Br. J. Pharmac. (1983), **78**, 405–416

Evidence that ionic channels associated with the muscarinic receptor of smooth muscle may admit calcium

T.B. Bolton & K. Kitamura

Department of Pharmacology, St. George's Hospital Medical School, London SW17 0RE

1 The actions of carbachol on the membrane potential and conductance of smooth muscle of the guinea-pig intestine were investigated using microelectrode recording and the double sucrose-gap method in solutions in which calcium was the only cation creating an inwardly-directed electrochemical gradient.

2 In a calcium chloride solution containing a small amount of potassium but no sodium and buffered to physiological pH (Ca Locke) the membrane was hyperpolarized to more than −80 mV. Carbachol (2×10^{-7}–10^{-4}M) depolarized the membrane and increased the membrane conductance.

3 By passing current the membrane potential of the smooth muscle cells could be varied. In Ca Locke the depolarization produced by carbachol was shown to be reduced if the membrane was depolarized. The relationship between the size of the carbachol depolarization and the membrane depolarization was linear, giving an apparent reversal potential for carbachol depolarization some 20 mV positive to the resting membrane potential, as measured by extracellular electrodes in the sucrose gap.

4 Carbachol depolarization was reduced if the calcium concentration was reduced below 2.5 mM by replacing calcium with Tris, but the depolarization in 2.5 mM Ca and in Ca Locke (100 mM Ca) were of similar size. In Ca-free Na-free solution with EGTA, carbachol depolarization was soon abolished.

5 In the sucrose-gap when the chloride gradient across the cell membrane was reversed by replacing the chloride of Ca Locke by an impermeant anion the membrane depolarized. Carbachol now contracted the muscle but produced a hyperpolarization.

6 These results are consistent with the hypothesis that activation of muscarinic receptors opens ionic channels which at least in the solutions used, can admit sufficient calcium ions to depolarize the cell and cause tension development.

Introduction

The identification of the sources of calcium for contraction of smooth muscle, is of pharmacological importance as these sources may be targets for the action of drugs which reduce smooth muscle tension. Already it seems likely that one class of drugs, the so called 'Ca-antagonists', may exert a major part of their action at one relevant site, viz. channels opened by depolarizing the cell and admitting calcium (see Bolton, 1979; van Breemen, Mangel, Fahim & Meisheri, 1982). Thus, pharmacological interference with these sources is possible and we may anticipate further advances in this area given that inappropriately high smooth muscle tension makes a contribution to several important diseases e.g. asthma, essential hypertension. However, we need more information about these sources.

Present ideas are that stimulant drugs may increase smooth muscle tension by releasing calcium from bound sites in the cell, or by opening membrane pores which allow it to enter down its electrochemical gradient. These pores, or ion channels, may be of two types: one which opens as the membrane is depolarized and which contributes to a major extent to the action potential in excitable smooth muscles, and another which may admit calcium even when the membrane potential does not significantly change (Evans, Schild & Thesleff, 1958; Durbin & Jenkinson, 1961). This latter type is suggested to be rather

0007-1188/83/020405-12 $01.00 © The Macmillan Press Ltd 1983

closely associated with receptors for stimulant substances and therefore has been called a 'receptor-operated channel' (Bolton, 1979; Bolton, Clark, Kitamura & Lang, 1981). We show here that, in high-Ca solutions, calcium may enter the cell through receptor-operated channels in these muscles, which may imply that it can do so under more normal conditions both in this muscle and in others.

Methods

Guinea-pigs, of either sex (300–500 g), were stunned and bled. Strips of ileal longitudinal muscle were separated from the underlying circular muscle as previously described by Bolton (1972a).

For intracellular microelectrode recordings of membrane potential, small portions of ileal longitudinal muscle (5 × 5 mm) or of taenia caeci (2 × 5 mm) were mounted in a chamber with a small volume (0.1 ml). Glass microelectrodes filled with 3M KCl and having resistance of 30–50 Mohm were used to penetrate cells from the serosal side (Bolton 1972b). In Cl-free, Na-free solution, a 2M K citrate microelectrode was used. To minimize the liquid junction potential, an agar bridge (3M KCl Agar–3M KCl –Ag/AgCl) as indifferent electrode was used. Some experiments were done in hypertonic solution, made by the addition of 100 g/l sucrose, to depress contraction (Tomita, 1967; Kuriyama, 1981).

For double sucrose gap recordings, the ends of a strip of taenia caeci (0.5 × 15 mm) were tied with fine threads. One end of the strip was fixed in the isotonic KCl compartment and the other was connected to an isometric force transducer as described by Bolton, Lang & Ottesen (1981). The apparatus for double sucrose gap method was similar to that described by Bübring & Tomita, (1969). The width of test node was about 1 mm.

The temperature was maintained at 35–36°C for microelectrode recording and at 32–33°C for double sucrose gap method throughout the experiments and the rate of flow of solutions was kept constant at about 3 ml/min.

Carbachol chloride (Sigma) was applied by adding it to the solution in the reservoirs or by slow injection using a motor driven syringe (Harvard infusion/withdrawal pump, Model 944A) into the flowing solution. In the latter case, final concentrations of carbachol were calculated from the flow rate of solution. The compositions of the solutions used are shown in Table 1. Experiments in Ca Locke solution were mainly done by using Tris buffered solution. Sometimes 146 mM Cl-containing Ca Locke solution was created by using Ca acetate or propionate. The pH of solutions was adjusted to 7.3–7.4 by bubbling with 95%O_2:5%CO_2 gas for HCO_3^- containing solutions or with 100% O_2 for Tris buffered (Tris-Cl or Tris-malate) solutions. Sometimes the pH of the solutions was checked before and/or after application to the tissue.

Table 1 Ionic composition (mM) of the solutions used for perfusion

	Na	*K*	*ca*	*Mg*	*Cl*	*HCO_3*	Tris	*Propionate* or acetate	*Benzene* sulphonate	Malate	Glucose
Locke	143	5.9	2.5	—	148	5.9	—	—	—	—	11.4
Ca Locke	—	5.9	102	—	204	5.9	—	—	—	—	11.4
*	—	5.7	100	—	209	—	3.8	—	—	—	11.1
+	—	5.7	100	—	146	—	3.8	63	—	—	11.1
Cl-free Ca Locke	—	5.6	97	—	—	—	7.3	195	5.6	7.3	11.0
Na-free (Tris) Locke											
0 Ca	—	5.9	—	—	146	5.9	146	—	—	—	11.4
2.5 Ca	—	5.9	2.5	—	147	5.9	142	—	—	—	11.4
10 Ca	—	5.9	10	—	152	5.9	131	—	—	—	11.4
Cl-free Na-free (Tris) Locke											
0 Ca	—	5.9	—	—	—	—	146	—	5.9	146	11.4
2.5 Ca	—	5.9	2.5	—	—	—	142	5.0	5.9	142	11.4
Mg Locke											
0 Ca	—	5.9	—	98	195	5.9	—	—	—	—	11.4
2.5 Ca	—	5.9	2.5	95	195	5.9	—	—	—	—	11.4

* Ca Locke solution adjusted to pH 7.4 by Tris-Cl.
+ Ca Locke solution in which the Cl concentration was 146 mM.

