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The effects of phospholipase C on excitation-contraction coupling mechanisms in smooth muscle

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The technique of incubation with phospholipase C has been used as a means of investigating the role of phospholipids in the excitation-contraction coupling mechanisms of smooth muscle. The effects of this incubation procedure were observed upon the interactions of calcium with the contractile responses of the guinea-pig ileum longitudinal muscle preparation under a number of conditions. The reduction in the acetylcholine responses of both normal and potassium depolarized tissues after incubation was partially restored by a ten-fold increase in the calcium concentration of the bathing medium. A faster rate of decline of contractile responses upon immersion in calcium-free solutions and a slower rate of restoration upon the reintroduction of calcium were seen in both normal and depolarized tissues. The relation between the external calcium concentration and the size of the contractile responses to a given stimulus was also changed. The size of the response to added calcium obtained in a potassium depolarized preparation was also reduced after phospholipase C incubation. These results and their implications are discussed.

Previous studies in this laboratory have shown that incubation of the guinea-pig ileum longitudinal muscle preparation with phospholipase C results in an irreversible loss of contractile responses (Leach & Lodge, 1966). Evidence was presented to support the hypothesis that the action of phospholipase C is due to the hydrolysis of membrane phospholipids which are essential for the normal activity of smooth muscle excitatory mechanisms.

In view of the many roles which have been suggested for the phospholipid components of cell membranes, as structural components (Robertson, 1960), ion-exchange components (Tobias, 1964), and essential components of enzyme systems (Green & MacLennan, 1969), it is possible for phospholipase C to exert its inhibitory action in a number of ways. One possibility is that it causes an impairment of excitation-contraction coupling mechanisms.

Ample evidence now exists to show that calcium ions (Ca^{2+}) function as an important component of the excitation-contraction coupling processes in smooth muscle (Bohr, 1964). We might therefore expect that any change in excitation-contraction coupling mechanisms would be reflected as changes in the interactions of calcium with muscle contractile responses. The present investigation deals with the changes that were observed in these properties after incubation of the muscle preparation with phospholipase C.

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METHODS

The experimental preparation was the guinea-pig ileum longitudinal muscle strip described by Rang (1964). Usually, two separate preparations (test and control) from adjacent pieces of ileum were mounted in 3 ml organ baths and were bathed in Tyrode solution at 32° gassed with air, the initial resting tension was adjusted to 0·3 g. Contractile responses were recorded on a smoked drum by means of isotonic levers.

The Tyrode solution had the following composition in g litre^{-1}; NaCl, 8·1; KCl, 0·2; $NaHCO_3$, 1·0; $MgCl_2$, 0·2; Na_2HPO_4, 0·065; $CaCl_2$, 0·4; glucose, 1·0. 'Potassium depolarizing Tyrode' was prepared by substituting an equivalent amount of potassium chloride for the sodium chloride. All drugs were from BDH Chemicals Limited. Analar chemicals were from Hopkin Williams Limited. Phospholipase C (*Clostridium welchii*) was from Koch-Light Laboratories Limited.

Drugs were administered as concentrated solutions in 0·01 to 0·05 ml of Tyrode and were washed out by upward displacement. Drug solutions were prepared in the Tyrode solution identical in composition to that used for the tissue incubation medium.

From preliminary experiments it was decided that repeated incubation of the preparation for 5 min in Tyrode solution containing phospholipase (100 μg ml^{-1}) was the most suitable means of studying the enzyme action. Under these conditions, the initial contraction which usually accompanied phospholipase action was not maintained on washing out the enzyme, and recovery of the tissue to its original resting length could be hastened by repeated application of doses of acetylcholine. Furthermore, the muscle preparations varied in their susceptibility to enzyme action and in most experiments it was desirable that a fairly constant degree of inhibition of the contractile responses should be obtained. The use of repeated 5 min incubations allowed the action of the enzyme to be terminated when a suitable loss of response had been obtained. Experimental results were obtained from three to six preparations in each series.

RESULTS

The effect of a high calcium ion concentration upon tissue responses

(a) *Responses in normal Tyrode.* If phospholipase C causes a loss of contractile responses by an impairment in the activity of calcium binding sites, then increasing the calcium ion concentration of the medium might be expected to bring about a partial restoration of responses.

