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# EVIDENCE FOR MULTIPLE SOURCES OF CALCIUM FOR ACTIVATION OF THE CONTRACTILE MECHANISM OF GUINEA-PIG *taenia coli* ON STIMULATION WITH CARBACHOL

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**1** The evidence presented suggests there are three sources of Ca available for contraction of the smooth muscle of the guinea-pig *taenia coli* on stimulation with carbachol; the inward Ca current of the spike, a second voltage-dependent Ca channel and an internal Ca store.

**2** The initial increment of tension in response to carbachol is thought to be due to an increase in spike frequency which is probably the main source of Ca at low carbachol concentrations ($< 10^{-6}$ M).

**3** The maintained tension in the continuous presence of high concentrations of carbachol seems to involve continuous influx of membrane-bound Ca by a potential-dependent mechanism which can be very quickly deactivated, resulting in rapid relaxation. This mechanism can be blocked by $2 \times 10^{-7}$ M methoxyverapamil (D600).

**4** An internal Ca store can be released by high concentrations of carbachol ($< 10^{-6}$ M) and is probably responsible for the initial transient peak of tension, of about 5 min duration seen on continuous application of high concentrations of carbachol and for the tension increase in response to carbachol in tissues depolarized in high-K.

**5** Investigation of the properties of the store indicates that it; (i) is very rapidly filled by application of high extracellular Ca; (ii) empties after a few minutes in zero-Ca EGTA Krebs solution; (iii) can be refilled in depolarized tissues in the presence of low concentrations of D600 and Mn, but does not refill during application of carbachol at concentrations greater than $10^{-6}$ M; (iv) contains enough Ca for one near-maximal contraction and once emptied can assist relaxation by Ca re-uptake.

## Introduction

There is good evidence that activation of the contractile mechanism in smooth muscles is triggered by an increase in free Ca ions in the cytoplasm (see for example Filo, Bohr & Ruegg, 1965; Endo, Kitazawa, Yagi, Iino & Kakuta, 1977) and it is believed that the action of excitatory agonists is in some way to bring about such an increase. Depolarization of the smooth muscle membrane will also trigger contraction, and it was initially believed that the action of excitatory agonists was always mediated through their ability to depolarize the cells. Schild and his coworkers (Evans & Schild, 1957; Evans, Schild & Thesleff, 1958) demonstrated however, that smooth muscles depolarized by high K would contract further in response to some agonists, suggesting that there might be potential-dependent and potential-independent mechanisms involved. More recent work has indeed revealed that some smooth muscles normally contract in response to certain agonists without a change in membrane potential (e.g. Su, Bevan & Ursillo, 1964; Casteels, Kitamura, Kuriyama & Suzuki, 1977), and sometimes tissues actually hyperpolarize, and still contract (response of rabbit superior mesenteric artery to acetylcholine, Kuriyama & Suzuki, 1978).

The exact mechanisms by which drug-receptor interactions lead to an increase in free intracellular Ca, whether by potential-sensitive or potential-insensitive mechanisms have been the subject of numerous investigations, reviewed thoroughly by Hurwitz & Suria (1971) and Bolton (1979). Much evidence has come from studies of tissue responses to drugs in Ca-free media. The contractile responses of most smooth muscles are eventually lost under these conditions but there is considerable variability both in the speed with which different smooth muscles lose their ability to respond to an agonist, and in the susceptibility of the contractile responses to different agonists with the same tissue in Ca-free solutions. Such results have led to the suggestion, initially made by Edman & Schild (1962; 1963), that Ca may come not only from an extracellular source, but also from a cellular store which may be released by drug-receptor interaction, to the cytoplasm.

Entry of extracellular Ca may be activated by

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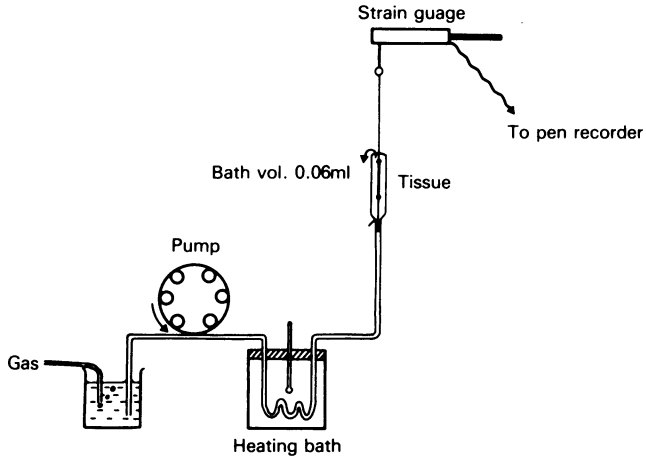


**Figure 1** Diagram of superfusion apparatus used for recording tension. In practice, four such pieces of apparatus were used so that four different pieces of taenia coli from the same animal could be studied simultaneously. Oxygenated solutions were pumped through an oil filled heating bath to the tissues at a flow rate of 1.6 ml/min by a peristaltic pump. To change the superfusion solution (for example, in order to apply a drug) the open end of the silicone rubber tubing was simply moved from one container to another, this resulted in introduction into the system of a small air bubble which could be followed to the organ bath to indicate when the new solution arrived. The tissues were suspended in glass tubes approx. 3 cm long with inside diameter 1.7 mm. Tension changes were monitored by a strain gauge approximately isotonically. The temperature of the superfusing solution in the organ bath was checked with a thermistor probe.

increased spike activity in spontaneously active tissues, since it is believed that Ca ions carry the inward current responsible for the upstroke (Tomita, 1975). There may also be other voltage-sensitive Ca channels which operate in K depolarized tissues or a direct increase in Ca permeability through the receptor operated channels (Bolton, 1979). Intercellular stores have been studied most in vascular smooth muscle, and are particularly well developed in arterial smooth muscle (Hudgins & Weiss, 1968), in which responses to noradrenaline may persist in Ca-free solutions long after the response to high K is abolished. More recently, intracellular Ca stores have been proposed to play a role in the more Ca labile responses of longitudinal gut muscles to muscarinic receptor activation (guinea-pig ileum: Hurwitz, 1975; guinea-pig taenia coli: Ohashi, Takewaki & Okada, 1974; Casteels & Raemaekers, 1979).

