or half that in normal (137 mmol/l sodium) solution. If the internal sodium falls in 17 mmol/l sodium the driving force will not be reduced so much. Providing the sodium conductance opened by carbachol is the same in 137 mmol/l as in sodium-deficient (17 mmol/l) solution, it would be expected that about half as much sodium should enter. (This implies a 50% reduction in the efflux of potassium also as apparently only sodium and potassium ions move through the channels opened by carbachol (Bolton 1972).) The very pronounced delay in repolarization is therefore remarkable. Szurszewski & Bülbring (this volume) have suggested that in sodium-deficient solutions, the increase in calcium permeability becomes important. It would seem necessary to invoke some additional mechanism of this type to explain the pronounced delay in repolarization which is observed.

The experiments described so far indicate that the after-hyperpolarization may result from the activity of the sodium pump but do not indicate that it is the electrogenic property of the pump which is responsible for the phenomenon rather than the pump's ability to restore sodium and potassium gradients. For this reason experiments were done in chloride-deficient solution, in which responses due to the electrogenic property of the pump would be expected to be potentiated. Following the application of acetylcholine (5.5×10^{-5} mol/l) for a brief period to 7 preparations in chloride-deficient (13 mmol/l) solution, the after-hyperpolarization averaged 13.8 ± 3.8 mV (mean ± S.E.) but in normal solution it averaged 3.8 ± 2.0 mV in these same tissues (Bolton 1971*b*).

Thus the after-hyperpolarization which is a feature of the response to acetylcholine was affected by a number of procedures in a way which would be expected if it resulted from the activity of an electrogenic sodium pump which acts to extrude the additional sodium entering during the action of acetylcholine and at the same time acts to draw potassium into the cell.

Effects of arresting sodium pump activity with ouabain during the action of carbachol

The results described so far suggest two possibilities: one is that during the action of acetylcholine or carbachol the internal concentrations of sodium and potassium are changed (and possibly that their external concentrations are also altered). The other is that even during the action of acetylcholine or carbachol, the pump contributes an electrogenic component to the membrane potential and hence modifies the depolarization which is produced. For these reasons experiments were done in which fairly prolonged application of carbachol was made in the presence of ouabain, 10^{-5} mol/l, a concentration sufficient to abolish, or at least severely impair, sodium pump activity. In these experiments carbachol was used in preference to acetylcholine because of its greater stability, and because measurements had previously been

made of the increase in conductance produced by some concentrations of carbachol in this tissue under the same conditions (Bolton 1972).

When carbachol (1.4 μmol/l) was applied for a period of two minutes in normal solution the membrane depolarized rapidly; its value measured 15 to 20 s after beginning carbachol application was -10.0 ± 1.5 mV (mean ± S.E., $n = 5$). After 2 min in carbachol the membrane potential fell slightly to a value of -6.3 ± 1.8 mV. When carbachol was applied to these same tissues in the presence of ouabain (10^{-5} mol/l), the potential 15 to 20 s after beginning its application was the same, -10.0 ± 2.2 mV, as in normal solution. With time in carbachol, however, the decay of the membrane potential was significantly ($p < 0.005$) faster, and its value after 2 min was -1.9 ± 2.3 mV. Presumably the more rapid decline in the presence of ouabain is a consequence of arresting sodium pump activity. Ouabain was applied for only 90 s before introducing carbachol, so that changes in the sodium and potassium gradients occurring before carbachol reached the tissue would be expected to be minimal. This was borne out by the observation that initially, depolarization in the presence of ouabain was to the same level of membrane potential as in ouabain-free solution.

The contribution of the electrogenic pump to the membrane potential in the presence of 10^{-5} mol/l ouabain must be negligible. Therefore, these results suggest that in ouabain-free, normal solution also, any electrogenic contribution to the membrane potential 15 to 20 s after applying carbachol must be also very small, as there was no effect of arresting sodium pump activity at this time. Presumably this is also true at longer times in the presence of carbachol, and the more rapid decline of the membrane potential in the presence of ouabain has some other explanation.

