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Carbachol causes rapid phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate and accumulation of inositol phosphates in rabbit iris smooth muscle; prazosin inhibits noradrenaline- and ionophore A23187-stimulated accumulation of inositol phosphates

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Rabbit iris smooth muscle was prelabelled with *myo*-[^{3}H]inositol for 90 min and the effect of carbachol on the accumulation of inositol phosphates from phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol (PtdIns) was monitored with anion-exchange chromatography. Carbachol stimulated the accumulation of inositol phosphates and this was blocked by atropine, a muscarinic antagonist, and it was unaffected by 2-deoxyglucose. The data presented demonstrate that, in the iris, carbachol (50 μM) stimulates the rapid breakdown of PtdIns(4,5)P_2 into [^{3}H]inositol trisphosphate (InsP_3) and diacylglycerol, measured as phosphatidate, and that the accumulation of InsP_3 precedes that of [^{3}H]inositol bisphosphate (InsP_2) and [^{3}H]inositol phosphate (Ins$P$). This conclusion is based on the following findings. (*a*) Time course experiments with *myo*-[^{3}H]inositol revealed that carbachol increased the accumulation of InsP_3 by 12% in 15 s and by 23% in 30 s; in contrast, a significant increase in Ins$P$ release was not observed until about 2 min. (*b*) Time-course experiments with ^{32}P revealed a 10% loss of radioactivity from PtdIns(4,5)P_2 and a corresponding 10% increase in phosphatidate labelling by carbachol in 15 s; in contrast a significant increase in PtdIns labelling occurred in 5 min. (*c*) Dose–response studies revealed that 5 μM-carbachol significantly increased (16%) the accumulation of InsP_3 whereas a significant increase in accumulation of InsP_2 and Ins$P$ was observed only at agonist concentrations > 10 μM. Studies on the involvement of Ca^{2+} in the agonist-stimulated breakdown of PtdIns(4,5)P_2 in the iris revealed the following. (*a*) Marked stimulation (58–78%) of inositol phosphates accumulation by carbachol in 10 min was observed in the absence of extracellular Ca^{2+}. (*b*) Like the stimulatory effect of noradrenaline, the ionophore A23187-stimulated accumulation of InsP_3 was inhibited by prazosin, an α_1-adrenergic blocker, thus suggesting that the ionophore stimulation of PtdIns(4,5)P_2 breakdown we reported previously [Akhtar & Abdel-Latif (1978) *J. Pharmacol. Exp. Ther.* **204**, 655–688; Akhtar & Abdel-Latif (1980) *Biochem. J.* **192**, 783–791] was secondary to the release of noradrenaline by the ionophore. (*c*) The carbachol-stimulated accumulation of inositol phosphates was inhibited by EGTA (0.25 mM) and this inhibition was reversed by excess Ca^{2+} (1.5 mM), suggesting that EGTA treatment of the tissue chelates extracellular Ca^{2+} required for polyphosphoinositide phosphodiesterase activity. (*d*) K^+ depolarization, which causes influx of extracellular Ca^{2+} in smooth muscle, did not change the level of InsP_3. We conclude that, in the iris, carbachol stimulates a rapid phosphodiesteratic breakdown of PtdIns(4,5)P_2 into InsP_3 and diacylglycerol, measured as phosphatidate, and that the accumulation of InsP_3 precedes that of InsP_2 and Ins$P$. In addition we conclude that the agonist-stimulated breakdown of PtdIns(4,5)P_2 in the iris is not regulated by intracellular Ca^{2+}.