Incubation of a longitudinal muscle preparation with phospholipase C for six, 5 min incubation periods almost completely and irreversibly eliminated the original maximal response to acetylcholine (1 μg); dose-dependent responses could still be obtained, however, to very high concentrations of acetylcholine (Fig. 1) and a response to potassium depolarizing Tyrode was still obtainable. A tenfold increase of the Ca^{2+} concentration of the Tyrode medium enhanced the phospholipase inhibited responses, the response to 1 mg of acetylcholine now being almost equal to the maximal response of the preparation before incubation (Fig. 1iii). The Ca^{2+}-induced increase in the size of the responses after incubation was in contrast to the usual depressant action of high Ca^{2+} concentration on the submaximal contractile responses obtained in a normal smooth muscle preparation.

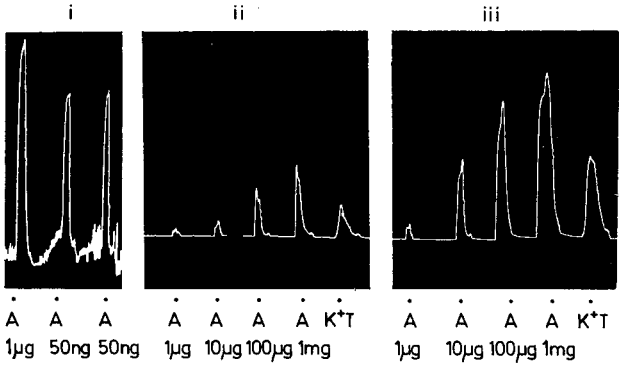

FIG. 1. The effect of a high calcium ion concentration upon the responses of a phospholipase C treated preparation bathed in normal Tyrode. (i), responses before incubation with phospholipase C; (ii), responses after six, 5 min incubations with phospholipase C (100 μg ml^{-1}) in normal Tyrode; (iii), responses in the presence of a tenfold increase of the calcium ion concentration. (A) acetylcholine, total injected dose; (K^+T) potassium depolarizing Tyrode. Doses as indicated in the figure.

The effect of phospholipase C on the responses of a potassium depolarized preparation to added calcium

When immersed in calcium-free, potassium depolarizing Tyrode at 32°, guinea-pig ileum longitudinal muscle will exhibit dose-dependent contractile responses to added calcium. Fig. 2 shows the effect of phospholipase C incubation upon these responses.

Incubations with phospholipase C caused an appreciable reduction in the size of the responses to acetylcholine and a slight reduction of the initial response to potassium depolarizing Tyrode (Fig. 2a). When transferred to potassium depolarizing Tyrode, the sub-maximal responses of the incubated tissue to Ca^{2+} (200 μg) were found to be reduced to about 15% of their former size (Fig. 2b), but these responses were now very much slower in their rate of development and did not attain their maximum height in the 2 min contact time allowed. An extension of the contact period to 10 min allowed the submaximal responses to attain their maximum heights (Fig. 2bii, 2nd panel), which were now approximately equivalent to 50% of the height of the response to the same dose of calcium before incubation with the enzyme. The maximal response of the

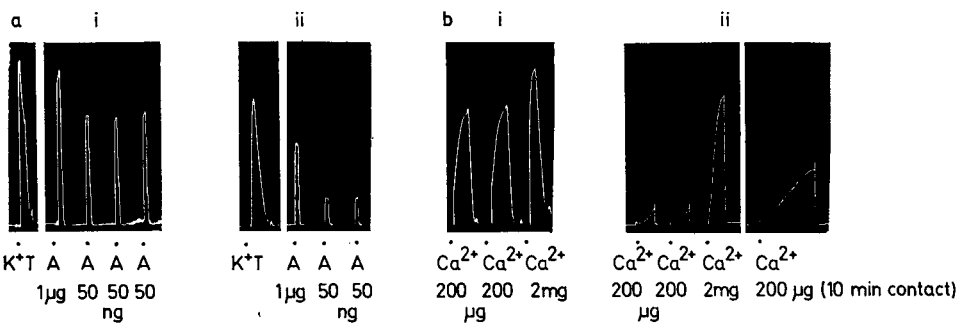

FIG. 2. The effect of phospholipase C incubation upon the calcium responses of a potassium depolarized preparation. Responses in normal Tyrode before (a. i) and after (a. ii) three, 5 min incubations with phospholipase C (100 μg ml^{-1}). Responses to Ca^{2+} in potassium depolarizing Tyrode, before (b. i) and after (b. ii)incubation with phospholipase C. (A) acetylcholine, total injected dose; (K^+T) potassium depolarizing Tyrode; (Ca^{2+}) calcium chloride, total injected dose. Doses as indicated in the figure. Ca^{2+} contact time 2 min unless otherwise specified.

preparation to Ca^{2+} (2 mg) was reduced after incubation to approximately 80% of its former size but little change was apparent in the rate of development of the response.