In this paper we have investigated the contractile responses of the guinea-pig *taenia coli* to carbachol under a variety of different conditions, to elucidate the mechanisms by which muscarinic receptor activation makes Ca available to the contractile apparatus, on the assumption that tension is a function of free $[Ca]_i$ and that this relationship is not significantly altered under the experimental conditions used. Our evidence supports the idea that at least three of the mechanisms postulated above are operating, namely increased spike activity, a maintained voltage sensitive Ca permeability, and release of Ca from an internal store. It has been possible to differentiate between these mechanisms by varying the concentration and duration of carbachol application, and through their varying susceptibility to the Ca-antagonist, methoxyverapamil (D600).

## Methods

A small piece of *taenia* (about 1 cm long and 1 mm wide) was dissected from the caecum of albino male guinea-pigs and mounted in the superfusion apparatus illustrated in Figure 1. This method allows rapid solution changes and accurate, short exposure times to drugs. Tension was monitored with a strain guage mounted so that tissue contraction was approximately isotonic. An automatic timing device operating relays was occasionally used for administration of drugs (Bell, 1973). Four tissues could be studied simultaneously by use of four sets of apparatus and contractions were recorded on a four channel Watanabe potentiometric pen recorder. In the majority of experiments a Krebs solution of the following composition was used (mM): Na 136.9, K 5.9, Ca 2.5, Mg 1.2, Cl 136.6, $HCO_3$ 15.5, $H_2PO_4$ 1.2, bubbled with 97% $O_2$, 3% $CO_2$, pH 7.4 at 36°C. The temperature of the solution bathing the tissue varied between 34.5 and 36.5°C. In solutions containing $Mn^{2+}$ ions or high $Ca^{2+}$, $NaHCO_3$ and $NaH_2PO_4$ were replaced by Tris chloride and the solution bubbled with 100% $O_2$. In

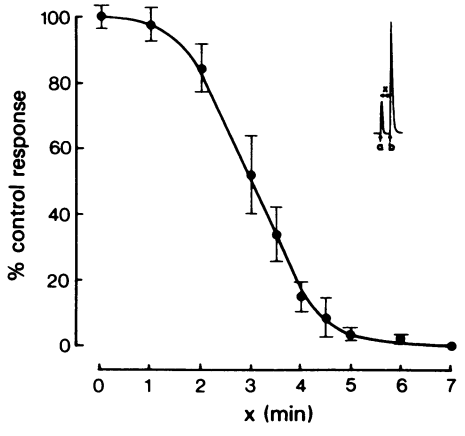


**Figure 2** Graph to illustrate the time course of loss of stored Ca in Ca-free EGTA (0.5 mM) solution. The size of the store is measured as the contractile response to $10^{-4}$ M carbachol (10 s) expressed as a % of the control response of the tissue to a 10 s application of $5 \times 10^{-5}$ M carbachol in normal Krebs. The procedure is shown in the inset. After carbachol no longer evoked a contractile response in Ca-free EGTA solution, a 5 s application of 87.5 mM Ca was given (a) and the response to carbachol tested (b) at an interval (x) afterwards. The procedure was repeated at different intervals given in a random order. Mean values are shown; vertical lines indicate s.e. mean ($n = 6$).

Ca-free solutions, $CaCl_2$ was replaced by $MgCl_2$. In high Ca solutions, $CaCl_2$ replaced NaCl isosmotically, or for hypertonic solutions, was added as the solid. EGTA when used was added as the Na salt. High K solutions were designed so that there were equal concentrations of Na and K (71.4 mM in normal Krebs and 63.1 mM in Tris buffered Krebs). Control responses to 10 s applications of $5 \times 10^{-5}$ M carbachol were established at the beginning of each experiment in normal Krebs solution and subsequent responses were expressed as a percentage of this control. Contractions were measured from the take off point to the peak of the response. A recovery period of at least 10 min was allowed between responses and normally doses were given at 15 min intervals in ascending order of concentration.

To test the possibility that transmitters released from nerve endings within the smooth muscle influenced the responses obtained, experiments were performed in the presence of tetrodotoxin ($5 \times 10^{-7}$ M), phentolamine ($5 \times 10^{-6}$ M) and propranolol ($5 \times 10^{-6}$ M). The responses obtained in the presence of these drugs were not substantially different from responses obtained in their absence.