In previous communications from this laboratory we have reported on the muscarinic cholinergic and α_1-adrenergic stimulation of PtdIns(4,5)P_2 breakdown into diacylglycerol and InsP_3 in the iris muscle (for reviews see Abdel-Latif, 1983; Abdel-Latif *et al.*, 1984). More recently, interest in this polyphosphoinositide effect was rekindled by the observation that the agonist-stimulated breakdown of PtdIns(4,5)P_2 is probably not controlled by Ca^{2+} and thus it might be involved in control of Ca^{2+} mobilization (Michell *et al.*, 1981). The renewed emphasis on the role of polyphosphoinositides, rather than phosphatidylinositol (PtdIns), in receptor function has led to demonstration of agonist-stimulated PtdIns(4,5)P_2 breakdown in a wide variety of tissues (for more recent reviews see Downes & Michell, 1982; Fisher *et al.*, 1984; Marx, 1984; Berridge, 1984); however, the requirement for Ca^{2+} in this phenomenon remains controversial at the present time (Hawthorne, 1983). In our earlier studies, which were designed to demonstrate the agonist-stimulated PtdIns(4,5)P_2 breakdown in the iris (Abdel-Latif *et al.*, 1977) and to understand the molecular mechanism underlying this phenomenon (Akhtar & Abdel-Latif, 1980), incubations were carried out at time intervals which ranged from 2.5 to 10min (Abdel-Latif & Akhtar, 1976) and were conducted either under breakdown or under incorporation conditions (Abdel-Latif *et al.*, 1977). In view of the recent findings that this phenomenon occurs rather rapidly both in isolated cells (Kirk *et al.*, 1981; Weiss *et al.*, 1982; Billah & Lapetina, 1982; Agranoff *et al.*, 1983; Creba *et al.*, 1983) and in intact tissue (Berridge, 1983; Berridge *et al.*, 1983), we have decided to re-investigate it at short time intervals in the iris. To achieve this we have studied the effects of carbachol, a muscarinic agonist, on the accumulation of the water-soluble metabolites of phosphoinositides, namely Ins*P*, InsP_2 and InsP_3, at short time intervals and at various concentrations of the agonist. In addition we have re-investigated the role of Ca^{2+} in this phenomenon (Akhtar & Abdel-Latif, 1978).

Part of this work has already appeared in preliminary form (Akhtar & Abdel-Latif, 1984).

Materials and methods

Chemicals

myo-[^{3}H]Inositol (sp. radioactivity 15.5Ci/mmol) was from Amersham Corp. and was purified by passing it through the anion-exchange column and eluting it with deionized water. $^{32}P_i$, carrier-free, was obtained from New England Nuclear. Carbachol (carbamylcholine chloride), L-noradrenaline (norepinephrine) bitartrate and ionophore A23187 were purchased from Sigma Chemical Co. Prazosin hydrochloride was a gift from Pfizer Inc., New York, NY, U.S.A. PtdIns4*P* and PtdIns(4,5)P_2 were prepared from bovine brain. A crude phosphoinositide fraction was prepared according to the method of Folch (1949), and PtdIns4*P* and PtdIns(4,5)P_2 were isolated by means of DEAE-cellulose column chromatography as described by Hendrickson & Ballou (1964).

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins*P*, *myo*-inositol phosphate; InsP_2, *myo*-inositol bisphosphate; InsP_3, *myo*-inositol trisphosphate.

Preparation and incubation of iris muscle

Albino rabbits of either sex weighing approx. 2kg were used in the present work. The unanaesthetized rabbits were killed by a blow to the head followed by decapitation. The eyes were enucleated immediately and irises were removed and placed in pairs from the same animal in tubes containing 2ml of a modified Krebs–Ringer bicarbonate buffer of the following composition: 118mM-NaCl, 25mM-$NaHCO_3$, 4.7mM-KCl, 1.2mM-KH_2PO_4, 1.2mM-$MgSO_4$, 1.25mM-$CaCl_2$, 1.6mM-cytidine, 5μM-inositol and 10mM-D-glucose. The pH of the modified Krebs–Ringer was adjusted to 7.4 with O_2/CO_2 (97:3). In our studies we routinely employ the whole iris-ciliary body, which is an instant slice and weighs about 42mg.

To label the phosphoinositides and the tissue inositol phosphates, the irises were incubated singly (of the pair, one was used as control and the other as experimental) for 90min at 37°C in 1ml of the modified Krebs–Ringer bicarbonate buffer that contained 6–8μCi of *myo*-[^{3}H]inositol. Carbachol and/or other agents were then added and incubations continued for various time intervals as indicated. All incubations were gassed with O_2/CO_2 (97:3) before use and periodically during the experiments. Incubations were terminated with 1ml of 10% (w/v) ice-cold trichloroacetic acid.

In experiments where ^{32}P was used as a precursor for phospholipids, the experimental procedure was as described above except that 30μCi of $^{32}P_i$/ml was added instead of *myo*-[^{3}H]-inositol.

