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The sources of calcium for carbachol-induced contraction in the circular smooth muscle of guinea-pig stomach

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1 The action of carbachol on the mechanical activity of circular muscle from guinea-pig upper stomach was studied. High concentrations of carbachol (e.g. 10^{-4} M) produced a rapid phasic contraction followed by a smaller, sustained tonic contraction. Low concentrations (e.g. 10^{-7} M) caused a contraction which did not generally show marked distinction between phasic and tonic components.

2 The response to 10^{-7} M carbachol was very sensitive to 10^{-5} M nifedipine as was the phasic response to 10^{-4} M carbachol. The tonic contraction to the latter, however, was only slightly reduced by nifedipine.

3 The carbachol-induced contractions remaining in the presence of nifedipine were dose-related and very dependent on the presence of external calcium.

4 Carbachol, 10^{-7} M, did not produce a contraction after 4 min exposure to calcium-free solution whereas 10^{-4} M carbachol did and this was phasic in nature but much reduced relative to the control in normal Ca.

5 A phasic followed by a small tonic contraction to 10^{-4} M carbachol was seen superimposed on the K contracture in tissues depolarized with 100 mM K, whereas only a small tonic response occurred for 10^{-7} M carbachol.

6 In the absence of a functional carbachol-sensitive intracellular store, 10^{-4} M carbachol was unable to trigger a contraction in calcium-free solution. However, when calcium was simultaneously readmitted with carbachol after exposure to calcium-free solution, a contraction occurred.

7 Carbachol, 10^{-7} M, did not significantly increase inositol polyphosphate levels, whereas 10^{-4} M carbachol did. The increase with 10^{-4} M occurred rapidly peaking within 2 min and was undetectable after 5 min, in the absence of lithium.

8 It is concluded that low concentrations of muscarinic agonist trigger a contraction predominantly through a nifedipine-sensitive route whereas higher concentrations further utilize intracellular calcium release and a receptor-operated extracellular calcium-dependent pathway. The former is probably associated with the phasic component and the latter with the tonic one.

Keywords: Guinea-pig stomach; smooth muscle; calcium stores; carbachol; nifedipine; inositol polyphosphates

Introduction

Muscarinic receptors mediate several distinct responses in various tissues including smooth muscles (Bolton, 1979). For example, in the longitudinal smooth muscle of guinea-pig ileum, muscarinic receptor activation increases spike frequency and depolarizes the plasma membrane (Bolton, 1972), stimulates inositol polyphosphate production (Best *et al.*, 1985) and increases guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels (Lee *et al.*, 1972). This diversity presumably results either from several distinct subtypes of muscarinic receptor present or linkage of one receptor to several different transduction mechanisms. The existence of subtypes was suggested by binding studies with the antagonist pirenzepine which revealed the existence of binding sites with different affinities (Hammer *et al.*, 1980). Recent progress in molecular biology using receptor cloning techniques has demonstrated the presence of several muscarinic subtypes and it appears that different muscarinic subtypes are encoded by separate genes (Bonner *et al.*, 1987). These subtypes are linked to different transduction mechanisms although there is some overlap (Lechleiter *et al.*, 1989). In the guinea-pig stomach, the muscarinic agonist carbachol produces a contraction consisting of an initial phasic and a late tonic component. From the sensitivities of these components to nifedipine, Golenhofen (1976) postulated two mechanisms of contraction in smooth muscle namely the P and T systems. The phasic (initial component) was nifedipine-sensitive whereas the tonic (later) component was nifedipine-resistant. However, this generalization seems a little too simplistic. Carbachol can mobilize intracellular calcium stores in visceral smooth muscle (Brading & Sneddon, 1980) and the role of this in the P and T system is not clear.

The aim of this study was to investigate the sources of calcium mobilized by different concentrations of carbachol in guinea-pig stomach and how these contractions were affected by nifedipine. We conclude that high concentrations of carbachol trigger and maintain contraction using mechanisms in addition to those used by low concentrations.

Methods

Isometric contraction

Guinea-pigs of either sex were killed by stunning and bleeding. Whole stomachs were rapidly removed, opened along the greater curvature and the contents washed out in physiological saline solution (PSS, composition described below). Both halves of the stomach wall were then incubated in PSS for about 10 min at 37°C before the mucosa was removed completely under an optical microscope. Muscle strips (about 1 mm wide and 10 mm long) from the fundus were cut along the direction of the circular fibres and then mounted for isometric tension as previously described (Syed *et al.*, 1990; Brading & Sneddon, 1980). The bath temperature was maintained at 35–38°C.

Inositol polyphosphates

Dissected tissues were finely minced with microscissors and then loaded with 30 μCi [^{3}H]-myoinositol for 1.5 h. The suspension was then washed several times in warm PSS.

