Muscarinic-receptor stimulation enhances polyphosphoinositide breakdown in guinea-pig ileum smooth muscle

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Muscarinic-receptor stimulation by 0.1 mM-carbachol in longitudinal muscle of the guinea-pig ileum increases the incorporation of [^3H]inositol into inositol-containing phospholipid. This effect was blocked by 16 μM -atropine. After 60 min incubation, carbachol increased the accumulation of total inositol phosphates 20-fold in the presence of 10 mM- Li^+ . Less than 20% of the total inositol phosphate corresponded to inositol 1-phosphate by ion-exchange chromatography, whereas of the remainder about two-thirds corresponded to inositol bisphosphate and one third to inositol trisphosphate. It is concluded that stimulation of muscarinic receptors in guinea-pig ileum enhances breakdown of polyphosphoinositides, suggesting that this may be a primary event associated with Ca^{2+} mobilization in the guinea-pig ileum.

Most of the *myo*-inositol in cells exists in the form of phosphatidylinositol (PI) and its two phosphorylated derivatives, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). Whereas PI is distributed in both intracellular membranes and plasma membrane (Rouser *et al.*, 1968; Michell, 1975), the less-abundant lipids, PIP and PIP_2 , appear to be present mainly on the cytosolic side of the plasma membrane (Buckley & Hawthorne, 1972; Soukup *et al.*, 1978). It has been previously shown that stimulation of muscarinic receptors in longitudinal smooth muscle of guinea-pig ileum increases labelling of PI with [^{32}P]PO $_4^{3-}$ (Jafferji & Michell, 1976; Sekar & Roufogalis, 1982).

Recently, evidence has been presented in platelets (Billah & Lapetina, 1982; Agranoff *et al.*, 1983; Imai *et al.*, 1983), hepatocytes (Michell *et al.*, 1981; Thomas *et al.*, 1983; Creba *et al.*, 1983), parotid gland (Weiss *et al.*, 1982; Downes & Wusteman, 1983; Berridge *et al.*, 1983), superior cervical sympathetic ganglia (Michell *et al.*, 1983), lacrimal acinar cells (Godfrey & Putney, 1984), exocrine pancreas (Putney *et al.*, 1983), insect salivary glands (Berridge, 1983; Berridge *et al.*, 1983) and

pituitary cells (Rebecchi & Gershengorn, 1983) that enhanced PI labelling probably reflects receptor stimulation of the breakdown of polyphosphoinositide rather than PI, through activation of phosphoinositide phosphodiesterase (Michell *et al.*, 1981). Berridge *et al.* (1982) found that the presence of Li^+ led to the accumulation of inositol 1-phosphate in brain or parotid slices, most probably due to the inhibition of inositol 1-phosphatase by Li^+ (Allison & Blisner, 1976; Sherman *et al.*, 1981). They suggested that Li^+ can therefore be used to amplify the primary step of phosphoinositide breakdown after receptor stimulation.

In the present paper we report the effect of carbachol on PI turnover and polyphosphoinositide breakdown in the guinea-pig ileum in the presence of Li^+ . We show that carbachol stimulates the accumulation of inositol phosphates, which most probably arise from breakdown of polyphosphoinositides, and that this effect is amplified in the presence of Li^+ .

Experimental

Materials

[^3H]Inositol (15.8 Ci/mmol) and Econofluor scintillation fluid were from New England Nuclear. Carbamylcholine chloride, atropine sulphate, inositol 2-monophosphate and LiCl were

Abbreviations used: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate.

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from Sigma. Dowex-1 (X8; formate form) resin was from Bio-Rad. Glass-distilled chloroform and methanol were obtained from Caledon Laboratories, Georgetown, Ontario, Canada. All inorganic salts were of analytical-reagent grade. ACS scintillation fluid was from Amersham International.

Methods

Dissection of longitudinal smooth muscle. For each experiment the ileum was obtained from one or two female guinea pigs (350–400g). Longitudinal smooth muscle was removed as described by Rang (1964) and stored in cold buffer under an O_2/CO_2 (19:1) atmosphere until incubation. All incubations were carried out in Krebs–Ringer bicarbonate buffer adjusted to pH 7.4 with O_2/CO_2 (19:1) at 37°C.