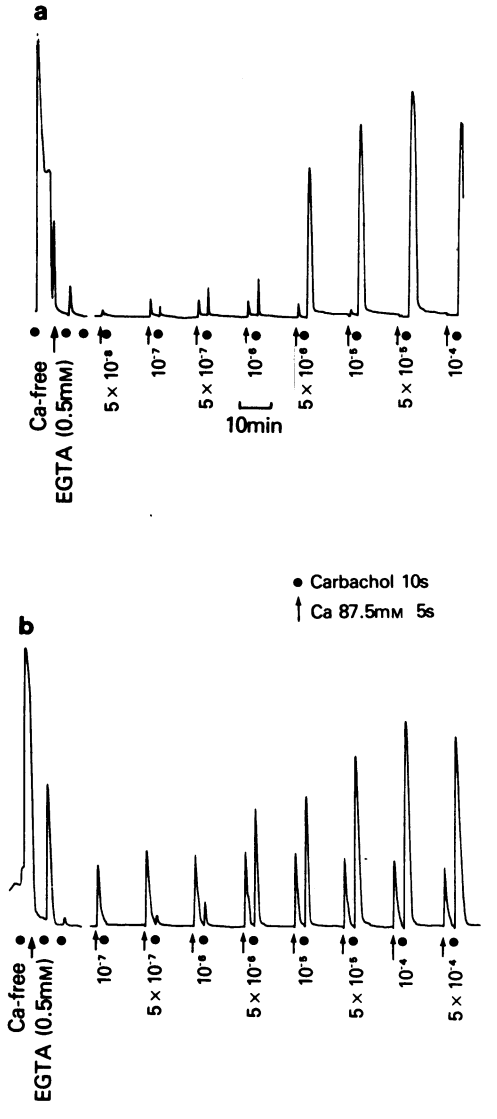


**Figure 3** Experiments to determine the relationship between the concentration of carbachol and release of Ca from the internal store. The control responses of the two tissues to a 10 s application of $5 \times 10^{-5}$ M carbachol in normal Krebs are shown on the left. The tissues were then exposed to zero Ca EGTA (0.5 mM) solution until the test dose of carbachol produced no response. To fill the store a 5 s application of 87.5 mM Ca was made (at small arrows) 3 min before each experimental dose of carbachol (10 s). In (a), high Ca application initially produced a small tension response, but towards the end of the experiment this disappeared, although the store was still filled. In (b) the tissue responded to Ca application with a larger response throughout. This is more typical.

## Results

### *Release of Ca from an internal store*

The contractile response to carbachol in Ca-free solution was lost within minutes whether or not EGTA was included in the medium. If a solution of high Ca concentration was then applied for a few seconds the tissue normally responded with a small contraction and then relaxed rapidly. Subsequent application of carbachol in this Ca-free solution induced a contraction, the size of which depended on the time interval between the Ca and drug application. It appears that the transient application of Ca fills a store which can be released by carbachol to produce contraction. Initial experiments have shown that this store can be partially filled by short application of quite low concentrations of Ca (3 s of 10 mM) if there is no EGTA in the Ca-free solution. If 0.5 mM EGTA is included, higher concentrations of Ca are required. Initial experiments were performed in which the concentration and duration of Ca applications were varied, and the size of the response to $10^{-4}$ M carbachol in Ca-free EGTA (0.5 mM) solution 3 min after Ca application was recorded. It could be shown that a 5 s application of 87.5 mM Ca (all NaCl replaced by $CaCl_2$) was supramaximal for filling the store, and this duration and concentration were used in all subsequent experiments. Carbachol $10^{-4}$ M was used to release the store since this concentration produced the maximum contractile response possible under these conditions. In Figure 2 the size of the carbachol response is plotted against the time interval between the 5 s application of 87.5 mM Ca and the subsequent drug application. The store was refilled with Ca before each carbachol application, as shown in the inset of the figure. This result shows that the Ca-store is lost in Ca-free solution within 5 to 6 min. In subsequent experiments 3 min were allowed between the Ca and carbachol application in order to give ample time for washing Ca from the extracellular space. Figure 3 illustrates two experiments in which the dose-response curve for release of the store was measured. The two experiments illustrate the variability of the response of tissues to applied Ca. As can be seen in some cases the store was filled without any response of the tissue to Ca whereas more often calcium application initiated a contractile response. The full dose-response curves for the contractile response to carbachol in normal Krebs and for the release of Ca from the store are shown in Figure 4. It can be seen that the dose-response curve for Ca release from the store is shifted to the right, low doses of carbachol being unable to release this store. The maximum contraction produced by releasing the store is probably somewhat underestimated in this experiment since there will clearly be some decline in store size during the 3 min period in Ca-free EGTA Krebs.

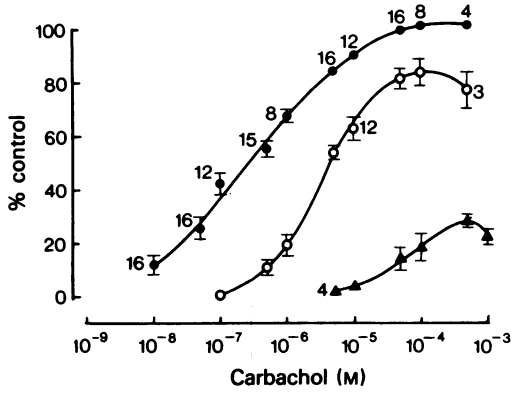


**Figure 4** Dose-response curves to 10 s application of carbachol under different experimental conditions. The responses are given as a percentage of the control response to 10 s application of $5 \times 10^{-5}$ M carbachol in normal Krebs. Values are mean; vertical lines show s.e. mean. (●) Responses in normal Krebs, (*n*) as shown. (○) Release of Ca store, response to carbachol in 0 Ca EGTA (0.5 mM) solution 3 min after a 5 s application of 87.5 mM Ca; $n = 9$ unless otherwise shown. (▲) Responses in 71.4 mM K solution. These responses are superimposed on a large maintained tension. $n = 6$ unless otherwise shown.

A similar shift to the right of the dose-response curve has recently been observed by Casteels & Raeymaekers (1979), who fill the store by exposing the tissue for 5 min to a 42 mM K solution containing 2.5 mM Ca.