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In some experiments (see below) 10 mM Li-containing PSS was added to inhibit inositol-1-monophosphatase (Berridge *et al.*, 1982). The labelled tissue suspension was taken and added to tubes containing PSS with vehicle or agonist. Reactions were terminated after different times (see text) with 940 μl of chloroform: methanol: HCl (50 : 100 : 1 v/v), 310 μl chloroform and 310 μl distilled water, vortexed and then centrifuged at 1000 *g* for 5 min; 800 μl of the upper (cytoplasmic) phase was diluted with 1 ml water and then passed through a Dowex column (50% slurry). Inositol was eluted from the column by applying 60 mM ammonium formate/5 mM sodium tetraborate. Total [^{3}H]-inositol phosphates (IP_1, IP_2 and IP_3) were then eluted with 0.8 M ammonium formate/0.1 M formic acid (Guard *et al.*, 1988). This fraction was collected in scintillation vials and then counted for radioactivity after addition of scintillation fluid.

Of the lower organic lipid phase, 200 ml was removed and counted for radioactivity as above. This was done to normalize each assay with respect to the amount of tissue, assuming that the amount of lipid is proportional to the amount of tissue.

Solutions

The composition of the PSS was (mM): NaCl 130, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 5, glucose 11, HEPES 5; pH was adjusted to 7.35–7.40 with Tris base. The solutions were gassed with 100% oxygen. In experiments where high K was used, NaCl was replaced isosmotically with KCl to the desired concentration. Calcium-free solutions contained 1 mM EGTA and Ca was isosmotically replaced with Mg.

Drugs used were carbachol chloride, propranolol hydrochloride, caffeine, procaine, and nifedipine (all from Sigma).

Statistics

Results are expressed as the mean ± s.e.mean. The significance was assessed by a paired *t* test when responses were compared in the same strip and an unpaired *t* test when the inositol polyphosphates were analysed. Values of *P* less than 0.05 are shown with a single * and less than 0.001 with two **.

Results

Responses in the presence of extracellular calcium

General patterns of response Application of carbachol resulted in a concentration-dependent contraction, the pattern of which depended on the concentration of agonist. A maximal dose of carbachol (10^{-4} M) resulted in a rapid phasic (initial) contraction followed by a maintained tonic (later) component which was 51.9 ± 2.8% of the peak phasic contraction ($P < 0.001$, $n = 48$ strips from 10 animals). For a low concentration of agonist (10^{-7} M, the threshold for a detectable response being about 10^{-8} M), there was generally no clear demarcation between phasic and tonic components. In Figure 1e, dose-response curves are shown for the contraction after 2 and 15 min applications of carbachol. There was a significant difference between the response to 10^{-4} and 10^{-7} M carbachol after a 2 min application ($P < 0.05$).

Sensitivity of contractions to nifedipine The 1,4 dihydropyridine, nifedipine, blocks the L-type calcium channel in smooth muscle. Nifedipine concentration-dependently reduced both the phasic (initial) (measured after 2 min) and tonic (later) responses to a low dose of carbachol (10^{-7} M) but was able to attenuate markedly only the phasic response to a high concentration of agonist (10^{-4} M). The tonic component produced by 10^{-7} M carbachol was more-or-less totally abolished by 10^{-5} M nifedipine whereas the tonic component produced by

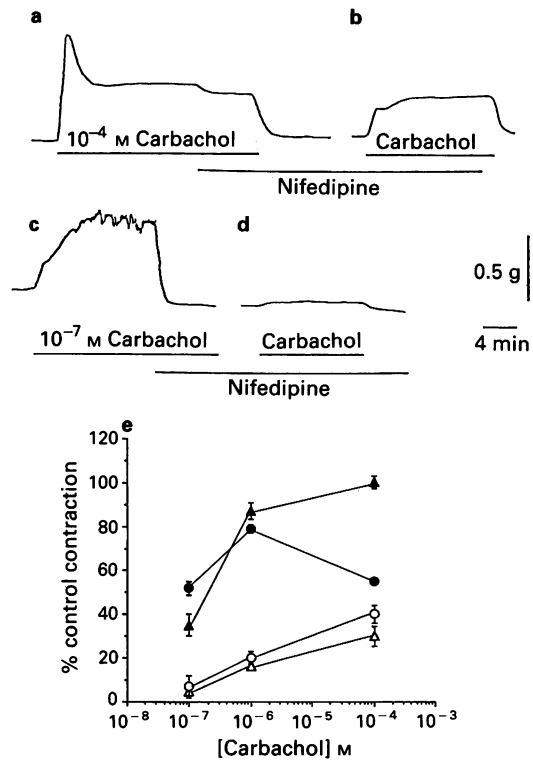

Figure 1 Effects of 10^{-5} M nifedipine on carbachol contractions. Nifedipine applied during contraction to (a) 10^{-4} M and (c) 10^{-7} M carbachol. Responses in the presence of nifedipine after 15 min pretreatment to (b) 10^{-4} M and (d) 10^{-7} M carbachol. (e) Concentration-response curves after 2 min exposure to carbachol in the absence (▲) and presence (△) of nifedipine (10 strips from 2 animals) and responses after 15 min application of carbachol in the absence (●) and presence (○) of nifedipine (20 strips from 4 animals).