Measurement of inositol incorporation into lipid. Pieces of longitudinal guinea-pig ileum (15–25 mg wet wt.) were combined and preincubated for 30 min with 30 μ Ci of undiluted [3H]inositol in 15 ml of Krebs buffer under an O_2/CO_2 atmosphere. Individual pieces were then separated and incubated separately for a further 30 min period with 1 μ Ci of [3H]inositol in the presence of various agents, in a total volume of 4 ml. The reaction was stopped by adding 2 ml of 20% (w/v) trichloroacetic acid. The tissue was removed and homogenized, and the lipids extracted by the method of Bligh & Dyer (1954). The lower organic phase was separated by centrifugation, washed twice with methanol/0.1 M-HCl/chloroform (48:41:3, by vol.), and the solvent finally removed by evaporation. The residue was dissolved in chloroform (2 ml) and two 75 μ l aliquots were taken for total-phosphorus determination. The radioactivity was determined in the remaining portion, after removal of the solvent and addition of 10 ml of Econofluor scintillation fluid. Incorporation is calculated as d.p.m./ μ g of phosphorus and expressed as a percentage of the control value.

Inositol phosphate accumulation in the presence of Li^+ . After prior treatment with ion-exchange resin to remove anionic contaminants, 30 μ Ci of [3H]inositol was added to 14 ml of Krebs buffer saturated with O_2/CO_2 (19:1). Guinea-pig ileum longitudinal-muscle pieces were incubated with 1 μ Ci of [3H]inositol in 1 ml of Krebs–Ringer bicarbonate buffer in the presence or absence of 0.1 mM-carbachol, 10 mM-LiCl and 6.4 μ M-atropine sulphate, as required, for a period of 1 h under O_2/CO_2 (19:1). The smooth-muscle pieces were transferred into 1 ml of chloroform/methanol (1:2, v/v), the method originally described by Berridge *et al.* (1982). Tissues were homogenized and the lipids separated as described above. Inositol phosphate accumulated in the aqueous phase was determined by the simplified assay procedure

described by Berridge *et al.* (1982). A 1 ml slurry of 50% (w/v) Dowex-1 (X8; 100–200 mesh; formate form) resin was added to the aqueous phase. The supernatant was discarded after the resin had been allowed to settle. After washing the resin five times with 4 ml of 5 mM-inositol, inositol phosphates were eluted with 0.8 ml of 0.1 M-formic acid plus 1 M-ammonium formate. Half of this eluate (0.4 ml) was used for the determination of radioactivity after adding 10 ml of ACS scintillation fluid.

Separation of inositol phosphates. Washed resin containing adsorbed inositol phosphates was successively eluted in a test tube with four 2 ml portions of: (i) 5 mM-sodium tetraborate plus 60 mM-ammonium formate; (ii) 5 mM-sodium tetraborate plus 150 mM-ammonium formate; (iii) 0.1 M-formic acid plus 0.4 M-ammonium formate; and (iv) finally 0.1 M-formic acid plus 1 M-ammonium formate. In preliminary experiments we confirmed that only inositol monophosphate was adsorbed to the anion-exchange resin from a mixture of [3H]inositol and standard inositol monophosphate (results not shown), and it was eluted from the resin with 5 mM-sodium tetraborate plus 150 mM-ammonium formate, as expected (Berridge *et al.*, 1982, 1983). The identity of the inositol phosphates was further characterized by ascending paper chromatography (Sequi *et al.*, 1966) on Whatman no. 3 paper (20 cm) with propanol/ NH_3 (sp.gr. 0.88)/water (5:4:1, by vol.) (Grado & Ballou, 1961). Inositol phosphate spots were identified by a molybdate spray reagent (inositol 2-phosphate and inositol hexakisphosphate controls) or by counting 3H radioactivity in 1 cm $\times$ 1.5 cm strips after soaking in 1 ml of water with continuous shaking overnight and addition of ACS scintillation fluid.