It seems probable that even under normal conditions, carbachol can release Ca from the store if high enough concentrations are used. Figure 5 shows results from experiments in which carbachol at various concentrations was applied continuously to the tissues. In Figure 5a, the effects of successively higher carbachol concentrations were investigated. The contraction induced by $10^{-7}$ M carbachol rises to a plateau level without a marked transient peak, increasing the concentration to $10^{-6}$ M carbachol, which as shown earlier can release some of the store, causes a transient peak of contraction which declines to a steady level higher than in $10^{-7}$ M. Further increasing the carbachol concentration causes successively smaller transient contractions, so that $10^{-4}$ M after $10^{-5}$ M carbachol initiates only a small transient contraction. If, however, the tissue is put initially into $10^{-4}$ M carbachol as shown in Figure 5b, a very large transient peak is seen which is probably due to complete release of the internal store. These results suggest that during a continuous application of a concentration of carbachol high enough to release some

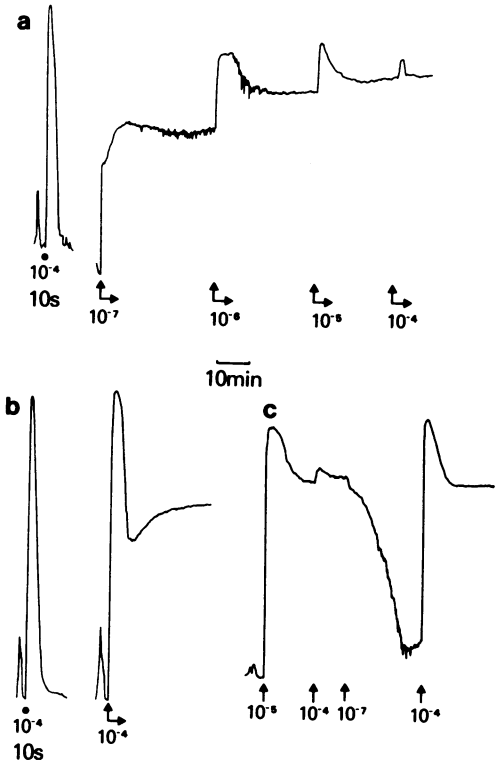


**Figure 5** Contractile responses to continuous application of various concentrations of carbachol showing the transient peaks which may be associated with release of Ca from the internal store. At concentrations less than $10^{-6}$ M there is little evidence of the store being released, since the tension rises to a steady state without a marked transient peak. Successive application of $10^{-6}$, $10^{-5}$ and $10^{-4}$ M carbachol produce successively smaller transients (a) but if $10^{-4}$ is added initially (b) or after $10^{-7}$ M carbachol (c) then the transient is much larger, indicating that the store can refill in the presence of low concentrations of carbachol but not in the presence of carbachol concentrations which can release the store. The maximum tension is indicated by the response to 10 s $10^{-4}$ M carbachol at the beginning of the trace.

of the calcium from the store, the store does not refill to its normal capacity. Figure 5c indicates, however that the store does refill in the continued presence of lower doses of carbachol. The transient peak in the response to $10^{-4}$ M carbachol is very small after continuous application of $10^{-5}$ M carbachol, but when the concentration is lowered to $10^{-7}$ M for 20 min, if $10^{-4}$ M carbachol is reintroduced, the transient peak is very much larger.

The contractile response to carbachol in tissues depolarized by high-K solutions may also be due to release of stored Ca. High-K does not appear to

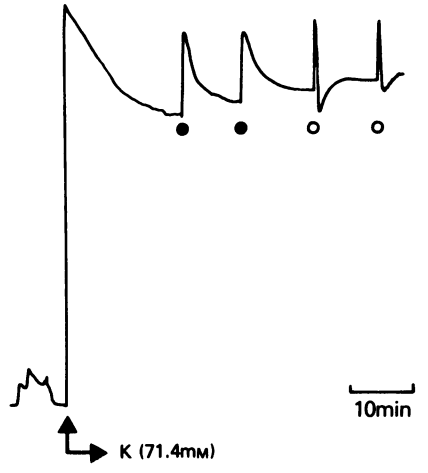


**Figure 6** Responses to Ca and carbachol applications of a tissue during K contracture. The tissue was exposed to 71.4 mM K to evoke the contracture: (●) 5 s application of 87.5 mM Ca; (○) 10 s application of $5 \times 10^{-5}$ M carbachol. Note the slow relaxation after the contractile response to Ca, whereas the carbachol response relaxes rapidly, with an undershoot.

release significant amounts of calcium from the store itself (Brading, 1977), and Ohashi *et al.* (1974) have studied the filling and emptying of the store at 20°C in K-depolarized tissue. In the presence of high-K the dose-response curve to carbachol is shifted to the right, as shown in Figure 4. In this experiment, tissues were depolarized in solutions containing equal amounts (71.4 mM) of Na and K. No response to carbachol could be initiated at concentrations below $5 \times 10^{-6}$ M. In high-K solutions, the contractile responses are superimposed on a large maintained tension (see Figure 6) and since the $[Ca]_i$ versus tension relationship is non-linear (Endo *et al.*, 1977) the size of the contractions is not a good reflection of the amount of calcium released from the store (i.e., the amount of Ca released will be greater than the further increase in tension indicates). The maximum absolute size of contraction reached at $5 \times 10^{-4}$ M carbachol was between 76 and 95% (mean 83%, $n = 6$) of the control response to $5 \times 10^{-5}$ M carbachol, which is very similar to the maximum for the release of the store. The dose-response curve to carbachol of tissues depolarized in Na-free high-K solutions (143 mM K) was similar to that in 71.4 mM Na and K medium. Further evidence that the contractile response to carbachol in high-K is due to release of Ca from a store can be seen in Figure 6 which shows the contractile response to 5 s application of 87.5 mM Ca and to 10 s of $5 \times 10^{-5}$ M carbachol of tissues depolarized by a 50/50 K/Na solution. The response to Ca declines very slowly in comparison to a similar sized response