Extraction and separation of myo-[^{3}H]inositol phosphates by anion-exchange chromatography

Irises labelled with *myo*-[^{3}H]inositol were homogenized in 5% (w/v) trichloroacetic acid and the homogenate was centrifuged at 3000***g*** for 15min. The supernatant was analysed for *myo*-[^{3}H]inositol phosphates by a slight modification of the anion-exchange method described by Berridge *et al.*

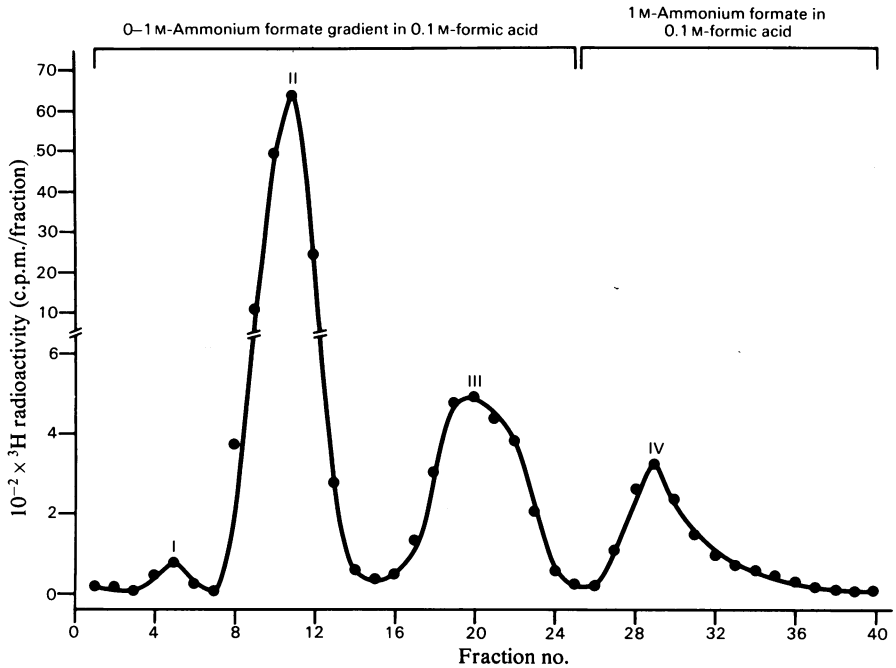

Fig. 1. *Separation of myo-[^3H]inositol phosphates from iris smooth muscle by anion-exchange chromatography*
Iris smooth muscle was incubated for 60min at 37°C in 1ml of modified Krebs–Ringer bicarbonate buffer (pH7.4) containing 5 μM-*myo*-[^{3}H]inositol (7.8 μCi/ml). The incubation was terminated by adding 10% (w/v) trichloroacetic acid. The water-soluble inositol phosphates were extracted and applied to an AG1 X4 (formate form) column. The column was washed with 5mM-*myo*-inositol until no radioactivity (free *myo*-[^{3}H]inositol) was detected in the eluate. This was followed by elution of the column first with a linear 0–1 M-ammonium formate gradient in 0.1 M-formic acid (usually about 30ml), and then with 15ml of 1 M-ammonium formate in 0.1 M-formic acid. The peaks were identified as: I, glycerophosphoinositol; II, Ins*P*; III, InsP_2, and IV, InsP_3.

(1983), and the pellet was analysed for labelled phosphoinositides. The supernatant (2ml), which contains the *myo*-[^{3}H]inositol phosphates, was extracted five times with 4ml of diethyl ether and then diluted with equal volume of distilled water and neutralized with 0.1 M-NaOH. The neutralized extract was applied to a Bio-Rad AG1 X4, formate form, column (0.7cm × 8cm). The resin was washed with water containing 5mM-*myo*-inositol until no free *myo*-[^{3}H]inositol was detected in the eluate. The column was then eluted with 30ml of a linear gradient of ammonium formate (0–1 M) in 0.1 M-formic acid and 1.2ml fractions were collected. This was followed by elution of the column with 15ml of 1 M-ammonium formate dissolved in 0.1 M-formic acid and 1.2ml fractions were collected again. A 1ml portion from each fraction was taken for determination of radioactivity and the data thus obtained was used for construction of the elution profile of inositol phosphates (Fig. 1). For identification, each peak was first desalted, freeze-dried, mixed with known standards of inositol phosphates, which were prepared by alkaline hydrolysis of Folch fraction I/II (Folch, 1949), then subjected to low-voltage paper electrophoresis and identified as previously described (Akhtar & Abdel-Latif, 1980). As shown in Fig. 1, elution of the column with a linear gradient of ammonium formate results in distinct separation of glycerophosphoinositol (peak 1, fractions 1–6), Ins*P* (peak II, fractions 7–15) and InsP_2 (peak III, fractions 16–25). Finally, the InsP_3 (peak IV, fractions 26–36) was eluted with 1 M-ammonium formate in 0.1 M-formic acid. From the elution profile it was possible to determine the volume of the gradient required for elution of each of the inositol phosphates. Routinely, the first two samples in an experiment were processed in this manner whereas the eluates from the remaining samples were collected into four fractions corresponding to glycerophosphoinositol, Ins*P*, InsP_2, and InsP_3. The total radioactivity in each fraction was determined and corrections for quenching were made by a quench curve based on the external-

standard ratio. The counting efficiency of ^3H in the present work was 44%.