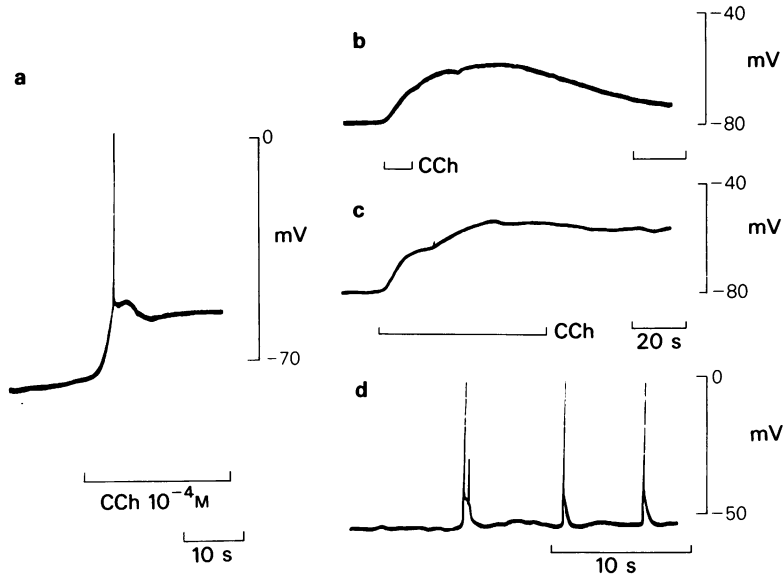


Figure 1 Depolarization of guinea-pig ileal longitudinal muscle by carbachol (10^{-4}M) in isotonic or hypertonic Ca Locke solution recorded by microelectrode. (a) Carbachol (CCh) was applied 5 min after starting perfusion with isotonic Ca Locke solution; (b) 10 s application of carbachol 5 min after starting perfusion with hypertonic Ca Locke solution; (c) 1 min application of carbachol 10 min after starting perfusion with hypertonic Ca Locke solution; (d) spontaneous action potentials in hypertonic Ca Locke solution which appeared 3 min after the record shown in (c) was obtained.

Results

The membrane potential in smooth muscle, as in other excitable cells, is believed to be determined mainly by the potassium diffusion potential and in guinea-pig ileal longitudinal muscle or taenia caeci in normal Locke solution it is generally negative to −50 mV. The application of carbachol or other muscarinic agonist depolarizes the muscle in this solution. This depolarization is believed to result largely from increased fluxes of sodium across the membrane, but there are also increased fluxes of other ions, especially potassium (see Bolton, 1979).

In a solution which contains calcium as the main cation (Ca Locke, Table 1) the inwardly directed calcium gradient is the only cationic gradient capable of depolarizing the membrane should this occur upon application of carbachol. In this solution, the sodium gradient is reversed and the potassium gradient is also outward. The outward movement of potassium, and of sodium should the cells still contain this cation, will result in hyperpolarization.

Experiments in Ca Locke solution

In Ca Locke solution, the membrane was hyperpolarized and spontaneous action potentials were abolished as described by Sakamoto (1971). The mean membrane potential obtained from guinea-pig ileal longitudinal muscle was -81.9 ± 7.1 mV (s.d.; $n = 44$) 5–30 min after perfusion with Ca Locke solution. In this solution carbachol depolarized the muscle (Figure 1a). In the experiment of Figure 1a the membrane potential was −78 mV. Carbachol (10^{-4}M) depolarized the membrane and produced an action potential with overshoot. As it was difficult to maintain the microelectrode in the cell during contraction, sucrose hypertonic Ca Locke solution was used in further experiments. The membrane potential of ileal longitudinal muscle in hypertonic Ca Locke solution muscle was -83.0 ± 11.2 mV ($n = 45$), this value being nearly the same as that in isotonic solution. Short application (10 s) of 10^{-4}M carbachol depolarized the membrane. Repolarization was slow (Figure 1b). Longer application of 10^{-4}M carbachol (1 min) depolarized the membrane but complete repolarization did not occur (Figure 1c). We did not see an initial spike upon application of carbachol in hypertonic Ca Locke solution, but spontaneous spike discharges appeared after 3 min in the same cell, as shown in Figure 1d. The membrane potential at this time was nearly −50 mV and spikes reached 0 mV.

The mean of the peak amplitude of depolarization

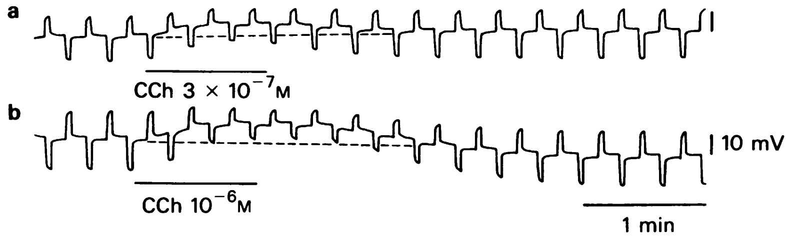


Figure 2 Effects of low concentrations of carbachol (CCh) in Ca-Locke solution on taenia caeci recorded by the double sucrose gap method. In (a) and (b) 3×10^{-7} and 10^{-6}M carbachol were applied respectively for 1 min. Broken lines show the resting membrane potential before application of carbachol. Vertical calibration applies to all records. In these and other double sucrose gap experiments, alternate depolarizing and hyperpolarizing rectangular current pulses were applied to evoke electronic potentials. The change in size of these was used to detect any change in membrane resistance. Because of a high threshold, depolarizing electronic potentials did not elicit action potentials in this preparation.

by 10^{-4}M carbachol in hypertonic solution was 24.6 ± 5.3 mV ($n = 16$). Following application of 10^{-4}M carbachol the membrane usually remained partially depolarized in the microelectrode-penetrated cell, but other cells still recovered a high membrane potential of about -80 mV. Presumably the combined effects of microelectrode penetration and application of a high concentration of carbachol had a permanently deleterious effect. The mean membrane potential of taenia in Ca Locke solution was -73.8 ± 7.8 mV $n = 12$) in isotonic solution and -76.3 ± 4.8 mV ($n = 10$) in hypertonic solution. Carbachol (10^{-4}M) depolarized the membrane, and the average amplitude of depolarization was 21.4 ± 4.8 mV ($n = 7$) in hypertonic Ca Locke solution. These observations are similar to those in ileal longitudinal muscle.