10^{-4} M carbachol was only slightly but significantly reduced from 58.8 ± 5.9% to 41.1 ± 4.6% ($P < 0.05$). Typical results are shown in Figure 1. Nifedipine was applied during the tonic component of the contraction to a high (10^{-4} M) and low (10^{-7} M) concentration of carbachol (Figure 1a and c respectively). The contraction to 10^{-4} M carbachol was slightly reduced whereas nifedipine almost totally abolished the tonic contraction to 10^{-7} M carbachol. In another set of experiments, preparations were exposed to nifedipine for at least 15 min before carbachol was applied in the maintained presence of nifedipine. As is evident from Figure 1, the contraction after 2 min exposure to agonist was greatly reduced for both a high and a low concentration of carbachol (Figure 1b and d respectively) whereas the tonic component to a low concentration was more sensitive to nifedipine.

Dependence of the nifedipine-insensitive component on extracellular calcium concentration The size of the tonic (later) component of contraction to 10^{-4} M carbachol in the presence of nifedipine was dependent on the concentration of extracellular calcium (Figure 2a and b). Similar results were obtained for both 10^{-7} M and 10^{-6} M carbachol (8 strips from 2 animals, data not shown). La, 1 mM, which blocks the calcium-dependent contraction to noradrenaline in rat vas deferens (Swamy *et al.*, 1976), rapidly abolished the tonic response to 10^{-4} M carbachol (5 strips from 1 animal, data not shown).

Responses in Ca-free solution

To study the role of intracellular calcium stores in the response to carbachol, external calcium was removed by perfusion with calcium-free solution. Such a method has been

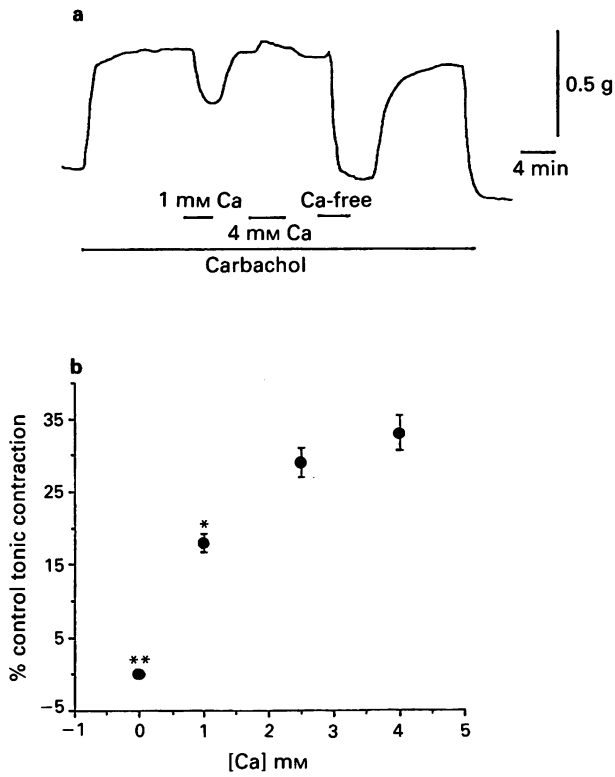

Figure 2 Dependency of tonic contraction on external calcium. (a) Changing extracellular calcium produced changes in the tonic contraction to carbachol (10^{-4} M) in the presence of nifedipine (10^{-5} M). (b) Graphical representation of the data, given as mean of 12 strips from 2 animals with s.e.mean shown by vertical bars. The tonic response in 2.5 mM Ca in the absence of nifedipine was taken as 100%. The significance of changing extracellular calcium on steady-state tone was determined relative to the contraction to 2.5 mM Ca in the presence of nifedipine.

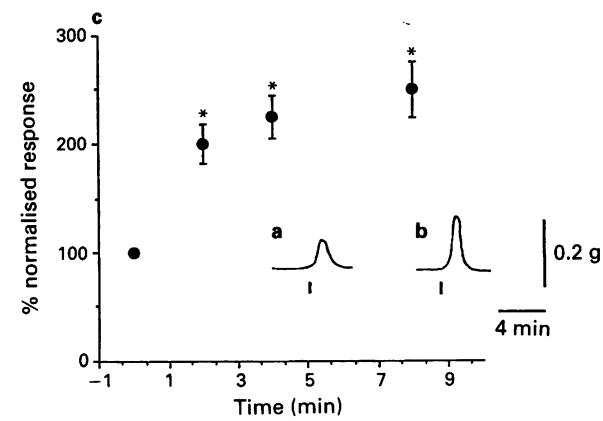

Figure 3 Effect of pretreatment with 40 mM K solution on the contractile response to 10^{-4} M carbachol in Ca-free solution. Tissues were exposed to high K for the times indicated (c) and then superfused with Ca-free (5.9 mM K) solution for 4 min before exposure to carbachol in Ca-free solution for 2 min. Points are mean of 31 strips from 6 animals; s.e.mean shown by vertical bars. Response in the absence of pretreatment was taken as 100%, and significance was determined relative to this. Inset: (a) contraction to carbachol without 40 mM K pretreatment; (b) contraction after 4 min 40 mM K pretreatment.

widely used in smooth muscle (Casteels & Raeymakers, 1979; Brading & Sneddon, 1980; Ashoori & Tomita, 1983; Lalanne *et al.*, 1984).