Extraction and analysis of phospholipids

Extraction and separation of iris phospholipids by two-dimensional (Abdel-Latif *et al.*, 1977) and one-dimensional (Akhtar *et al.*, 1983) t.l.c. were as described previously. Briefly, the trichloroacetic acid-insoluble pellet from the iris homogenate was extracted once with chloroform/methanol/conc. HCl (200:200:1, by vol.) and once with chloroform/methanol/conc. HCl (400:200:1.5, by vol.). The extracts were combined, evaporated under N_2 and the residue dissolved in 2ml of chloroform. After washing the extract with methanolic 0.1M-HCl, the lipids were concentrated in small volume (60μl) of chloroform. Carrier PtdIns4*P* and PtdIns(4,5)P_2 were added to this extract and the phospholipids were separated by one-dimensional t.l.c. on silica gel 60 high-performance thin-layer plates as previously described (Akhtar *et al.*, 1983). The phospholipids were visualized by exposure to I_2 vapour and spots corresponding to PtdIns4*P*, PtdIns(4,5)P_2 and PtdIns were scraped into vials and their radioactive content determined by liquid-scintillation counting. The data were corrected for silica-gel quenching of the ^3H label as described above. The ^{32}P-labelled phospholipids were separated by one- and two-dimensional t.l.c. The data presented in the Figures are representative of three or more similar experiments and error bars represent the standard errors calculated from at least three separate experiments. Statistical analysis of the data was done by Student's *t* test for paired differences.

Results

Time course of incorporation of myo-[^3H]inositol into phosphoinositides and accumulation of myo-[^3H]inositol phosphates

In the iris, the γ-phosphate of ATP and the ^{32}P labelling of PtdIns(4,5)P_2 reach isotopic equilibrium with extracellular $^{32}P_i$ in about 30–60min (Akhtar & Abdel-Latif, 1982). To determine the time interval at which *myo*-[^3H]inositol, phosphoinositides and *myo*-inositol phosphates reach isotopic equilibrium, the irises were incubated with the isotope for intervals which ranged from 5 to 120min, and the radioactivities in phosphoinositides and *myo*-inositol phosphates were determined (Fig. 2). The labelling of PtdIns increased at all time intervals investigated; in contrast, that of polyphosphoinositides reached a maximum between 90 and 120min (Fig. 2*a*). About 74% of the ^3H label was found in PtdIns, and this was followed by PtdIns(4,5)P_2 (15%) and PtdIns4*P* (11%). A similar time course for the accumulation