Using the double sucrose gap, carbachol (3×10^{-7}M or more) depolarized the membrane in Ca Locke solution and reduced the size of the electronic potential (Figure 2). The membrane repolarized rapidly after application of these lower concentrations of carbachol. The depolarization was reduced when carbachol ($> 10^{-6}$M) was applied several times for 1 min each. A contraction always accompanied the depolarization. Figure 4a shows the relationship of the size of the depolarization to the concentration of carbachol in Ca Locke solution obtained by the

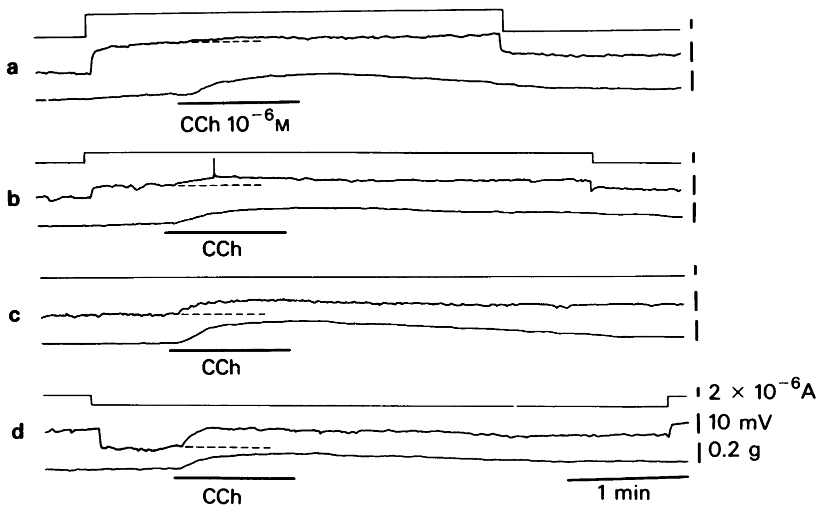


Figure 3 Changes in the amplitude of carbachol (CCh) depolarization after shifting the membrane potential by constant current in Ca Locke solution. Recorded by the sucrose gap method. Carbachol 10^{-6}M was applied for 1 min. Bottom trace shows tension recorded. (a) and (b) The membrane potential was displaced by depolarizing current; (c) control; (d) the membrane potential was displaced by hyperpolarizing current. Dotted lines show the membrane potential before application of carbachol. Vertical calibrations apply to all records.

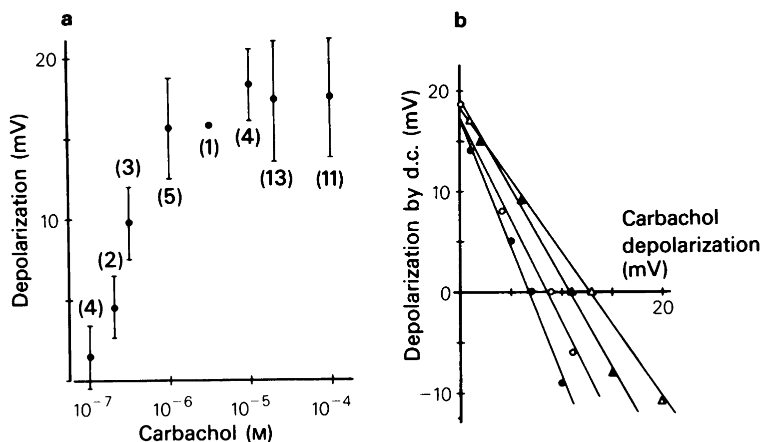


Figure 4 (a) The relationship between depolarization (mV) and concentration of carbachol in Ca Locke solution; (●) mean amplitude of depolarization, s.d. shown by vertical lines. The numbers of observations are indicated in parentheses. The individual amplitudes of depolarization were normalized by comparison with the maximum depolarization by carbachol (10^{-4}M) in normal Locke solution using the following equation (normalized V_c) = (V_c)($\bar{V}$)/(V). V_c: amplitude of depolarization at some concentration of carbachol in Ca Locke solution. V: amplitude of depolarization produced by 10^{-4}M carbachol in normal Locke solution on the same preparation. $\bar{V}$: the mean amplitude of carbachol depolarization (10^{-4}M) in normal Locke solution (23.7 mV). (b) The relationship between carbachol depolarization and the membrane potential shifted by constant currents using the double sucrose gap method. (○, ●) Amplitude of response to 10^{-6}M carbachol in Ca Locke solution; (△, ▲) amplitude of response to 2×10^{-5}M carbachol in Na-free (Tris) Locke solution (2.5 mM Ca^{2+}). Open and filled symbols represent observations obtained from different preparations. Straight lines were drawn by eye.

double sucrose gap technique. The individual depolarizations were normalized in each tissue using the response to 10^{-4}M carbachol in normal Locke solution as a standard (see Figure 4). The mean amplitude of depolarization by 10^{-4}M carbachol in normal Locke solution was 23.7 ± 7.9 mV ($n = 38$). In Ca Locke solution 10^{-7}M carbachol did not significantly depolarize the membrane, but 2×10^{-7}M carbachol depolarized the membrane slightly. Carbachol (10^{-6}M) depolarized the membrane submaximally (15.6 ± 3.2 mV; $n = 5$) and the average of the maximum depolarization obtained by using 10^{-4}M carbachol in Ca Locke solution was 17.4 ± 3.6 mV ($n = 11$). Thus this value was slightly smaller than that in normal Locke solution.