After exposure to calcium-free solution for 4 min, high K calcium-free solution did not produce a contraction (13 strips from 3 animals). This suggests that, after this time, most of the external calcium had been removed. Under these conditions, 10^{-7} M carbachol failed to produce a contraction but 10^{-4} M carbachol produced a phasic response which was much smaller than the phasic contraction in the presence of calcium (Figure 3a); the tonic component was negligible.

For ease of analysis, the size of the carbachol-induced contraction in calcium-free solution was elevated by imposing a calcium load on the tissue. This was achieved by exposure to 2.5 mM calcium in 40 mM K solution (this contained 0.1 μg ml^{-1} tetrodotoxin to abolish action potential-mediated release of endogenous neurotransmitters) for different times followed by calcium-free 1 mM EGTA solution for 4 min then agonist challenge in calcium-free solution. Under these conditions the 10^{-4} M carbachol-induced contraction was increased 2.2 $\pm$ 0.2 fold (27 of 31 strips from 6 animals, Figure 3b), whereas 40 mM K in calcium-free solution still did not produce a contraction (10 strips from 2 animals). This is probably a consequence of increased calcium uptake into the carbachol-sensitive intracellular store. The contraction to carbachol increased as a function of exposure time to 40 mM K solution as shown in Figure 3c and as observed by Casteels & Raeymakers (1979) in guinea-pig taenia. For subsequent experiments, 4 min high K pretreatment was used. Carbachol, 10^{-7} M, did not trigger a contraction in calcium-free solution even if a calcium load had been imposed (10 strips from 2 animals).

The carbachol response was lost rapidly after stimulation. After a contraction to 10^{-4} M carbachol had been obtained in calcium-free solution, preparations were maintained in calcium-free solution for a further 4 min and then challenged again with 10^{-4} M carbachol. No contraction occurred. This is unlikely to reflect receptor desensitization because 10^{-4} M carbachol still did not produce a contraction in calcium-free solution if the initial concentration of carbachol applied was lower (10^{-5} M, 10 strips from 2 animals). This suggests that the calcium released by carbachol is not taken back up into the store from which it was released.

Effects of caffeine and procaine Agents known to interfere with calcium release from internal stores were studied in calcium-free solution.

Procaine is thought to inhibit calcium-induced calcium release (Endo, 1977). It also produced a dose-dependent reduction of the 10^{-4} M carbachol-induced contraction in a concentration-dependent way with an IC_{50} of 0.1 mM (Figure 4a; 14 strips from 3 animals).

To enhance the effects of the methylxanthine, caffeine, on calcium release from the sarcoplasmic reticulum (Iino, 1989) experiments were conducted at room temperature (20–24°C) as the contractile response is increased in this temperature range (Endo, 1977). Caffeine produced a small, transient contraction (about 30% of the carbachol-induced contraction in calcium-free solution) at concentrations >1 mM and this was enhanced after a calcium load (data not shown). Caffeine pretreatment resulted in a concentration-dependent reduction in the carbachol contraction, with an IC_{50} of 1 mM (Figure 4b; 14 strips from 3 animals).

Effects of nifedipine on store refilling in the presence of extracellular calcium

After prolonged treatment with nifedipine the small contraction to carbachol in calcium-free solution was reduced (15 strips from 3 animals). This probably did not reflect non-specific rundown of the tissues because these contractile responses in control preparations were not reduced with time. It was difficult to quantify these contractions as they were small if a calcium load was not imposed as discussed above (e.g. with high K, but this potentiation is nifedipine-sensitive). This raises the key question of the role of the nifedipine-sensitive route in the response to carbachol. It is possible that