Results

In preliminary results we found that 98% of the total [3H]inositol label after carbachol stimulation was incorporated into inositol lipids. Therefore [3H]inositol incorporation was routinely determined in the total-lipid fraction. Carbachol (0.1 mM) increased the incorporation of [3H]inositol, which was completely blocked by 16 μ M-atropine (Table 1).

The effect of carbachol on the accumulation of inositol phosphates was examined in the presence of 10 mM-LiCl for 60 min, according to the conditions previously used by Downes & Michell (1981) and Berridge *et al.* (1982). Linear inositol phosphate accumulation was observed after a lag period of 5 min (results not shown). In the presence of Li^+ , 0.1 mM-carbachol produced a 20-fold higher accumulation of radioactivity compared with the effect of carbachol or Li^+ alone (Table 2). The

Table 1. *Effect of carbachol on [³H]inositol incorporation into phospholipid*

Guinea-pig ileum pieces were incubated with [³H]inositol in the presence or absence of 0.1 mM-carbachol for 30 min as described under 'Methods'. Atropine (16 μM) was added 5 min before the addition of carbachol. The percentage of radioactivity in the phospholipid fraction was calculated after subtraction of the counts incorporated during the initial 30 min preincubation of the tissue with [³H]inositol ('basal'). The results are the mean incorporations of [³H]inositol (± S.E.M.). The numbers of guinea-pig ileum pieces are shown in the parentheses. Similar results were obtained in four other experiments.

Treatment	Incorporation	
	(d.p.m./μg of total phosphorus)	(%)
Basal	182 ± 27 (4)	—
No addition	368 ± 32 (5)	100
Carbachol (0.1 mM)	553 ± 29 (6)	199
Carbachol (0.1 mM) + atropine (16 μM)	386 ± 34 (4)	110

Table 2. *Accumulation of labelled inositol phosphates after stimulation of guinea-pig ileum with carbachol in the presence of Li⁺*

Guinea-pig ileum pieces were incubated for 60 min in a medium containing [³H]inositol, with or without 0.1 mM-carbachol, and in the presence or absence of 10 mM-LiCl (see under 'Methods'). Total accumulated radioactivity was eluted from the Dowex (formate-form) resin with 0.1 M-formic acid plus 1 M-ammonium formate. The phosphorus content was determined in portions of the organic phase. Results are given as means ± S.E.M. The numbers of guinea-pig ileum pieces are shown in parentheses. Similar results were obtained in several other experiments.

Treatment	Accumulated radioactivity
	(d.p.m./μg of total phosphorus)
No addition	3.21 ± 1.9 (4)
Li ⁺ (10 mM)	31.0 ± 2.6 (4)
Carbachol (0.1 mM)	23.5 ± 2.1 (4)
Carbachol (0.1 mM) + Li ⁺ (10 mM)	492 ± 34.0 (6)

accumulation of inositol phosphates by carbachol in the presence of Li⁺ was dose-dependent, as shown in Fig. 1. The threshold for activation was around 0.1 μM-carbachol, whereas half-maximal activation (ED₅₀) calculated from double-recipro-

Fig. 1. *Dose-response curve for carbachol-stimulated inositol phosphate accumulation in the presence of 10 mM-LiCl, with (■) or without (●) 6.4 μM-atropine*

Each point is the mean for duplicate incubations from a single experiment. Similar results were obtained in a second experiment.

cal plots was 2–10 μM (range in two separate experiments). The response to 10 μM- or 100 μM-carbachol was blocked by the muscarinic antagonist atropine (6.4 μM) (Fig. 1), suggesting that the response results from muscarinic-receptor stimulation.

Less than 20% of the inositol phosphate radioactivity that accumulated after incubation of guinea-pig ileum with carbachol and Li⁺ was eluted in a peak corresponding to inositol monophosphate (Table 3). The remainder, eluted with higher-ionic-strength solutions, corresponded to about 50% inositol bisphosphate and around 24% inositol trisphosphate (Table 3). Accumulation of both inositol poly- and mono-phosphate was confirmed in four other experiments by an ion-exchange-column method and by paper chromatography (results not shown). Although the absolute proportions varied somewhat according to the method used, probably due to differences in sample-processing time, in no case was the content of inositol monophosphate determined by ion-exchange greater than that of inositol polyphosphate.