Taking into account the standard errors of these results, we can set a possible upper limit of about 1 mV to the electrogenic contribution of the sodium pump to the membrane potential in the presence of 1.4 μmol/l carbachol. It could well be less than this, but it is unlikely to be greater. On this basis, an upper limit for sodium pump activity can be set if the assumption is made that the rises in internal sodium and external potassium concentrations in the presence of carbachol are sufficient to produce maximum pump activity. Calculations based on the value of the membrane conductance in the presence of carbachol $\Delta G = 2.5 \times 10^{-4}$ S cm^{-2} (for 1.4 μmol/l carbachol, Bolton 1972), the resting membrane conductance, $G = 2.5 \times 10^{-5}$ S cm^{-2} (Tomita 1970) and the contribution of sodium to the membrane conductance in the presence of carbachol (Bolton 1972), show that the maximum rate of sodium pumping probably does not exceed 45×10^3 sodium ions s^{-1} μm^{-2} in this tissue. This estimate assumes that one-third of the sodium is extruded electrogenically. The value obtained is close to the estimate made by Landowne & Ritchie (1970) for maximum sodium pump activity of unmyelinated nerve membrane. Alternatively, we may say that if the maximum sodium pumping ability of the membrane of longitudinal ileal muscle is similar to that of unmyelinated nerve membrane, then the contribution of the electrogenic pump to the membrane potential in the presence of 1.4 μmol/l carbachol would be less than 1 mV, and hence undetectable in these experiments.

We are left, therefore, with explaining the more rapid decay of the membrane potential in the presence of carbachol, when sodium pump activity is arrested by ouabain. It is plausible that the more rapid decline results from the running down of the ionic gradients to sodium and potassium. To test this explanation two models were considered. In the first, exchange of sodium and potassium ions between the bathing solution and the extracellular space was assumed to be very rapid compared with their exchange across the cell membrane. In the other

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model, the converse was assumed; that the exchange of these ions across the cell membrane was much more rapid than their exchange between the extracellular space and the bathing solution. In this model the intracellular space was assumed to be twice the extracellular space. Both models can be regarded as unrealistic and the true situation must lie between these extremes.

If the membrane potential, V, in the presence of concentrations of carbachol 10^{-6} mol/l or greater is close to the equilibrium potential for the channels operated by the muscarinic receptor (Bolton 1972) we may write

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

where ΔG_K and ΔG_{Na} are the contributions of potassium and sodium to the increase in membrane conductance, ΔG, produced by the presence of carbachol. The assumptions involved in the application of the equation to this situation have been previously discussed (Bolton 1972, 1973). Now if the sodium and potassium currents are given by

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

and only sodium and potassium ions are involved we may write *in the absence of sodium pumping*

$$\frac{d(Na^+)_i}{dt} = -\frac{d(K^+)_i}{dt} = \Delta G_{Na} \frac{V - E_{Na}}{Fv}$$

(inward current negative) where F is the Faraday constant, and v is the volume (l) of cell interior in which the sodium entering through 1 cm² of cell membrane is distributed.

Numerical solutions to these equations were computed to obtain the value of the membrane potential, V, at different times after applying carbachol by inserting the following values at $t = 0$: $\Delta G = 2.5 \times 10^{-4}$ S cm^{-2}, $\Delta G_{Na} = 1.3 \times 10^{-4}$ S cm^{-2} (Tomita 1970; Bolton 1972), $E_{Na} = 0.050$ V, $E_K = -0.084$ V (Casteels & Kuriyama 1965, 1966; Casteels 1966, 1969, 1971; Bülbring *et al.* 1968), $v = 1.5 \times 10^{-7}$ l (assuming a cell diameter of 6 μm). It was taken that ΔG and ΔG_{Na} were constant in the presence of carbachol. When it was assumed that the rates of exchange of sodium and potassium across the cell membrane were negligible compared with their rates of exchange between extracellular and bathing solution, the predicted time course differed widely from that observed. On the other hand, when exchange across the cell membrane, rather than with the bathing solution, was assumed to be the main factor determining the extracellular concentrations of sodium and potassium, the predicted time course was close to that observed.

Conclusions

The sodium pump acts at all times to restore the ionic gradients to sodium and potassium and to generate an electrogenic potential. Pump activity is important in retarding the decline of the sodium and potassium gradients during the action of carbachol, but the electrogenic contribution to the membrane potential is small at this time. When sodium pump activity was reduced or abolished by 10^{-5} mol/l ouabain, the membrane potential in the presence of carbachol declined at an increased rate which could be predicted from calculations based on available estimates of membrane conductance and internal ion concentrations, if it was assumed that the rate of exchange of sodium and potassium ions across the cell membrane was a more

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important determinant of their extracellular concentration than exchange with the bathing solution.