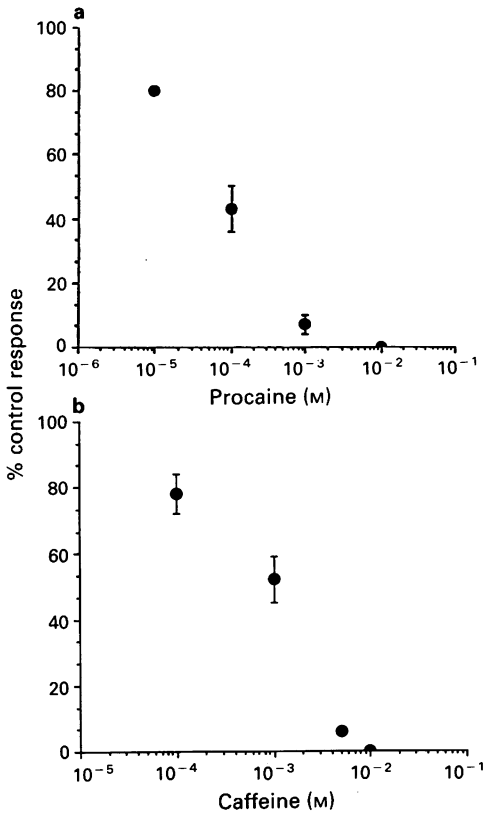

Figure 4 Effects of agents interfering with intracellular calcium release on the contraction to 10^{-4} M carbachol. After high K pretreatment, tissues were exposed to Ca-free solution and drug for 4 min before 10^{-4} M carbachol was applied in the maintained presence of these drugs: (a) procaine; (b) caffeine. Points are mean of 14 strips from 3 animals each; vertical bars show s.e.mean. The contraction after high K treatment was taken as 100%.

calcium through this route could directly contribute to activation of the contractile system or that this calcium first passes into the carbachol-sensitive store and is then released by the agonist or triggers a form of calcium-induced calcium release. The following experiments were designed to gain some information on the contribution of the nifedipine-sensitive route and intracellular calcium release on the phasic component of the carbachol contraction.

Phasic (initial) response in the absence of the nifedipine-sensitive route

As mentioned above, we believe the carbachol-sensitive store is at least partly refilled through a nifedipine-sensitive route. To study the effects of nifedipine on store release, we applied 10^{-5} M nifedipine for 10 s before giving 10^{-4} M carbachol. This short time was chosen because it did not seem to affect calcium store filling (as judged by the contraction to carbachol in calcium-free solution) yet the time was sufficient to block the 100 mM K-induced contraction (in the presence of nifedipine, 2 out of 2 experiments, data not shown). Under these conditions, the phasic response was significantly reduced to $67.8 \pm 6.1\%$ ($P = 0.002$) and also declined more rapidly. This phasic contraction was larger, however, than the early peak response to carbachol obtained after prolonged nifedipine treatment (data not shown).

Responses in depolarized tissues

The nifedipine-sensitive calcium channel in other smooth muscles shows both voltage- and calcium-dependent inactivation (Ohya *et al.*, 1988; Bean, 1989). Therefore in the presence of high external K (which would both depolarize and increase cytoplasmic free calcium) one would anticipate some inactivation and therefore reduced availability of the nifedipine-sensitive channel for contribution to the phasic response of carbachol. Thus preparations were depolarized by exposure to 100 mM K which resulted in a phasic followed by a tonic contraction (Figure 5a). Once a steady-state had been reached, application of 10^{-4} M carbachol resulted in a rapid phasic contraction and this was followed by a slowly developing tonic component superimposed on the tonic component of 100 mM K (Figure 5b). For 10^{-7} M carbachol, only a slowly developing tonic component was observed without any phasic response (10 strips from 2 animals, Figure 5c). This suggests that the phasic response to 10^{-4} M carbachol under these conditions is due to calcium release from well-loaded intracellular stores (due to the high K) while the lack of a phasic response to 10^{-7} M carbachol is due to an inability to release calcium from this store.

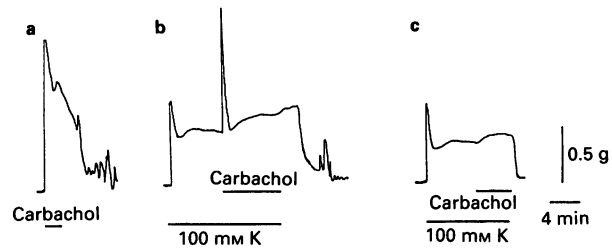

Figure 5 Responses to carbachol in the presence of 100 mM K. (a) Control 10^{-4} M carbachol response in normal solution; (b) in response to 10^{-4} M and (c) to 10^{-7} M carbachol in high K.

Responses in the absence of a functional intracellular store

Preparations were exposed to calcium-free 1 mM EGTA solution for 10 min and then calcium-free solution in the absence of EGTA for 5 min. Subsequent application of 10^{-4} M carbachol (in calcium-free solution) did not produce a contraction. This indicates functional loss of the carbachol-sensitive intracellular calcium pool. If, after the calcium-free perfusion described above, 10^{-4} M carbachol was applied simultaneously with 2.5 mM Ca for 10 s before returning the preparations to calcium-free 1 mM EGTA, a transient phasic contraction occurred (Figure 6) and this was $28.7 \pm 5.1\%$ of the control phasic response (10 strips from 2 animals). If 10^{-5} M nifedipine was added during the calcium-free 1 mM EGTA perfusion and then maintained, the response to simul-