Table 3. Ion-exchange separation of inositol phosphates accumulated after incubation of guinea-pig ileum with carbachol and Li^+

Guinea-pig ileum fragments were incubated in a medium containing [^3H]inositol with 10 mM- LiCl and 0.1 mM-carbachol for 60 min. Inositol phosphates accumulated in the aqueous phase were separated on a Dowex-1 (X8) resin as described under 'Methods'. The results are expressed as mean $\pm$ S.E.M. for four tissue samples in a single experiment. Similar results were obtained in other experiments.

Elution medium	Radioactivity eluted (%)
5 mM-Sodium tetraborate + 60 mM-ammonium formate	6.4 ± 0.4
5 mM-Sodium tetraborate + 150 mM-ammonium formate	16.9 ± 1.7
0.1 M-Formic acid + 0.4 M-ammonium formate	50.8 ± 0.9
0.1 M-Formic acid + 1.0 M-ammonium formate	24.3 ± 0.7

Discussion

Muscarinic agonists, including carbachol and *cis*-1,3-dioxolanes, produce a biphasic contraction of the guinea-pig ileum longitudinal smooth muscle (Chang & Triggle, 1973; James-Kracke & Roufogalis, 1981) that is mediated by the mobilization of Ca^{2+} . The pools of Ca^{2+} mobilized by the phasic and tonic components of the contraction appear, however, to be different (Chang & Triggle, 1973; Rangachari *et al.*, 1982). Enhanced labelling of PI by [^3H]inositol after carbachol stimulation (Table 1) is consistent with PI turnover occurring in response to muscarinic-receptor stimulation, since the stimulation was blocked completely by atropine. To determine if carbachol-stimulated PI turnover reflected polyphosphoinositide breakdown (Michell, 1982), the inositol phosphate products of phosphoinositide breakdown were separated by ion-exchange chromatography. Although the accumulation of inositol phosphates by 0.1 mM-carbachol was greatly enhanced by the presence of 10 mM- Li^+ (Table 2), even after 30–60 min incubation with 0.1 mM-carbachol only a small portion (17%) of the inositol phosphate accumulated corresponded to inositol monophosphate, whereas, of the remainder, two-thirds corresponded to inositol bisphosphate and one-third to inositol trisphosphate (Table 3). A similar increase of inositol bis- and tris-phosphate with 1 mM-carbachol was observed by Downes & Wusteman (1983) in rat parotid glands in the absence of Li^+ and to a lesser extent by Godfrey & Putney (1984) after stimulation of rat lacrimal acinar cells

by 0.1 mM-carbachol for 30 min in the presence of 10 mM- Li^+ . Taken together with those from recent experiments on other tissues, the present results suggest that muscarinic-receptor stimulation in the longitudinal muscle of the guinea-pig ileum is accompanied by polyphosphoinositide breakdown. The products of this breakdown, inositol bis- or tris-phosphate, were found to be elevated even after 30–60 min incubation, probably reflecting continued breakdown and re-synthesis of polyphosphoinositides (Aub & Putney, 1984). It is not possible to conclude decisively from these experiments whether the accumulation of inositol phosphates in the presence of Li^+ is due solely to the known inhibition by Li^+ of *myo*-inositol 1-phosphatase (Allison & Blisner, 1976; Sherman *et al.*, 1981; Berridge *et al.*, 1982) or to the additional inhibition by Li^+ of inositol bis- and tris-phosphatase recently described in hepatocytes (Thomas *et al.*, 1984). However, the present results are consistent with recent studies suggesting that receptor-mediated breakdown of polyphosphoinositides in the plasma membrane is an early event in the coupling of receptor occupation to Ca^{2+} mobilization (see Michell, 1982), especially since the ED_{50} of carbachol-stimulated inositol phosphate accumulation (Fig. 1) is similar to the IC_{50} for muscarinic-receptor occupation in the guinea-pig ileum (Furchgott & Burtszyn, 1967). The nature of the mediator or mediators associated with Ca^{2+} mobilization in the ileum remains to be determined.

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