The effect of phospholipase C on the rate of loss of responses on removal of calcium and on the rate of recovery on restoring calcium

The drug-induced contractions of smooth muscle immersed in either normal or potassium depolarizing Tyrode require the presence of Ca^{2+}. The substitution of a calcium-free medium results in a gradual loss of the contractile responses; re-introduction of calcium causes a rapid restoration of the responses (Edman & Schild, 1962). Any observed changes in the rates of loss and restoration of tissue responses under these conditions could indicate an impairment in the activity of calcium uptake or binding sites.

(a) *Acetylcholine responses in normal Tyrode.* Incubation of a longitudinal muscle strip preparation for three, 5 min periods in normal Tyrode produced a complete loss of responses to submaximal doses of acetylcholine. After incubation, the responses to a previously maximal dose of acetylcholine (1 μg) were approximately 70% of their original size and these responses were compared with an equivalent response obtained to acetylcholine (50 ng) in an untreated preparation and the effects of immersion for 1, 3, and 6 min in Ca^{2+}-free Tyrode solution were observed on the acetylcholine responses of the two preparations. To allow for the possibility that the larger dose of acetylcholine used in the phospholipase C-treated preparation might accelerate the rate of decline of the responses, both tissues were equilibrated with normal Tyrode for 5 min before further immersion in a calcium-free medium. It can be seen from Fig. 3 that the responses of the phospholipase C-treated tissue declined to zero after 3 min exposure whilst those of the control tissue were still present after 6 min.

In the same tissues, the rate of restoration of the responses was also dependent upon the reintroduction of calcium. The test doses of acetylcholine were applied after 1, 2 and 4 min exposure to normal Tyrode. After each exposure to normal Tyrode, the

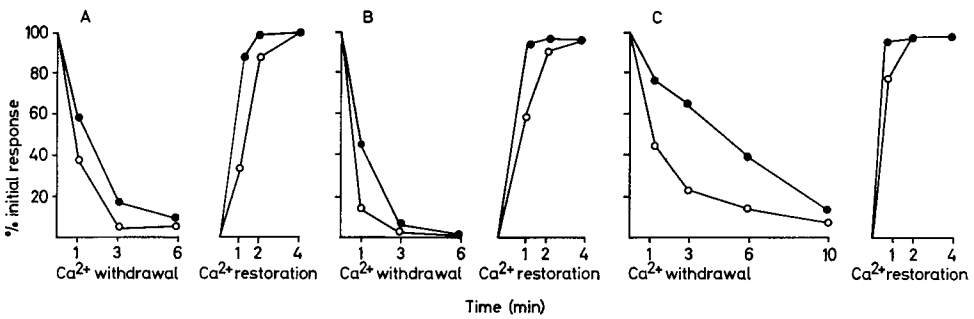

FIG. 3.A. The effect of removal and restoration of Ca^{2+} upon the standard acetylcholine responses. Left hand section: effect of immersion in calcium-free solution; right hand section: effect of immersion in normal calcium Tyrode. Ordinate: response height expressed as a percentage of the initial response before removal of calcium. (●) control tissue; (○) phospholipase C-treated tissue. Each point represents the mean of three responses.

B. The effect of removal and reintroduction of calcium ions upon the initial response to potassium depolarizing Tyrode. Left hand section: effect of immersion in calcium free solution; right hand section: effect of immersion in normal calcium Tyrode. Other details as for A.

C. The effect of removal and reintroduction of calcium ions upon standard acetylcholine responses obtained in potassium depolarizing Tyrode. Other details as for B.

tissues were returned to calcium-free solution until the responses had fully declined. In the control tissue, the responses almost fully recovered after 2 min exposure to normal Tyrode whereas in the phospholipase C-treated tissue, 4 min were required for full recovery (Fig. 3A).

(b) *The initial response to potassium depolarizing Tyrode.* A similar action of phospholipase C on the contractile responses to potassium depolarizing Tyrode was also observed. The incubation of a longitudinal muscle preparation with phospholipase C for two, 5 min incubations reduced the initial response to potassium depolarizing Tyrode to 90% of its former value. The effects of removal and reintroduction of Ca^{2+} on these contractile responses were then observed by a similar technique to that described in the preceding experiment.

After immersion in Ca^{2+}-free Tyrode for 1 min, the response of the phospholipase C-treated tissue had been reduced to 14% of its post-incubation size whereas that of the control tissue had only been reduced to 46% of its original size. After 3 min exposure, the responses of both tissues had almost completely disappeared (Fig. 3B).

On reintroduction of calcium, the response of the control tissue was almost fully recovered after 1 min whereas the phospholipase C-treated tissue required 4 min for its full restoration.