To study whether carbachol depolarization in Ca Locke solution was voltage-dependent or not, the membrane potential was displaced positively and negatively by constant current as shown in Figure 3. Carbachol (10^{-6}M) was used so that consistent responses could be repeatedly obtained. The carbachol depolarization was almost abolished when the membrane was strongly depolarized (Figure 3a). Moderate depolarization also reduced the amplitude of the carbachol response and a small spike appeared during the carbachol depolarization (Figure 3b). Membrane hyperpolarization by constant current increased the amplitude of carbachol depolarization compared with the control (Figure 3d). These results show that the depolarization by carbachol in Ca Locke solution is affected by the level of the membrane potential. Figure 4b shows the relationship between the carbachol depolarization and membrane potential shifted by constant currents in Ca Locke and in Na-free (Tris) Locke solutions. There was a linear relationship between the size of the carbachol depolarization and the membrane potential changed by passing current. A similar relationship was also observed in Na-free (Tris) Locke solution (2.5 mM Ca) using 2×10^{-5}M carbachol. Although in the present experiments we could not obtain a reversal of carbachol depolarization by passing current, the reversal potential level for carbachol in Ca Locke and Na-free (Tris) Locke solutions could be obtained by extrapolating the lines. The reversal potential was about 17 mV depolarized to the resting membrane potential. This value was close to the membrane potential level during maximal carbachol depolarization (Figure 4a).

Experiments in Na-free (Tris) Locke

The influence of external Ca concentration on the carbachol depolarization was studied in Na-free Locke. Upon changing to this solution the membrane potential was transiently hyperpolarized and then repolarized slowly. The mean membrane potential as measured by microelectrode in taenia caeci in Na-

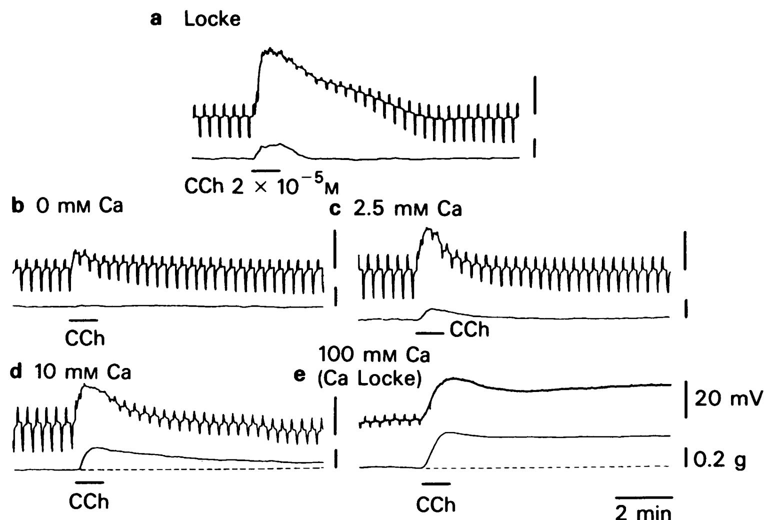


Figure 5 Changes in carbachol (CCh) depolarization in Na-free Locke solutions containing various concentrations of calcium (sucrose gap recording). (a) Control in normal Locke solution (2.5 mM Ca); (b) 30 min after perfusion with 0 mM Ca (without EGTA) Na-free (Tris) Locke solution; (c) 30 min after perfusion of 2.5 mM Ca Na-free (Tris) Locke solution; (d) 30 min after perfusion of 10 mM Ca, Na-free (Tris) Locke solution. (a–d) were obtained from the same preparation and carbachol (2×10^{-5}M) was applied for 1 min. (e) Twenty min after perfusion of Ca Locke solution. Carbachol 10^{-4}M was applied for 1 min. (e) was obtained from a different preparation from (a–d). Broken lines in (d) and (e) show the resting tension. Vertical calibrations are 20 mV and 0.2 g throughout.

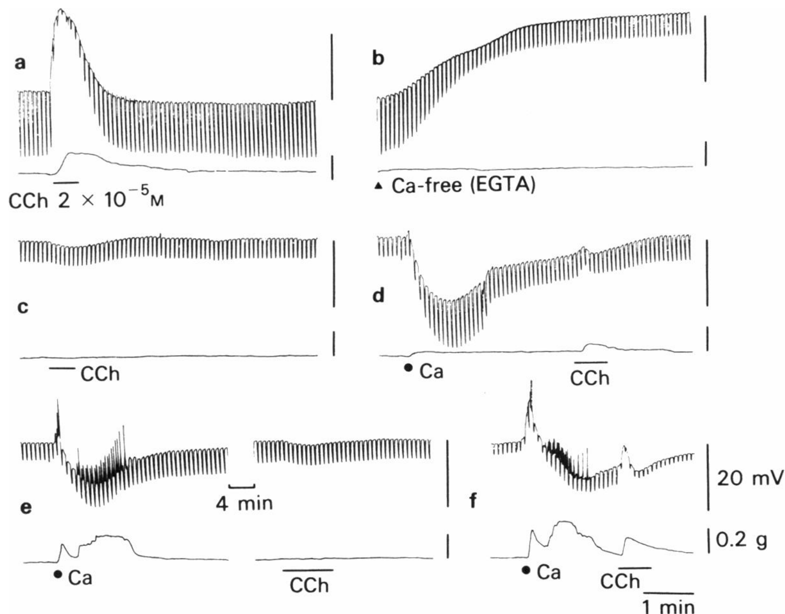


Figure 6 Carbachol (CCh) action in Ca-free (0.5 mM EGTA) Na-free (Tris) Locke solution. (a) Carbachol (2×10^{-5}M) was applied in 2.5 mM Ca Na-free Locke solution; (b) at the beginning of the record, Ca-free (EGTA) Na-free (Tris) Locke solution was superfused; (c) carbachol (2×10^{-5}M) was applied 40 min after (b) and produced very slight hyperpolarization; (d–f) carbachol (2×10^{-5}M) was applied 3 min, 8 min and 2 min respectively after exposure to high Ca solution. Ca indicates the application of Ca Locke solution (100 mM Ca) for 10 s. Carbachol was applied for 30 s (a, c), 40 s (d,f) or 1 min (e). Before readmission of Ca in (d), (e) and (f), carbachol (2×10^{-5}M) neither depolarized the membrane nor elicited contraction; (a–f) were obtained from same preparation in the sucrose gap.

free (Tris) Locke solution at steady state was -43.7 ± 4.5mV ($n = 40$; in Locke solution -46.5 ± 6.6 mV; $n = 35$). However, by the double sucrose gap method, the membrane always hyperpolarized to nearly the same extent as in Ca Locke solution and this hyperpolarization was maintained for 2–3 h in Na-free (Tris) Locke solution.