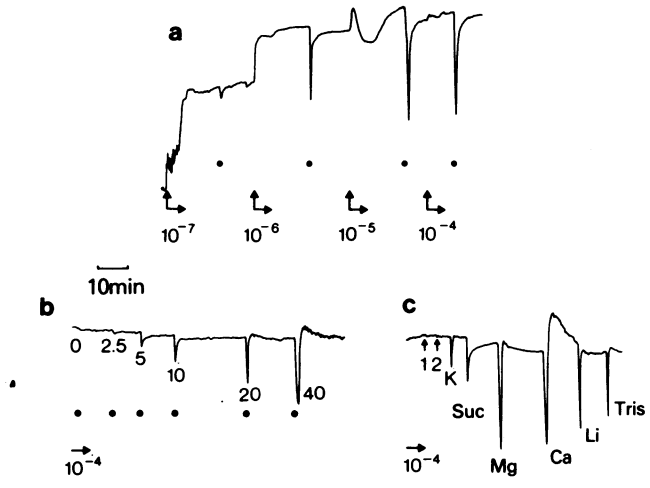


**Figure 7** Transient relaxations induced in tissues contracted by continuous application of carbachol (in the concentrations indicated at the arrows): (a) 5 s applications of 25 mM Ca ($CaCl_2$ replacing NaCl isosmotically); (b) 10 s applications of various hypertonic Ca concentrations (mM) made by adding $CaCl_2$ solid to isotonic NaCl; (c) 10 s exposures to Na free solutions in which Na was replaced isosmotically by the substitutes indicated. At (1) removal of Ca for 10 s and at (2) removal of carbachol for 10 s had no effect on the tension.

to carbachol. The rapidity of relaxation after the carbachol contraction is consistent with the idea of calcium released from a store since re-uptake into the store would allow rapid relaxation whereas Ca entering the tissue with a full store is clearly only extruded from the tissue slowly.

*Entry of Ca from the external medium*

Tension is well maintained at a submaximal level in tissues continuously perfused with carbachol. During this time it is reasonable to assume that the tension is due to an increased level of free Ca available to the myofilaments as a result of an increase in Ca entry from the external medium, and that the tension is maintained at a steady level due to increased extrusion across the membrane.

If the Ca permeability in the presence of carbachol is high it might be expected that increasing the extracellular Ca concentration would lead to further contraction, as occurs in tissues depolarized in high-K solutions (Figure 6). However, it was found experimentally, that transient application of high Ca, which caused contraction in the high-K depolarized tissues, caused a transient relaxation in tissues exposed to various concentrations of carbachol, see Figure 7a. Relaxation occurred if Ca replaced Na or if it was added hypertonically (see Figure 7b). This result was unexpected and seemed inconsistent with an increased $P_{Ca}$, but further investigation showed (see Figure 7c) that similar relaxations could be caused by transient reduction of extracellular Na (using Li, sucrose, Tris or Mg as substitutes) and it is possible that the relaxation was secondary to repolarization of the membrane and the closing of potential dependent Ca channels. Webb & Bohr (1978) have also reported Ca-induced relaxation of contraction maintained by noradrenaline in vascular smooth muscle.

A short application of Ca-free solution had no effect on the tension response of tissues continuously perfused with carbachol, and if the perfusing solution

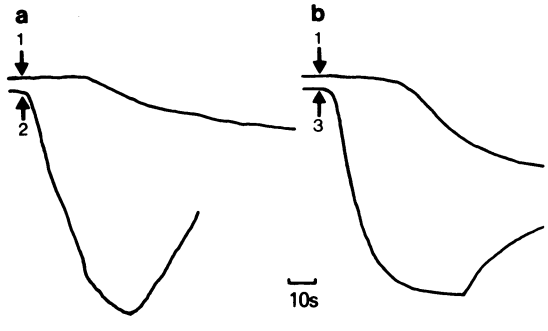


**Figure 8** Comparison of the time course of relaxation of tissues in which the tone had been raised by $10^{-4}$ M carbachol (a) or 71.4 mM K (b). In both cases removal of Ca and introduction of 0.5 mM EGTA, (1) produced a slow relaxation after a delay of nearly 30 s. Rapid immediate relaxation was produced in the presence of carbachol by 10 s application of 40 mM Ca (2) and in the K depolarized tissue by re-introduction of Krebs solution (3). Both procedures probably repolarize the tissues.

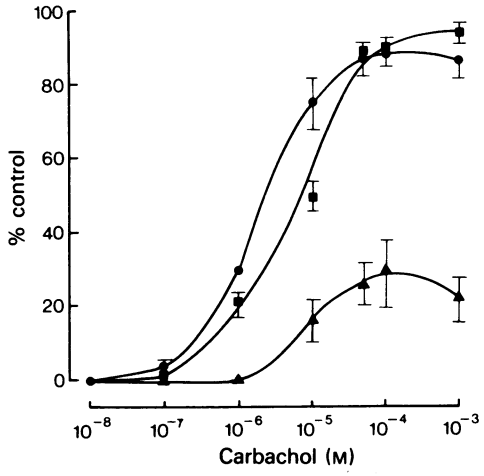


**Figure 9** Dose-response curves to 10 s simultaneous application of carbachol at 3 different Ca concentrations to tissues superfused with Ca-free EGTA (0.5 mM) Krebs: (▲) 2.5 mM Ca; (●) 25 mM Ca; (■) 75 mM Ca. Values are expressed as a % of the control response to 10 s application of $5 \times 10^{-5}$ M carbachol in normal Krebs solution. Values are mean; vertical lines show s.e. mean. ($n = 4$).