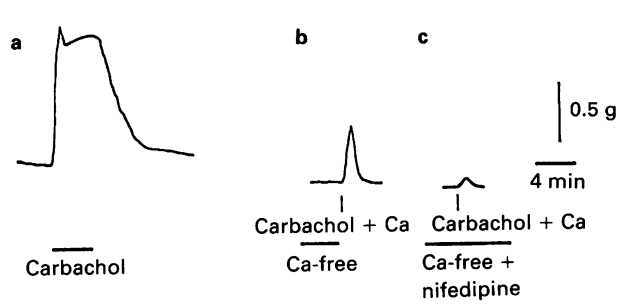

Figure 6 Responses to 10^{-4} M carbachol in the absence of its functional intracellular store. (a) Control contraction. After Ca-free exposure (see text) carbachol was applied with 2.5 mM Ca for 10 s in the absence (b) or presence (c) of nifedipine. Nifedipine was applied 10 min before carbachol application. Calcium readmission alone for 10 s did not produce a contraction.

taneous carbachol and calcium application was significantly reduced to 7.9 ± 3.2% ($P = 0.001$, 10 strips from 2 animals). A 10 s application of calcium alone did not result in contraction. This suggests that (1) a phasic nifedipine-sensitive contraction can occur in the absence of a functional carbachol-sensitive intracellular pool and (2) this contraction is not a consequence of calcium-induced calcium release.

We cannot discriminate between direct activation of the contractile system by this calcium influx or whether it passes first into an intracellular pool and is then released by a second messenger. From the results described below with inositol polyphosphate measurements, we favour the former possibility.

Effects on inositol polyphosphate production

The effects of carbachol on total inositol phosphate levels (IP_{1-3}) were studied. Carbachol, 10^{-7} M, did not produce a significant increase in IP_{1-3} levels whereas 10^{-6} and 10^{-4} M carbachol triggered dose-dependent increases. The results are depicted graphically in Figure 7. In two larger animals it was possible to compare different doses of carbachol. Here 10^{-7} M carbachol still did not cause a significant increase, yet, in the same animal, 10^{-6} M did. There was some variation in the IP_{1-3} response to 10^{-7} M carbachol and the range was 90–130% of the mean. In 11 out of 28 assays from 7 animals, application of 10^{-7} M carbachol resulted in no increase relative to control. In those 17 assays where an increase was observed, the range was 105–127% (mean 117 ± 4%, control 100%). In those animals that produced a large response to 10^{-4} M carbachol, the tendency was that a small increase was seen with 10^{-7} M. This suggests that 10^{-7} M carbachol may be the critical concentration such that beyond this increases in inositol polyphosphates are obtained.

These results were obtained by exposing the preparations to agonist for 15 min in the presence of 10 mM Li to inhibit inositol-1-monophosphatase (Berridge *et al.*, 1982) in order to amplify and maintain increases. To gain more insight into the role of IP_{1-3} in contraction it was therefore necessary to measure the time-course of change in IP_{1-3}.

Time-course of change in IP_{1-3}

To monitor the rate of change of IP_{1-3} levels, carbachol was applied for different times. As seen in the experiment shown in Figure 8, the production of IP_{1-3} occurred rapidly and there was no further significant increase between 2 and 15 min. This suggests that IPs are rapidly produced and, in the absence of their breakdown, the rate of synthesis returns to basal levels despite the maintained presence of agonist. In longitudinal muscle of ileum, however, IP_{1-3} production increases linearly over 20 min (Best *et al.*, 1985). It is not clear why gastric fundus is atypical, but one possibility may be that phosphatidyl inositol 4,5, bisphosphate is substrate limiting.

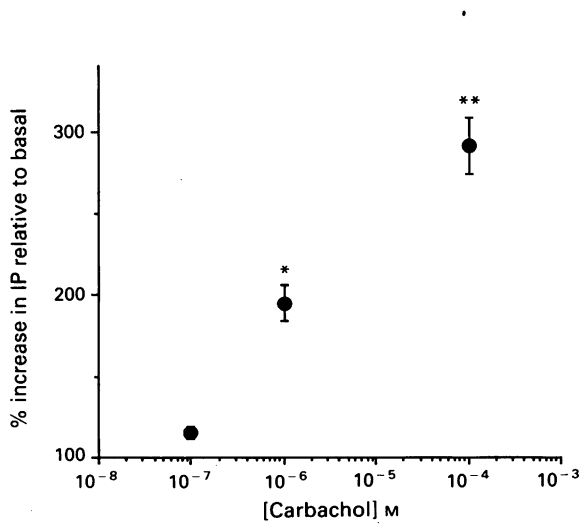

Figure 7 The effect of carbachol on inositol polyphosphate production. The total polyphosphates produced during 15 min exposure to carbachol are expressed relative to basal production. Points are the means of 5 experiments with s.e.mean shown by vertical bars.