Figure 5 shows the effects of 0 mM, 2.5 mM, 10 mM and 100 mM Ca on carbachol depolarization. Carbachol (2×10^{-5}M) depolarized the membrane about 32 mV in this preparation in normal Locke solution (Figure 5a). When 0 mM Ca Na-free (Tris) Locke solution was perfused, carbachol (2×10^{-5}M) depolarized the membrane slightly and during the depolarization the membrane resistance was decreased (Figure 5b). By increasing the Ca concentration to 2.5 mM (Figure 5c), the amplitude of depolariazation and the effect on the membrane conductance were increased. Contraction also appeared. Further increasing the Ca concentraction to 10 mM hardly changed the amplitude of depolarization but still increased the effect on the membrane conductance and amplitude of contraction (Figure 5d). The membrane rapidly repolarized even during the application of carbachol in these solutions containing low Ca concentrations, but contraction was prolonged presumably because of lack of a Ca extrusion mechanism such as the Na-Ca exchange mechanism. (Brading, 1981a). In Ca Locke solution an even higher concentration of carbachol (10^{-4}M) depolarized the membrane nearly to the same extent as in 2.5 mM and 10 mM Ca Na-free (Tris) Locke solution (Figure 5e). However, neither relaxation nor repolarization followed return to carbachol-free solution, and the electrotonic potential was too small to be detected.

Figure 6 shows the effects of carbachol in Ca-free (0.5 mM EGTA) Na-free (Tris) Locke solution. The membrane depolarized in this solution, and after 40 min carbachol (2×10^{-5}M) did not depolarize the membrane (Figure 6b and c). Short application of 100 mM Ca (10 s) depolarized the membrane transiently and then hyperpolarized it (Figure 6d–f). In some cases, during the changes in membrane potential, spontaneous spikes were observed which were associated with large contractions (Figures 6e and f). The hyperpolarization brought about by Ca application was followed by slow depolarization. Three minutes after application of 100 mM Ca when the membrane potential had not yet returned to its previous level, carbachol (2×10^{-5}M; 40 s) depolarized the membrane slightly (Figure 6d). When the interval between Ca and carbachol application was longer (8 min), carbachol did not depolarize the membrane and did not produce contraction (Figure 6e). If the interval was shortened (to 2.0 min) a large depolarization and contraction were observed (Figure 6f). A small hyperpolarization followed the initial depolarization (Figure 6d, f). The amplitude of carbachol depolarization also varied with the Ca concentration in Mg Locke solution. The mean membrane potential of taenia in Mg Locke solution measured by microelectrode was -51.8 ± 4.8 mV ($n = 15$), slightly higher than in normal Locke solution

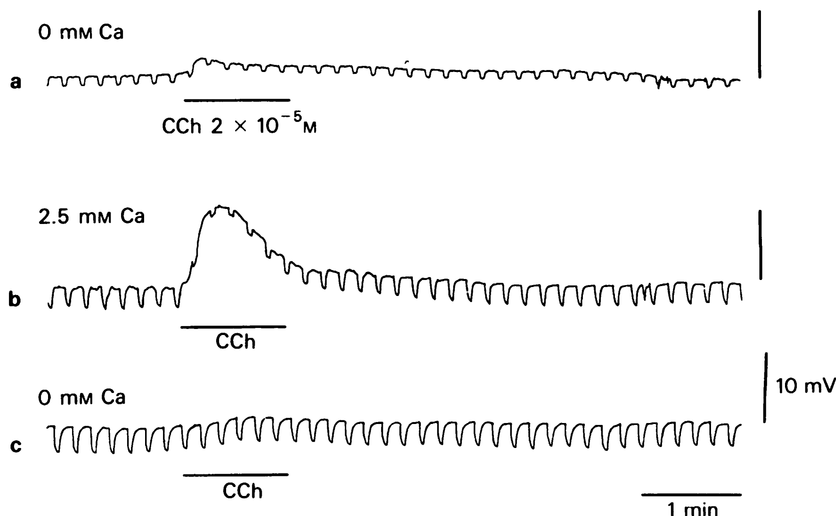


Figure 7 Effect on depolarization by carbachol (CCh, 2×10^{-5}M) of varying the Ca concentration in Mg Locke solution. (a) Carbachol was applied for 1 min, 30 min after starting perfusion with 0 mM Ca, Mg Locke solution; (b) carbachol was applied for 1 min, 30 min after starting perfusion with 2.5 mM Ca, Mg Locke solution; (c) Response to carbachol 20 min after returning to 0 mM Ca, Mg Locke solution. (a–c) were obtained from the same preparation in the sucrose gap.

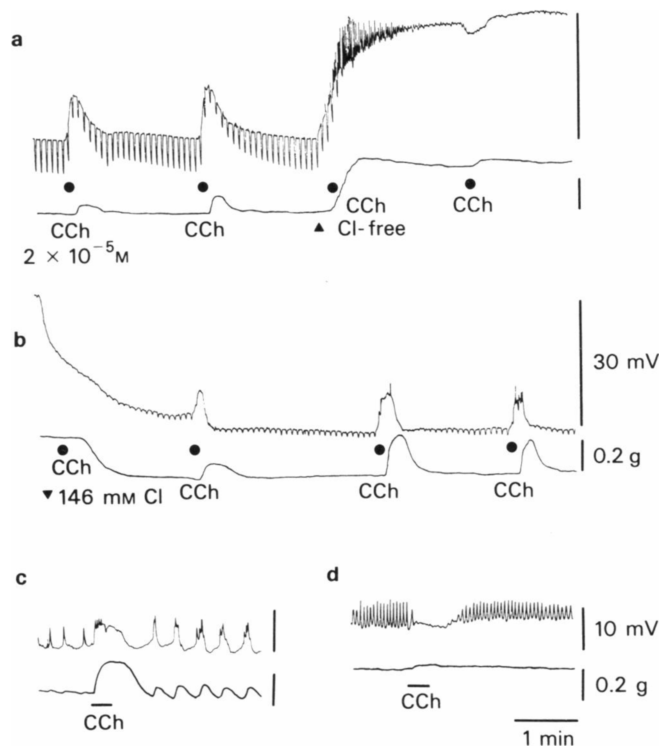
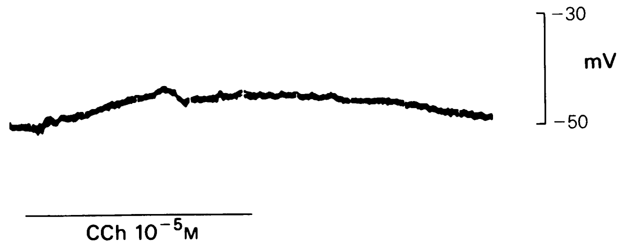


Figure 8 Hyperpolarizing responses to carbachol (CCh) in Cl-free, Na-free (Tris) Locke solution (a, b) and in Na-free (Tris) Locke solution containing 2.5 mM Ca (c, d). (a,b) The preparation was in Na-free (Tris) Locke solution; carbachol (2×10^{-5}M) was applied at (●) for 5 s every 2 min. Cl-free, Na-free (Tris) Locke solution was superfused between (▲) and (▼). (a) and (b) are continuous recordings; (c,d) carbachol (2×10^{-5}M) was applied for 20 s; (d) was recorded 30 min after (c). All records were obtained from the same preparation in the sucrose gap.