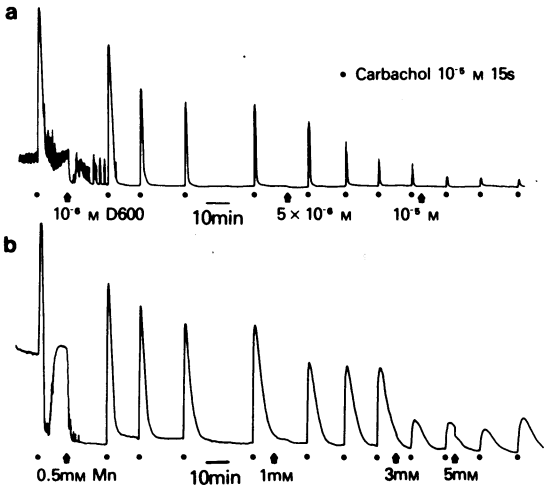


**Figure 10** The effect of various concentrations of Ca antagonists on the response to a 15 s application of $10^{-5}$ M carbachol: (a) D600; (b) Mn. A control response in Krebs was obtained at the beginning of each trace. Notice that both blockers abolished spontaneous activity and reduced the responses to carbachol. Mn also reduced the rate of relaxation of the tissues.

was changed to Ca-free (carbachol containing) solution there was a time lag of 30 s before tension began to decline, even if EGTA was included in the solution. In contrast, increasing the Ca concentration produced an immediate and much more rapid decline in tension, as shown in Figure 8. The fast time course of the relaxation suggests that the mechanism by which Ca moves into the cells can be very quickly deactivated, and the slow time course of the tension decline in zero Ca suggests that the calcium moving into the cell when the mechanism is activated, must be bound in some way, possibly to fixed negative sites in the membrane, and cannot easily be washed off. Figure 8 also shows similar effects in tissues depolarized with 63.1 mM K. Repolarization by readmitting Tris buffered Krebs leads to an instantaneous rapid relaxation, while removal of Ca again only slowly relaxes the tissues after a time lag.

Another approach to studying this problem is to deplete the tissues of Ca in a Ca-free EGTA containing solution and re-apply Ca and carbachol simultaneously for a few seconds. Under these conditions, whatever the concentration of calcium used, the dose-response curve is shifted to the right compared with the control in Krebs (see Figure 9) and little response is obtained to concentrations of carbachol less than $10^{-6}$ M, i.e., contractions are only elicited by concentrations of carbachol that are capable of releasing the internal store. It is also apparent that with 2.5 mM Ca the maximum contraction induced by $10^{-4}$ M carbachol is far less than the control response to carbachol ($5 \times 10^{-5}$ M) in normal Krebs (which also contains 2.5 mM Ca). These results imply that in order to obtain a maximal contraction to a 10 s application of carbachol it is not sufficient to have Ca present throughout the period of stimulation but that Ca must be present for some time prior to carbachol application possibly so that Ca ions occupy fixed binding sites before translocation into the cell through the voltage-sensitive pathway as previously suggested. This implies that for Ca entry during the maintained response to carbachol, a longer period of exposure to Ca is required to re-occupy the necessary sites than to refill the internal store (see Figure 3).

*Effects of Mn and D600 on responses to carbachol*

Electrophysiological recordings suggest that application of carbachol leads to a burst of high frequency spike activity superimposed on the early depolarization phase of the response. As depolarization continues spike activity ceases (Bolton, 1972). With maintained application of carbachol, particularly at low concentrations, there is often a rapid, initial contraction (occurring earlier than the previously described transient peak at the higher carbachol concentrations associated with release of an internal store) which ends abruptly (see Figure 5a) sometimes with a small transient relxation. This is followed by a slower rise in tension to a steady level. This early, rapid phase of the

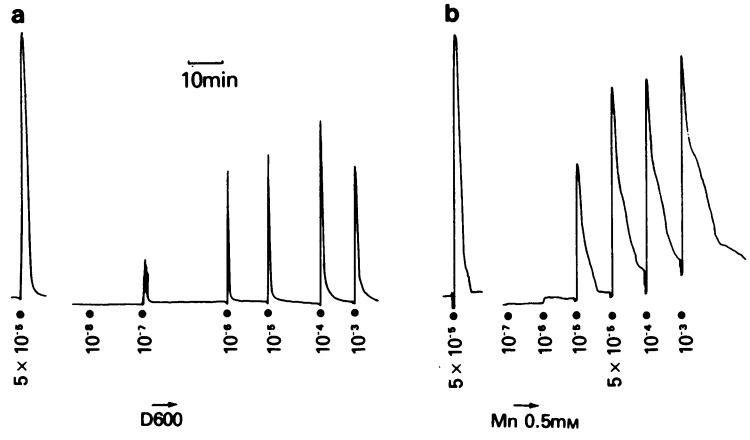


**Figure 11** The effect of $2 \times 10^{-6}$ M D600 (a) and 0.5 mM Mn (b) on the contractile response to various concentrations of carbachol (10 s). A control response in normal Krebs solution is shown at the beginning of each trace. Both D600 and Mn reduced the sensitivity of the tissue to carbachol but only Mn reduced the rate of relaxation.

response is probably mediated by spike activity. In order to investigate this possibility further, we looked at the actions of D600 and Mn, both of which are known to suppress spontaneous activity in smooth muscle. Figure 10 shows the effect of increasing concentrations of Mn and D600 on the contractile response to a 15 s application of $10^{-5}$ M carbachol. The contractile responses are progressively reduced. It is noticeable that in the presence of D600 the contractile responses relax rapidly whereas in the presence of Mn the relaxation is progressively slowed, suggesting that both substances reduce Ca entry but Mn has an additional action which prevents rapid reduction of elevated intracellular Ca levels. Figure 11 shows the effect of varying the concentration of carbachol in the presence of $2 \times 10^{-6}$ M D600 or 0.5 mM Mn. With