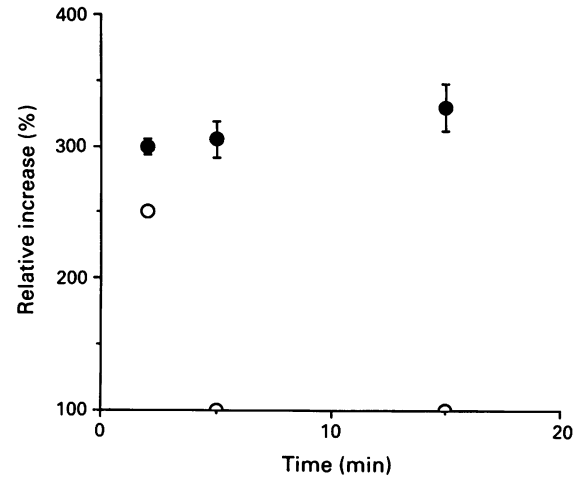

Figure 8 Time course of inositol polyphosphate production. Preparations were exposed to 10^{-4} M carbachol for the times indicated in the presence (●) or absence (○) of Li. Results are expressed relative to basal production.

To see if the rate of synthesis fell if breakdown of inositol polyphosphates can occur, experiments were performed in the absence of Li and the IP_{1-3} levels observed after different agonist exposure times. In all experiments (11 assays from 3 animals) 10^{-4} M carbachol did not produce a significant increase in IP_{1-3} relative to control after 15 min ($P > 0.05$; Figure 8). These results suggest that IP_{1-3} production has returned to basal values before 15 min.

Discussion

The main finding of this study is that there are at least three sources of calcium mobilized by carbachol for contraction. These are: (1) a nifedipine-sensitive component, (2) a nifedipine-insensitive but extracellular calcium-dependent component and (3) a carbachol-sensitive intracellular store. We have found that different concentrations of carbachol can activate these components differentially.

Low concentrations of carbachol

Low concentrations of carbachol did not elicit a contraction under calcium-free conditions suggesting weak mobilization of an intracellular store. Consistent with this was the finding that such concentrations did not significantly elevate inositol polyphosphates, which would be presumed to mobilize such stores (Streb *et al.*, 1983). The methodology for inositol polyphosphate measurements, however, is very crude in that it does not allow localized changes to be resolved. Only net inositol phosphate changes in the whole cytoplasm are detected. As there are reports of superficial intracellular calcium stores in rapid exchange with the extracellular solution (e.g. Brading & Sneddon, 1980; Casteels & Droogmans, 1981), it is possible that low concentrations of carbachol mobilize these stores under normal conditions through a localized increase in inositol polyphosphates. Dilution with the cytoplasm may render this increase undetectable.

The contraction to low concentrations of carbachol was very sensitive to nifedipine. Both the early portion as well as

the tonic component were significantly reduced. The small contraction remaining in the presence of nifedipine was dependent on external calcium. This suggests that the main source of calcium for low concentrations of carbachol is through a nifedipine-sensitive route but there is also a small contribution during the tonic phase through a nifedipine-insensitive route. This is similar to the observations of Cauvin *et al.* (1984) in rabbit mesenteric resistance vessels. These workers found that low concentrations of noradrenaline did not release calcium from intracellular stores but utilized a diltiazem-sensitive route for contraction. As in other gastrointestinal smooth muscle (e.g. ileum, Bolton, 1972), 10^{-7} M carbachol can produce significant depolarization in gastric fundus (unpublished observation). It seems reasonable to suppose that low concentrations of carbachol act largely through electromechanical coupling.

High concentrations of carbachol

The contractions to higher concentrations of carbachol were complex and consisted of an initial phasic component which rapidly declined to a later tonic phase. For analytical convenience let us consider each component in turn.

The tonic component was only slightly reduced by nifedipine treatment but was critically dependent on the presence of external calcium. These results are consistent with the ^{45}Ca uptake data of Lammel (1977) who found both a nifedipine-sensitive and a nifedipine-insensitive component of calcium uptake in guinea-pig gastric fundus in response to a high muscarinic agonist concentration. Recently, Lammel *et al.* (1991) have shown acetylcholine depolarizes single gastric fundus smooth muscle cells by inhibiting an outward I_K, thus depolarizing the cells, and presumably increasing L-type calcium channel activity. A role for non-selective cation channels was not revealed in this study. Gastric fundus circular muscle is therefore different from longitudinal muscle of ileum where muscarinic receptors activate non-selective cation channels which are permeable to calcium (Inoue & Isenberg, 1990) and hence may contribute to a nifedipine-insensitive contraction. It seems reasonable to suppose that the nifedipine-insensitive tonic response is due to a receptor-operated calcium influx pathway (Bolton, 1979), although we cannot determine whether this calcium activates the contractile system directly or indirectly via activation of some mediating intracellular factor. As the inositol polyphosphates declined rapidly after stimulation, it seems unlikely that intracellular calcium mobilization by these messengers is involved in the tonic response.