It follows that acetylcholine or carbachol in the concentrations used, increased the internal concentration of sodium, and probably also increase the external concentration of potassium. These increases stimulate the activity of the electrogenic sodium pump so that, when the membrane resistance recovers, there is an increased electrogenic contribution to the membrane potential. This produces the after-hyperpolarization which is a feature of the response to acetylcholine.

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References (Bolton)

Axelsson, J. & Holmberg, B. 1971 *Acta physiol. Scand.* **82**, 322–332.
Bolton, T. B. 1971*a* *J. Physiol., Lond.* **216**, 403–418.
Bolton, T. B. 1971*b* *J. Physiol., Lond.* **218**, 58–59*P*.
Bolton, T. B. 1972 *J. Physiol., Lond.* **220**, 647–671.
Bolton, T. B. 1973*a* The permeability change produced by acetylcholine in smooth muscle. In *Drug receptors* (ed. H. P. Rang). London: Macmillan.
Bolton, T. B. 1973*b* *J. Physiol., Lond.* **228**, 693–712.
Bolton, T. B. 1973*c* *J. Physiol., Lond.* **228**, 713–731.
Bülbring, E. 1954 *J. Physiol., Lond.* **125**, 302–315.
Bülbring, E. 1955 *J. Physiol., Lond.* **128**, 200–221.
Bülbring, E. 1957 *J. Physiol., Lond.* **135**, 412–425.
Bülbring, E. & Burnstock, G. 1960 *Br. J. Pharmac.* **15**, 611–624.
Bülbring, E., Casteels, R. & Kuriyama, H. 1968 *Br. J. Pharmac.* **34**, 388–407.
Bülbring, E. & Kuriyama, H. 1963 *J. Physiol., Lond.* **166**, 59–74.
Burnstock, G. 1958 *J. Physiol., Lond.* **143**, 165–182.
Casteels, R. 1966 *J. Physiol., Lond.* **184**, 131–142.
Casteels, R. 1969 *J. Physiol., Lond.* **205**, 193–208.
Casteels, R. 1971 *J. Physiol., Lond.* **214**, 225–243.
Casteels, R., Droogmans, G. & Hendrickx, H. 1971*a* *J. Physiol., Lond.* **217**, 281–295.
Casteels, R., Droogmans, G. & Hendrickx, H. 1971*b* *J. Physiol., Lond.* **217**, 297–313.
Casteels, R. & Kuriyama, H. 1965 *J. Physiol., Lond.* **177**, 263–287.
Casteels, R. & Kuriyama, H. 1966 *J. Physiol., Lond.* **184**, 120–130.
Connelly, C. M. 1959 *Rev. mod. Phys.* **31**, 475–484.
Harris, E. J. & Maizels, M. 1951 *J. Physiol., Lond.* **113**, 506–524.
Hidaka, T. & Kuriyama, H. 1969 *J. gen. Physiol.* **53**, 471–486.
Hurlbut, W. P. 1963 *J. gen. Physiol.* **46**, 1191–1222.
Landowne, D. & Ritchie, J. M. 1970 *J. Physiol., Lond.* **207**, 529–537.
Paton, D. M. 1971 *Comp. Biochem. Physiol.* **40**A, 751–759.
Rang, H. P. & Ritchie, J. M. 1968 *J. Physiol., Lond.* **196**, 183–221.
Ritchie, J. M. & Straub, R. W. 1957 *J. Physiol., Lond.* **136**, 80–97.
Schatzmann, H. J. von 1953 *Helv. physiol. pharmac. Acta* **11**, 346–354.
Straub, R. W. 1961 *J. Physiol., Lond.* **159**, 19–20*P*.
Szurszewski, J. H. & Bülbring, E. 1973 *Phil. Trans. R. Soc. Lond.* B **265**, 149 (this volume).
Taylor, G. S., Paton, D. M. & Daniel, E. E. 1970 *J. gen. Physiol.* **56**, 360–375.
Taylor, G. S., Paton, D. M. & Daniel, E. E. 1971 *Comp. Biochem. Physiol.* **38**A, 251–264.
Thomas, R. C. 1972 *Physiol. Rev.* **52**, 563–594.
Tomita, T. 1970 Electrical properties of mammalian smooth muscle. In *Smooth muscle* (ed. E. Bülbring *et al.*), pp. 197–243. London: Arnold.
Tomita, T. & Yamamoto, T. 1971 *J. Physiol., Lond.* **212**, 851–868.

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