(-46.4 ± 3.4 mV; $n=11$). When Ca was omitted from Mg Locke solution, the membrane potential slightly depolarized but the average of the membrane potential had nearly the same value (-48.0 ± 5.7 mV $n=12$). Thirty min after perfusion with 0 mM Ca Mg Locke solution, carbachol (2×10^{-5}M depolarized the membrane and increased the membrane conductance slightly (Figure 7a). In 2.5mM Ca Mg Locke solution, carbachol (2×10^{-5}M) produced a large depolarization (Figure 7b). After returning to 0 mM Ca Mg Locke solution, carbachol produced only a small depolarization (Figure 7c). These results suggest that even small amounts of Ca, which remained near to the membrane or bound to the membrane, were sufficient to produce depolarization.

In the experiments described so far, it was observed that carbachol application depolarized the membrane in solutions where calcium was the only cation exerting an inwardly directed electrochemical gradient. The reduction in the size of the electronic potential produced by carbachol application implied that activation of muscarinic receptors opens additional ion channels in the membrane. A simple explanation for the depolarization observed is that these additional channels allow increased inward Ca flux through them and so depolarization of the membrane. However, it is possible that the outward movement of an anion, e.g. chloride, through these channels could explain the observed depolarization. For this reason experiments were carried out in Cl-free solutions to investigate this possibility. In sucrose-gap experiments we were unable to obtain a depolarization to carbachol in Cl-free, Na-free (Tris) solution and in Cl-free, Ca-Locke solution, because of a large depolarization by Cl-free solution itself when the double sucrose gap method was used. Usually, Cl-free, Na-free solutions, which contained 0, 2.5, or 100 mM Ca, depolarized the membrane immediately by 20 to 30 mV. This depolarization easily exceeded the carbachol reversal potential and produced contraction. This depolarization in Cl-free, Na-free solution obtained using the double sucrose gap method did not subside unless chloride was readmitted. During this depolarization, carbachol (2×10^{-5}M) hyperpolarized the membrane but increased tension. Figure 8a and b shows a typical depolarization by



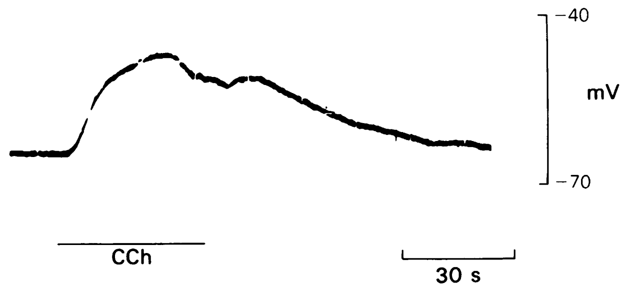


Figure 9 Effects of carbachol (CCh, 10^{-5}M) on taenia caeci after 40 min in hypertonic Cl-free, Na-free (Tris) Locke solution (2.5 mM Ca) (a) and after 70 min in hypertonic Cl-free, Ca Locke solution (b) recorded using microelectrodes filled with 2M K citrate.

Cl-free, Na-free (Tris) Locke solution. The amplitude of depolarization was 30 mV; it produced spontaneous spikes and contraction, and decreased the membrane resistance. Carbachol (2×10^{-5}M; 5 s) consistently depolarized the membrane in Na-free (Tris) Locke solution (Figure 8a). When carbachol was applied during the initial depolarization produced by Cl-free solution, no response to carbachol was seen. After about 2 min in Cl-free, Na-free (Tris) solution, carbachol hyperpolarized the membrane but still increased tension (Figure 8a). While repolarization was occurring after readmitting 146 mM Cl, carbachol depolarized the membrane slightly and 2 min after returning to Na-free (Tris) Locke solution, carbachol evoked depolarization (Figure 8b). Hyperpolarization by carbachol was also observed in the same preparation in Cl-containing, Na-free solution as shown in Figure 8c and d. In these records (c, d) the membrane potential was low due to repeated exposure to Cl-free solution, and spontaneous spikes were observed. Carbachol (2×10^{-5}M) depolarized the membrane (Figure 8c). However, 30 min later, when the membrane had further depolarized, carbachol hyperpolarized the membrane and inhibited action potential discharge (Figure 8d).

The large depolarization by Cl-free, Na-free solution, observed using the double sucrose gap method, was not seen if microelectrode recording was used. The mean membrane potential in Cl-free, Ca-Locke solution measured by microelectrode in taenia was 69.0 ± 3.6 mV ($n = 12$, in hypertonic solution using K-citrate electrode) and in Cl-free, Na-free (Tris) Locke solution was -42.4 ± 4.1 mV ($n = 9$; in hypertonic solution using K-citrate electrode). These values are nearly the same as in Cl-containing solutions (see above). Figure 9 shows the effects of carbachol (10^{-5}M) in Cl-free, Na-free (Tris) and Cl-free, Ca-Locke solutions. Carbachol depolarized the membrane slightly (7 mV) (Figure 9a) after 40 min in Cl-free, Na-free (Tris) Locke solution, and a large depolarization was observed after 70 min in Cl-free, Ca-Locke solution (Figure 9b).

Discussion

The depolarization and increase in membrane conductance produced by carbachol in Ca Locke solution could arise if additional channels were opened in the membrane by the activation of muscarinic recep-

tors which allowed calcium to enter the cell in increased amounts. Such a theory would also explain why activation of receptors can produce contraction of smooth muscle in a high potassium solution (Evans *et al.*, 1958) when there is no change in membrane potential (Bülbring & Szurszewski, 1974) and why there is an increased influx of calcium at this time (Durbin & Jenkinson, 1961). More recent work (Meisheri, Hwang & van Breemen, 1981) has also reinforced the hypothesis that calcium may enter ion channels in the membrane which are rather closely associated with receptors for stimulant substances ('receptor-operated channels', ROCs, Bolton, 1979) perhaps even with receptors for more than one type of stimulant (Bolton *et al.*, 1981) and which do not primarily open in response to depolarization of the membrane as do 'potential-sensitive' ion channels. The actions of calcium blocking agents (van Breemen, Hwang & Meisheri, 1981; Meisheri *et al.*, 1981; van Breemen *et al.*, 1982) also support the division of channels admitting calcium into two types.