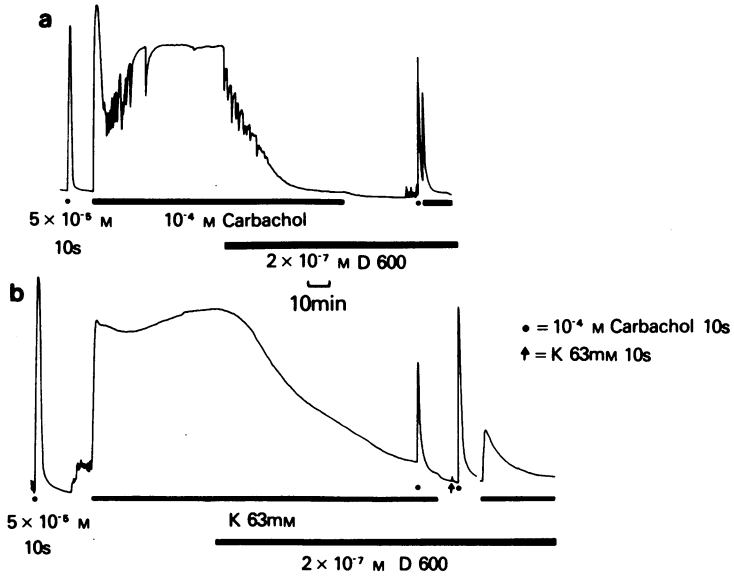


**Figure 12** Relaxation produced by D600 $2 \times 10^{-7}$ M during continuous application of $10^{-4}$ M carbachol (a) or 63.1 mM K (b). In both cases D600 produced complete relaxation but was unable to block the transient contraction produced on introduction of $10^{-4}$ M carbachol for 10 s. Note that re-application of $10^{-4}$ M carbachol continuously in the presence of D600 at the end of trace (a) initiated a transient peak, but no maintained tension.

these concentrations the lower doses of carbachol are ineffective and the dose-response curve is shifted to the right suggesting that under these conditions carbachol is again acting mainly by releasing Ca from an internal store. This interpretation is supported by the observation, illustrated in Figure 12, that in the continuous presence of $10^{-4}$ M carbachol, low concentrations of D600 can almost totally relax the tissue, even though at this concentration of D600 in normal Krebs large transient contractile responses can be elicited by $10^{-4}$ M carbachol. This is consistent with the suggestion previously put forward that the maintained tension in the continuous presence of $10^{-4}$ M carbachol is due to Ca influx, which D600 can block, but the transient response depends on Ca released from the internal store which is resistent to these antagonists. Mn only blocked the maintained tension at rather higher concentrations, at least 2.5 mM being needed for a maximal effect, but even at low concentrations (0.5 mM) when applied in $10^{-4}$ M carbachol, it had the additional ability to induce a transient relaxation of the tissue, reminiscent of the relaxations produced by high-Ca or Na-free solutions illustrated in Figure 7.

## Discussion

Our results suggest that there are at least three different mechanisms for increasing $[Ca]_i$ that are involved in the response of smooth muscle from the guinea-pig taenia to carbachol. Firstly calcium entering as inward current through the spike mechanism, following increased spike frequency on initial carbachol application. Secondly, a continued influx of calcium during prolonged action and thirdly the release of calcium from an intracellular store by higher concentrations of carbachol.

The least well defined on these possibilities is the spike component. However, since it can easily be shown by simultaneous electrical and mechanical recording (see for example, Bülbring, 1962) that each spike is normally associated with an increase in tissue tension, and that carbachol initially increases spike frequency (e.g. Bolton, 1972), it is logical to deduce that spike activity contributes to the tension response to carbachol, at least at lower concentrations. The mechanical counterpart appears to be an initial 'step' in the rising phase of tension development. At higher concentrations the depolarization occurs very rapidly and the tissue passes into a phase of 'depolarization block' when no spike activity is seen. Preliminary experiments with sucrose gap recording suggest that during long maintained application of high carbachol concentrations (even at $10^{-4}$ M) electrical activity may return with an oscillatory wave form occasionally associated with spikes.

It is not easy to differentiate between the spike component of tension and the maintained tension. Quite high concentrations of Ca antagonists are required to block spike activity. Brading, Bülbring & Tomita (1969) have shown that more than 2 mM Mn was needed to block evoked spikes, and Inomata & Kao (1976) have shown that $10^{-5}$ M D600 was needed to suppress the inward current to the same extent as 2 mM Mn. Such high concentrations also abolish the maintained tension response.

The maintained tension in response to prolonged application of carbachol has proved interesting. This state can be maintained for several hours at a level of tension depending on the carbachol concentration, and shows little sign of desensitization even at $10^{-4}$ M. Tracer studies have demonstrated that intracellular Ca does exchange with extracellular Ca in the taenia (see for example, Goodford, 1965), and it seems most likely that the tension is due to an increased influx of Ca, which would have to be balanced in the steady state by an increased efflux. Other mechanisms, such as a decreased efflux or decreased uptake into an internal store would be unlikely to sustain the response for such long periods. Data on the effect of carbachol on Ca fluxes have proved ambiguous (for references see Lüllmann, 1970; Setekleiv, 1970), but there is some evidence (e.g., Banerjee, 1972) that suggests that Ca influx may be affected by muscarinic agonists.

When tension was maintained in high-K solutions the addition of Ca caused a further contraction (Figure 6). This is consistent with the idea of an increased $P_{Ca}$ due to the opening of voltage dependent Ca channels as a result of the depolarization produced by high-K. Carbachol $10^{-4}$ M also depolarizes the tissue and should also open these channels, yet addition of Ca produced relaxation. This apparent inconsistency may be resolved if the mechanism of the depolarization is considered. In high-K the depolarization is due to a shift in the K equilibrium potential, and the voltage-dependent Ca channels are rapidly switched off when this is returned to normal, producing immediate relaxation (see Figure 8). In the presence of carbachol the depolarization is thought to be due to the opening of membrane channels with a high $P_{Na}$ and a reversal potential of about $-10$ mV (Bolton, 1973). In this case a repolarization (and subsequent switching off of the Ca channels), would be caused either by a reduction in the Na gradient or by blocking the channel. The finding that all the Na substitutes used, including Ca and K, produced rapid relaxation, suggests that the former mechanism is important, but the finding that hypertonic addition of Ca also caused a similar relaxation suggests that Ca can also directly block the channel opened by drug receptor interaction. Preliminary electrophysiological investigations suggest that these relaxations are

indeed associated with transient repolarization of the membrane. Unlike the K depolarized tissue, carbachol depolarized tissues often undergo transient spontaneous relaxations, particularly in the earlier stages of the maintained response, and these relaxations can also be triggered off by different procedures such as changing from $HCO_3/CO_2$ buffer to Tris buffer, or by addition of low Mn concentrations. It is as if the membrane is poised at a critical potential, and can undergo a kind of all or none transient repolarization. Bolton (1971) has shown that in guinea-pig ileal longitudinal smooth muscle, the channels activated by carbachol have unusual voltage sensitive properties which result in oscillatory changes of the membrane potential, and it is possible that the channels opened by carbachol in the taenia also have voltage sensitive properties (Bolton, 1975).