(c) *Acetylcholine responses in potassium depolarizing Tyrode.* The effects of phospholipase C incubation upon the acetylcholine responses of a potassium depolarized preparation were examined in tissues which had first been incubated with the phospholipase in normal Tyrode. This treatment reduced the maximal acetylcholine response obtainable in K^+ depolarized Tyrode to approximately 65% of its original size and the effect of Ca^{2+} withdrawal and restoration on this response was then compared with that of an equivalent sized response obtained on the normal unincubated control (Fig. 3C).

It can be seen that upon immersion in Ca^{2+}-free Tyrode (Fig. 3C) the rate of decline of the responses of the phospholipase C-treated tissue was faster than that of the control preparation; the difference being greatest at the 1 and 3 min immersion periods. On reintroducing calcium the response of the control tissue required only 1 min for full restoration whereas the enzyme-treated tissue required 2 min. Although the results presented in Fig. 3 represent results obtained in individual experiments, similar qualitative tendencies were always observed in all of the experiments of each group.

The effect of phospholipase C on the calcium concentration/response relation

Not only are smooth muscle responses dependent upon the presence of calcium, but within certain limits the magnitude of the response is a function of the calcium concentration (Bohr, 1964). Any changes in the properties of calcium binding or uptake sites may, therefore, be reflected in changes in the relation between the calcium ion concentration and the size of the tissue response.

(a) *Acetylcholine responses in normal Tyrode.* After incubation of a longitudinal muscle strip preparation treatment with phospholipase C, the response to acetylcholine (1 μg) was reduced to 80% of its preincubation size. The response to this dose of acetylcholine was compared with a similar sized response to acetylcholine (100 ng) obtained in a control untreated preparation. The relation between the Ca^{2+} concentration of the medium and the size of these responses was determined in 1·8 mM

(normal), 0·72, 0·36, and 0·18 mM of Ca^{2+} Tyrode containing solutions. A typical result is shown in Fig. 4A and a marked difference is seen in the responses of the two tissues. The greatest difference occurred at a concentration of 0·36 mM of calcium Tyrode when the response of the control tissue was 71% of that which was obtained in normal Tyrode as compared with a value of 28% in the phospholipase C-treated tissue.

(b) *Initial response to potassium depolarizing Tyrode.* Incubation of a longitudinal muscle preparation with phospholipase C reduced the size of the initial response to potassium depolarizing Tyrode. The previously described experimental procedure was then repeated to determine the relation between the response to potassium Tyrode and the Ca^{2+} concentration of the medium.

The results of this experiment again reveal a marked difference between the behaviour of the phospholipase C-treated tissue and the control preparation (Fig. 4B), indicating that treatment with the enzyme also impaired the interactions of calcium with the tissue and its responsiveness to immersion in potassium depolarizing Tyrode.

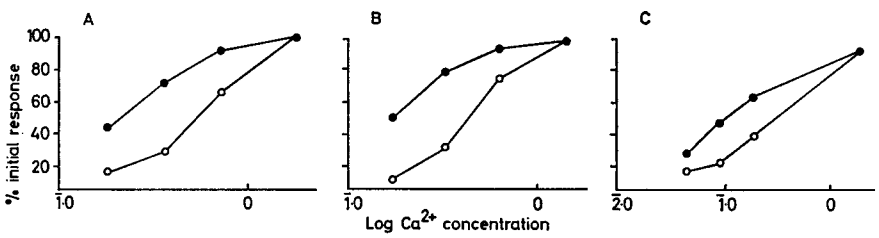

FIG. 4.A. The effect of phospholipase C upon the calcium ion concentration/standard acetylcholine response relation for preparation bathed in normal Tyrode. Ordinate: response height expressed as a percentage of the initial response obtained in 1·8mM calcium. (●) control tissue; (○) phospholipase C-treated tissue. Each point represents the mean of three responses.

B. The effect of phospholipase C upon the calcium ion concentration/standard potassium depolarization response relation for preparations bathed in normal Tyrode. Other details as for A.

C. The effect of phospholipase C upon the calcium ion concentration/standard acetylcholine response relation for preparations bathed in potassium depolarizing Tyrode. Other details as in B.

(c) *Acetylcholine responses in potassium depolarizing Tyrode.* On transferring the phospholipase C-incubated tissue to potassium depolarizing Tyrode, the acetylcholine responses were found to be reduced in comparison to their initial size. A suitable submaximal response (80% of maximum) could now only be obtained with larger doses of acetylcholine (10 μg) and the behaviour of this response to alteration of the Ca^{2+} concentration was compared with that of an equivalent sized acetylcholine response obtained in a control untreated tissue in the manner described for the preceding experiments. The Ca^{2+} concentrations used were 1·8, 0·18, 0·09 and 0·045 mM.