In the present work the idea that calcium can enter ROCs rests on several assumptions, not least that ROCs are essentially impermeable to the various foreign ions used to replace the common physiological ones, and that the depolarization seen upon application of carbachol does not result from the egress of anions from the cell. Removing chloride from the bathing solution reduces the internal chloride in these cells to very low levels (Casteels, 1971) but it is impossible to eliminate all other cellular anions which may conceivably pass through ROCs. However, the balance of probabilities seems to indicate that calcium may enter through ROCs under these albeit very artificial conditions, and by implication therefore may enter under more normal conditions and into other types of smooth muscle.

The almost complete loss of the conductance change upon carbachol application after a few minutes in Ca-free EGTA solution supports the suggestion (Bolton, 1979) that calcium is necessary for some part of the process whereby channel opening is brought about, either by enabling activation of the muscarinic receptor by carbachol or by enabling linkage of activated receptors to channel opening. Readmission of calcium for a brief period (Figure 6) restored the conductance change to carbachol and a small depolarization reappeared. Varying $[Ca^{2+}]_o$ in a Na-free solution revealed that the size of the depolarization by carbachol increased as $[Ca^{2+}]_o$ increased up to about 2.5 mM. Above this no further increase in size occurred but the duration continued to increase up to 100 mM. In $[Ca^{2+}]_o$ greater than 2.5 mM the increase in conductance produced by larger concentrations of carbachol was probably sufficient for the membrane potential to approach the equilibrium potential for the muscarinic ROCs (see Bolton, 1972a). Further support for this comes from experiments (Figures 3, 4b) in which the membrane potential was shifted by passing constant currents. The extrapolated equilibrium potential was some 17 mV or so depolarized to the resting membrane potential in the sucrose gap, and close to the membrane potential level during the peak depolarizing action of larger concentrations of carbachol (Figure 5). (This level was some 23 mV positive to the membrane potential when measured by microelectrode). Further support for the notion comes from the effects of Cl-free solution which depolarized the membrane in the sucrose-gap apparatus. In this solution the response to carbachol was reversed to a hyperpolarization at a level consistent with the presumed equilibrium potential for muscarinic ROCs. The position of this equilibrium potential presumably reflects the balance between outward movement of potassium ions and inward movement of calcium ions.

As the peak depolarization by carbachol was not appreciably affected by increasing $[Ca^{2+}]_o$ from 2.5 to 100 mM in Na-free solution, it is possible that the equilibrium potential for muscarinic ROCs does not change much despite the fact that the calcium equilibrium potential, E_{Ca}, presumably moves substantially more positively as $[Ca^{2+}]_o$ is increased. This may reflect a relative increase in K current, a relative decrease in Ca current, or a combination of these, occurring as $[Ca^{2+}]_o$ is increased. As the contraction increased as $[Ca^{2+}]_o$ was increased, the first possibility would seem the most likely.

The internal calcium concentration can be increased by carbachol through several mechanisms (Bolton, 1979; Brading 1981a,b; Brading & Sneddon, 1980). Our present observations combined with those of others suggests that calcium can move into the cell through ROCs. However, it is uncertain whether this calcium alone is sufficient to cause the observed rises in tension. Nevertheless, the contraction produced by carbachol application increased in size as $[Ca^{2+}]_o$ was increased and, although the exhaustive experiments were not done to study specifically the effect on conductance, it seemed that the increased size of contraction was paralleled by the effect on membrane conductance.

Recently it has been described how contractions to higher concentrations of carbachol persist for a few minutes in Ca-free EGTA solution before disappearing (Casteels & Raeymaekers, 1979; Brading, Burnett & Sneddon, 1980). The present results confirm this. In the works cited the carbachol response could be restored by brief readmission of calcium in Na-free (Tris) solution, but not in Mg Locke solution. We have shown that brief readmission of calcium hyperpolarized a depolarized membrane and that the depolarizing response to carbachol returns for a short

period associated with a brief return of the contractile response. These results also suggest an explanation for the relaxations of carbachol-induced tension which Brading & Sneddon (1980) could produce by application of sodium substitutes (Tris, Li, sucrose, Mg). Sodium substitutes will substantially reduce the depolarizing action of carbachol (Bolton, 1972a; 1973) firstly because Ca channels will close due to the repolarization brought about by the negative shift in E_{Na} and secondly because foreign ions or Na substitutes interfere with the ability of carbachol to activate its receptors; for example, in sucrose hypertonic solutions sensitivity to carbachol, measured by its effects on the membrane conductance, is drastically reduced (cf. Bolton 1971 and 1972a; also unpublished observations).

Two observations of discrepancies between sucrose gap and microelectrode experiments are worthy of note. In Na-free solution, taenia normally contracts and the membrane potential as measured by microelectrode is somewhat depolarized (cf. Kuriyama, 1963; Brading *et al.*, 1980; Hirata, Itoh & Kuriyama, 1981). However, in the sucrose-gap the muscle was relaxed and changing from normal Locke to Na-free solution hyperpolarized the membrane. In Cl-free solution our microelectrode measurements showed a normal membrane potential after 30 min, whereas in the double sucrose-gap, substantial depolarization occurred and persisted. The detailed explanation of these differences is obscure but it is almost certainly related to the polarizing effects of local circuit currents in the double sucrose gap which alters nodal potential (see Bolton, Tomita & Vassort, 1981). In the lobster giant axon when this method was used, similar polarizations were observed (Julian, Moore & Goldman, 1962).