There are some observations which suggest that the maintained tension in high-K and in carbachol may be due to activation of the same Ca channels. In both conditions the tension is relaxed by $2 \times 10^{-7}$ M D600, and changes in ionic concentration designed to cause repolarization (removal of Na in the presence of carbachol and re-introduction of normal Krebs in the case of high-K) lead to immediate and rapid relaxation, whereas removing extracellular Ca (even in the presence of 0.5 mM EGTA) leads to a much slower relaxation than occurs after a significant time lag of about 20 to 30 s. This suggests that the Ca needs to be bound to sites in the membrane before the voltage sensitive translocation can occur. Supporting evidence is the inability of the tissue to produce maximal tension when 2.4 mM Ca and $10^{-4}$ M carbachol are simultaneously applied for 10 s to tissues relaxed in Ca-free medium. The time lag is reminiscent of the loss of spike activity in zero Ca solution (Brading *et al.*, 1969) which also seems to depend on bound Ca, but it is unlikely that the Ca channels involved in spike generation are identical with those involved in the maintained tension response, since those involved in spike activity are likely to be inactivated by maintained depolarization (Inomata & Suzuki, 1977) and are far less susceptible to block with D600 than are the channels responsible for maintained tension, although they appear to have similar sensitivity to Mn ions. D600 ($10^{-6}$ M) has also been shown by Golenhofen and his colleagues to block the maintained tension response to acetylcholine in the *taenia*, in contrast to the situation in more 'tonic' smooth muscles such as stomach fundus and corpus, in which D600 does not abolish the maintained response to acetylcholine, but nitroprusside does (Boev, Golenhofen & Lukanow, 1976).

The presence of an internal Ca store in the guinea-pig *taenia* that can be released by carbachol has been reported by Ohashi and his co-workers. They used K-depolarized tissues at 20°C. After loss of the contractile response to carbachol in Ca-free solution, re-admission of Ca in these conditions leads to a Ca-contracture and after subsequent relaxation in 0 Ca for 10 min, carbachol ($10^{-3}$ M) would still evoke one transient contractile response (Ohashi *et al.*, 1974). $La^{3+}$ (0.5 mM) and D600 (10 μg/ml) would prevent filling of the store if applied before Ca re-admission, but not the release of the store by carbachol if the blockers were applied after the store had been refilled (Ohashi, Takewaki, Shibata & Okada, 1975).

Our experiments in 0 Ca EGTA solution are consistent with the proposal that there is a cellular Ca store that can be released to the contractile apparatus by carbachol, but they also show that the store can be filled very rapidly by extracellular Ca in polarized muscle at normal temperature, and that it can only be released by concentrations of carbachol higher than are needed to evoke a contractile response of the normal tissue. The store contains enough Ca to produce a transient response of nearly maximal size, can be filled and released in tissues depolarized by high-K and also in the presence of sufficient D600 to block the maintained tension response, although higher concentrations of D600 and Mn also block filling of the store. Recent experiments by Casteels & Raeymaekers (1979) have also shown that the dose-response curve for acetylcholine and store release is shifted to the right of the normal curve. These workers filled the store by depolarizing the tissues in high-K solutions containing 2.5 mM Ca. It is possible that the store could be the sarcoplasmic reticulum which has been shown to be present in *taenia* (Gabella, 1976), and to be in close association with the plasma membrane. In which case filling of the store from the outside could be via an ion exchange mechanism as has been proposed by Brading (1976), whereas filling from the cell could be by an ATP-dependent Ca pump. How carbachol could release the store has not yet been investigated.

We believe that in normal tissues the transient peak tension that develops in response to maintained high doses of carbachol is a manifestation of the release of this internal store of Ca and that decline in tension from the transient peak is due to emptying of the store rather than to a desensitization of the receptors. This is difficult to prove unequivocally but the maintained tension at $10^{-4}$ M carbachol shows little sign of declining even after several hours, and the transient peak shows the same relationship to the carbachol concentration as does release from the store. In the continued presence of a releasing concentration of carbachol the store does not refill, but can refill in the presence of lower concentrations of carbachol.

The contractile response to carbachol of K-depolarized tissues also appears to be due to release of the store, since high concentrations of carbachol are once again necessary to evoke the response. The fact that carbachol contractions in depolarized tissues relax

rapidly, whereas Ca contractions of a similar size only relax very slowly also suggests that Ca release and subsequent re-uptake into the store is involved in the carbachol response, since increase in $[Ca]_i$ due to influx in the presence of a full store is only slowly reversed, presumably by active extrusion.

We have not discussed the possibility that calcium ions can move through the channels opened by the carbachol receptor interaction. Any Ca movement through such channels is likely to be small, because in high-K solutions concentrations of carbachol up to $10^{-6}$ M, which clearly react with the receptors, do not initiate any extra tension development, presumably because little extra calcium permeability is induced beyond the voltage-sensitive mechanism which is already fully activated by the K depolarization. It is therefore possible to explain our results by invoking calcium influx associated with the action potentials, with another voltage sensitive but not depolarization-inactivated calcium channel, and release of calcium for an internal store.

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