The phasic response was more complex. As it was severely attenuated by prolonged pretreatment with nifedipine, it is tempting to attribute this to calcium influx through a nifedipine-sensitive route. However this may be too simplistic. We found that nifedipine, applied in the presence of extracellular calcium, was able to reduce the subsequent carbachol contraction in calcium-free solution suggesting that this store refills, at least in part, through a nifedipine-sensitive route. This is similar to observations in guinea-pig coronary artery where the related drug D-600 reduced responses dependent on intracellular calcium release at rest (Ganitkevich & Isenberg, 1990). This raises the possibility that nifedipine block of the phasic response is due indirectly to its ability to reduce store refilling and that intracellular calcium release is the cause of the phasic response. It was therefore necessary to determine whether the nifedipine-sensitive route could directly contribute to the phasic response. We think it can for the following reasons. First, exposure to nifedipine for a very short time (10 s, a time too short to compromise nifedipine-dependent refilling of the intracellular store) was sufficient to block the high K contraction. This duration of exposure did not completely block but significantly reduced the phasic response to carbachol. As the high K response was lost using this procedure, it seems likely that there is block of the L-type calcium channel which is widely thought to be the chemically sensitive site of nifedipine (Tsien, 1983; Bean, 1989 for reviews), and hence this channel is involved directly in the phasic contraction.

Second, after functional loss of the carbachol-sensitive intracellular pool by a long exposure to calcium-free solution, application of carbachol and calcium for a very short time resulted in a phasic contraction and this was much reduced by nifedipine. This may represent calcium influx through the L-type channel. We think it unlikely that this calcium first passes into an intracellular store (and is then mobilized) because a low concentration of carbachol did not increase inositol polyphosphates yet the contraction was very sensitive to the blocking action of nifedipine (see above), but we cannot exclude the possibility.

It seems probable that intracellular calcium release also contributes to the phasic response because short prior application of nifedipine reduced but did not abolish the phasic response. Furthermore, in depolarized tissues in which some of the calcium channels should be inactivated, only high concentrations of carbachol produced a phasic response and these concentrations also increased inositol polyphosphates (we are assuming inositol trisphosphate is the main mediator of intracellular calcium release; to our knowledge there is no other viable candidate released on muscarinic stimulation). Finally, only high concentrations of carbachol produced a small contraction in calcium-free solution and this was also phasic in nature and sensitive to agents that interfere with intracellular calcium store homeostasis.

Taken together we propose that intracellular calcium release and calcium influx through a nifedipine-sensitive route both contribute directly to the phasic component. These pathways interact in that intracellular calcium store requires, at least partly, the nifedipine-sensitive route for filling. The tonic component is a result of calcium influx through both a nifedipine-sensitive and a nifedipine-insensitive route and there is probably little contribution from IP_3-releasable stores.

Extreme caution must be applied to the interpretation of results when one agonist appears to utilize different mechanisms to elicit a response in a given tissue. Activation of the mechanisms may not be independent but rather one may be caused by another. For example, the transfected M_1 receptor-subtype can increase both IP_3 and cyclic AMP levels but the latter seems to be due to calcium-calmodulin activation of adenylate cyclase, the calcium arising from IP_3-induced store release (Felder *et al.*, 1989; Buck & Fraser, 1990). We believe the different transduction mechanisms in this paper are independent. Clearly, inositol polyphosphate production cannot be the cause of activation of the nifedipine-sensitive route because the latter was utilized by a low concentration of carbachol which did not affect inositol polyphosphate levels. Similarly, the nifedipine-sensitive route cannot be the mechanism of production of inositol polyphosphates because pretreatment with 10^{-5} M nifedipine did not affect inositol polyphosphate production by 10^{-4} M carbachol (data not shown).

It is therefore possible that each transduction mechanism is linked to a specific muscarinic receptor subtype, each subtype having different sensitivity to carbachol. If this is so, then it would appear that the subtype linked to the nifedipine-sensitive pathway has the highest relative sensitivity whereas that linked to inositol polyphosphate production has the lowest. Another possibility, however, is that one muscarinic receptor-type is linked to several different transduction pathways but with different efficacies. Hence the activated agonist-receptor complex would have high efficacy in activating the nifedipine-sensitive component but a low efficacy for inositol polyphosphate production. From studies on the effects of the transfected M_2 receptor subtype on IP_3 production and cyclic AMP inhibition, Ashkenazi *et al.* (1987) concluded that this subtype is linked to two different transduction mechanisms.

As the amount of acetylcholine released from a parasympathetic nerve terminal varies with the frequency of stimulation (Straughan, 1960), the concentration of acetylcholine to

which a tissue is exposed is variable. The physiological significance of this study then, may be as follows. Low sustained activity of parasympathetic nerves (presumably maintaining a low acetylcholine concentration in the synaptic cleft) can maintain a large contraction in the smooth muscle and an increase in nerve activity can induce a rapid further phasic contraction arising from a different transduction pathway.

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