Contractions of taenia to concentrations of carbachol as high as 10^{-3}M disappear or are very severely reduced within 5 min of immersing in a Ca-free EGTA solution (Casteels & Raeymaekers 1979; Brading & Sneddon, 1980). However, the rate of disappearance of this response is slower than the disappearance of the high-K contraction. For this reason, and to explain associated observations, it has been postulated that activation of muscarinic receptors releases calcium from storage sites in this muscle. Our experiments studied the electrophysical effects of Ca-free (EGTA) solution which was already sodium-free, so our results may not be an accurate indication of the electrophysiological effects of the Ca-free condition when sodium is present. Similarly, the effects of readmitting Ca which we observed are subject to the same proviso. Nevertheless, it was significant that loss of the contractile response to carbachol was associated with loss of an effect on the membrane conductance, and that the restorative effects of brief calcium readmissions were associated with a recovery of the effects on the membrane conductance and potential. It would seem that calcium stores in this muscle, such as exist, are associated with the receptor. Release of calcium from association with the receptor by the action of carbachol, serves to produce the effect on the membrane of opening ion channels. Moreover, the depolarization which occurs (e.g. Figure 6a, e) implies the existence of a calcium gradient which does not completely decay until 3–4 min have elapsed following Ca readmission. Whatever the detailed explanation, after Ca readmission there was a striking parallelism of the disappearance of the membrane and of the contractile responses.

This work was supported by the Medical Research Council.

References

BOLTON, T.B. (1971). On the nature of the oscillations of the membrane potential (slow waves) produced by acetylcholine or carbachol in intestinal smooth muscle. *J. Physiol.*, **216,** 403–418.

BOLTON, T.B. (1972a). The depolarizing action of acetylcholine or cabachol in intestinal smooth muscle. *J. Physiol.*, **220,** 647–671.

BOLTON, T.B. (1972b). The effects of varying the concentration of ions in the external solution on the oscillations of the membrane potential (slow wave) produced by carbachol in longitudinal ileal muscle. *Pflüger Arch.*, **335,** 85–96.

BOLTON, T.B. (1973). The permeability change produced by acetylcholine in smooth muscle. In *Drug Receptors*, ed. Rang, H.P. pp. 87–104. London, Basingstoke: MacMillan Press.

BOLTON, T.B. (1979). Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol. Rev.*, **59,** 606–718.

BOLTON, T.B. CLARK, J.P., KITAMURA, K. & LANG, R.J. (1981). Evidence that histamine and carbachol may open the same ion channels in longitudinal smooth muscle of guinea-pig ileum. *J. Physiol.*, **320,** 363–379.

BOLTON, T.B., LANG, R.J. & OTTESEN, B. (1981). Mechanism of action of vasoactive intestinal polypeptide on myometrial smooth muscle of rabbit and guinea-pig. *J. Physiol.*, **318,** 41–55.

BOLTON, T.B., TOMITA, T. & VASSORT, G. (1981). Voltage clamp and the measurement of ionic conductances in smooth muscle. In *Smooth Muscle: an Assessment of Current Knowledge*. ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp. 47–64. London: Edward Arnold.

BRADING, A.F. (1981a). Ionic distribution and mechanisms of transmembrane ion movements in smooth muscle. In *Smooth Muscle: an Assessment of Current Knowledge.* ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp. 65–92. London: Edward Arnold.

BRADING, A.F. (1981b). How do drugs initiate contraction in smooth muscle? *Trends in Pharmac. Sci.*, **26**, 261–265.

BRADING, A.F., BURNETT, M. & SNEDDON, P. (1980). The effect of sodium removal on the contractile responses of the guinea-pig taenia coli to carbachol. *J. Physiol.*, **306**, 411–429.

BRADING, A.F. & SNEDDON, P. (1980). Evidence for multiple sources of calcium for activation of the contractile mechanism of guinea-pig taenia coli on stimulation with carbachol. *Br. J. Pharmac.*, **70**, 229–240.

BÜLBRING, E. & SZURSZEWSKI, J.H. (1974). The stimulant action of noradrenaline (alpha-action) on guinea-pig myometrium compared with that of acetylcholine. *Proc. R. Soc. B.*, **185**, 225–262.

BÜLBRING, E. & TOMITA, T. (1969). Suppression of spontaneous spike generation by catecholamines in the smooth muscle of the guinea-pig taenia coli. *Proc. R. Soc. B.*, **172**, 103–119.

CASTEELS, R. (1971). The distribution of chloride ions in the smooth muscle cells of guinea-pig's taenia coli. *J. Physiol.*, **214**, 225–243.

CASTEELS, R. & RAEYMAEKERS, L. (1979). The action of acetylcholine and catecholamines on an intracellular calcium store in the smooth muscle cells of the guinea-pig taenia coli. *J. Physiol.*, **294**, 51–68.

DURBIN, R.P. & JENKINSON, D.H. (1961). The effect of carbachol on the permeability of depolarised smooth muscle to inorganic ions. *J. Physiol.*, **157**, 74–89.

EVANS, D.H.L., SCHILD, H.O. & THESLEFF, S. (1958). Effects of drugs on depolarized plain muscle. *J. Physiol.*, **143**, 474–485.

HIRATA, M., ITOH, T. & KURIYAMA, H. (1981). Effects of external cations on calcium efflux from single cells of the guinea-pig taenia coli and porcine coronary artery. *J. Physiol.*, **310**, 321–336.

JULIAN, F.J., MOORE, J.W. & GOLDMAN, D.E. (1962). Membrane potentials of the lobster giant axon obtained by use of the sucrose gap technique. *J. gen. Physiol.*, **45**, 1195–1216.

KURIYAMA, H. (1963). The influence of potassium, sodium and chloride on the membrane potential of the smooth muscle of taenia coli. *J. Physiol.*, **166**, 15–28.

KURIYAMA, H. (1981). Excitation-contraction coupling in various visceral smooth muscles. In *Smooth Muscle: an Assessment of Current Knowledge.* ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp. 171–198. London: Edward Arnold.

MEISHERI, K.D., HWANG, O. & VAN BREEMEN, C. (1981). Evidence for two separate Ca^{2+} pathways in smooth muscle plasma lemma. *J. membr. Biol.*, **59**, 19–25.

SAKAMOTO, Y. (1971). Electrical activity of guinea-pig taenia coli in calcium Locke solution. *Jap. J. Physiol.*, **221**, 295–306.

TOMITA, T. (1967). Current spread in the smooth muscle of the guinea-pig vas deferens. *J. Physiol.*, **189**, 163–176.

VAN BREEMEN, C., HWANG, O. & MEISHERI, K.D. (1981). Mechanism of inhibitory action of diltiazem on vascular smooth muscle contractility. *J. Pharmac. exp. Ther.*, **218**, 459–463.

VAN BREEMEN, C., MANGEL, A., FAHIM, M. & MEISHERI, K. (1982). Selectivity of calcium antagonistic action in vascular smooth muscle. *Am. J. Cardiol.*, **49**, 507–510.

(Received August 17, 1982.
Revised September 24, 1982.)