The results (Fig. 4C) again indicate a marked impairment in the interactions of calcium with the tissue after phospholipase C, the differences in the response size being most pronounced at calcium concentrations of 0·18 and 0·09 mM where a difference of 29% could be observed between the responses of the two preparations.

DISCUSSION

The ability of an elevation of the calcium ion concentration to partially restore the contractile responses of a phospholipase C-treated preparation may indicate that the enzyme is capable of impairing the interactions of calcium with the tissue. This conclusion is convincingly confirmed by the change in the rate of loss and the rate of restoration of responses upon removal and the reintroduction of calcium, by the changes in the calcium concentration response relation, and by the effect of the enzyme upon the responses of a depolarized preparation to added calcium.

As a consequence of the dual role of calcium in smooth muscle, the effects of phospholipase C on the interactions of calcium with the tissue could have two explanations.

Calcium has been shown to be essential for the normal membrane potential properties of smooth muscle in both the resting and excited state. The removal of calcium results in depolarization and changes in membrane potential responses, whilst an increase of calcium concentration results in hyperpolarization and increased stability (Burnstock, Holman & Prosser, 1963). If phospholipase C incubation caused some loss of membrane potential together with changes in membrane potential responses, then the effects of calcium removal may be enhanced, and an increase of the Ca^{2+} concentration may tend to restore normal membrane potential properties.

Calcium has also been shown to be an essential component of excitation-contraction coupling mechanisms (Bohr, 1964). Changes in the calcium ion concentration of the medium may, therefore, have abnormal effects if the properties of calcium binding sites which are associated with these activities are altered by the action of phospholipase C. The effects of phospholipase C on the properties of potassium depolarized preparations, in which the membrane potential responses have been eliminated, clearly show that the interactions of calcium with excitation-contraction coupling mechanisms have been impaired.

There is evidence to suggest that, in smooth muscle, there is probably more than one calcium binding or uptake site which is capable of contributing to excitation-contraction coupling (Edman & Schild, 1962). Thus, it has been shown that when calcium is removed from the medium of a depolarized smooth muscle preparation, the responses to different spasmogenic drugs decline at the same rate. This may indicate that, in the absence of membrane potential responses, only one type of calcium binding site is available for activation. In contrast, the responses of normal polarized smooth muscle to potassium depolarization, or agonist stimulation, decline at different rates when calcium is removed from the medium. This may indicate that under normal conditions an additional calcium binding site is available which is activated by membrane potential responses, and which is utilized to a different extent by different agents.

The observed effects of phospholipase C upon the behaviour of depolarized preparations provide strong evidence that the enzyme has impaired the activity of the "directly activated" calcium binding site. The changes in the behaviour of the acetylcholine responses in normal Tyrode may also suggest that phospholipase C is affecting the properties of a calcium binding site which is activated through membrane potential responses. Furthermore, the changes in the responses to potassium depolarizing Tyrode would appear to confirm this conclusion. The situation is complicated, however, by the possibility that membrane potential responses may also be affected by phospholipase C, an effect which may lead to abnormal behaviour of these responses on changing the calcium ion concentration of the medium. This evidence, therefore,

although strongly suggestive of an impaired activity of the "membrane potential activated" calcium binding sites, is rather less conclusive.

The results of these experiments allow no firm conclusions to be made as to the precise mechanism of action of phospholipase C on the calcium binding activity of smooth muscle. In skeletal muscle, the calcium binding properties of the sarcoplasmic reticulum have been correlated with the activity of an adenosine triphosphatase system (Martonosi, Donley & Halpin, 1968) which has been shown to depend upon the presence of phospholipids for its normal activity. In smooth muscle the sarcoplasmic reticulum is poorly developed (Bohr, 1964) or absent (Tomita, 1966) so that the location of the calcium binding sites which participate in excitation-contraction coupling cannot be definitely established.

Evidence has been discussed, however, which suggests that these calcium binding sites are located in the plasma membrane (Daniel, 1965) and the fact that excitation-contraction coupling seems to be an energy dependent process (Bülbring & Lullmann, 1957; Axelsson & Bülbring, 1961) raises the possibility that, in this tissue, as in skeletal muscle, an enzyme mechanism is involved. If this is so, then the explanation for the inhibitory action of phospholipase C on smooth muscle contraction may be that it is partly due to the hydrolysis of phospholipids which are essential for the normal activity of a calcium accumulating enzyme system.

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