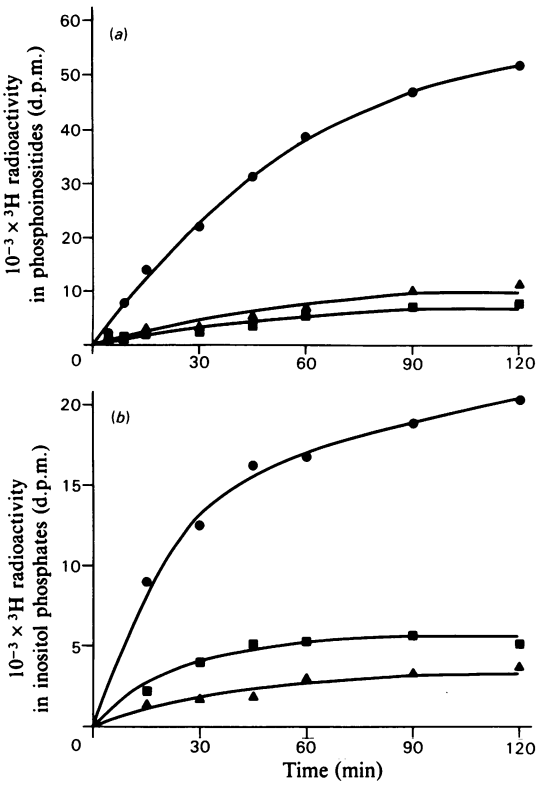

Fig. 2. *Time course of incorporation of myo-[^3H]inositol into phosphoinositides and inositol phosphates in irls muscle*
Irises were incubated in 1ml of modified Krebs–Ringer bicarbonate buffer (pH7.4) that contained 5μM-*myo*-[^3H]inositol for various time intervals as indicated. The incubations were terminated with 10% (w/v) trichloroacetic acid and the tissues were analysed for radioactivity in (*a*) phosphoinositides: ●, PtdIns; ■, PtdIns4*P*; ▲, PtdIns(4,5)P_2, and (*b*) inositol phosphates: ●, Ins*P*; ■, InsP_2; ▲, InsP_3. The results are the mean of two experiments conducted in duplicate.

of *myo*-inositol phosphates can be seen in Fig. 2(*b*). Thus after 90min of incubation the distribution of radioactivity in Ins*P*, InsP_2 and InsP_3 were 68%, 20% and 12%, respectively. As can be seen from Fig. 2, the labelling in PtdIns(4,5)P_2 > PtdIns4*P* whereas that of InsP_2 > InsP_3. Since the tissue apparently does not reach isotopic equilibrium under the present experimental conditions, the mechanism of formation of InsP_2 is unclear. However, it is possible that some of the InsP_2 could come from PtdIns4*P* and the rest of it comes from the hydrolysis of InsP_3 by *myo*-inositol trisphosphatase, an enzyme enriched in the microsomal fraction of the iris muscle (Akhtar & Abdel-Latif, 1980). Determination of free *myo*-[^3H]inositol in the tissue revealed that a maximal intracellular concentration of the isotope was reached between

Table 1. *Effects of atropine and 2-deoxyglucose on the carbachol-stimulated accumulation of myo-[^3H]-inositol phosphates in the iris muscle*

Irises (of the pair, one was used as control and the other as experimental) were first preincubated for 90min in 1 ml of modified Krebs–Ringer bicarbonate buffer (pH7.4) containing 5μM-*myo*-[^{3}H]inositol (7.3μCi/ml), then the pharmacological agents were added as indicated and incubations were continued for an additional 10min. The incubations were terminated with 10% (w/v) trichloroacetic acid and inositol phosphates were extracted and analysed for radioactivity as described in the Materials and methods section. The values are means ± S.E.M. obtained from three separate experiments conducted in triplicate. Significance of difference between paired data was calculated by using Student's *t* test; $*P<0.01$, $**P<0.02$.

	$10^{-2}\times{}^3$H radioactivity (d.p.m./iris)		
	Ins*P*	InsP_2	InsP_3
Control	399±45	79±5	40±2
Carbachol (50μM)	613±50**	121±9*	64±5*
Carbachol (50μM)+atropine (10μM)	415±47	87±5	42±2
2-Deoxyglucose (10mM)	402±48	75±6	39±3
2-Deoxyglucose (10mM)+carbachol (50μM)	610±45**	118±7*	67±7*

60 and 90min of incubation, and this concentration was about 8 times as high as that of the total inositol phosphates (results not shown). Thus in the following experiments the tissue was pre-incubated with *myo*-[^{3}H]inositol for 90min prior to the addition of the agonist.

Effects of atropine and 2-deoxyglucose on carbachol-stimulated accumulation of inositol phosphates

Atropine, a muscarinic antagonist, was shown previously to block the acetylcholine-stimulated loss of radioactivity from PtdIns(4,5)P_2 in ^{32}P-labelled iris (Abdel-Latif *et al.*, 1977). As can be seen from Table 1, carbachol stimulated the accumulation of inositol phosphates and this accumulation was inhibited by atropine. This suggests that the release of these water-soluble products from the membrane phosphoinositides is controlled by muscarinic receptors. 2-Deoxyglucose, which we employed in our earlier studies on the polyphosphoinositide effect in this tissue (Abdel-Latif *et al.*, 1977) had no effect on the carbachol-stimulated accumulation of inositol phosphates (Table 1). This finding answers the recent criticism made by Downes & Michell (1982) on the use of 2-deoxyglucose in these studies.

Time course of the effect of carbachol on the accumulation of inositol phosphates

We have already established a relationship between the decrease in labelling of PtdIns(4,5)P_2 with the increase in that of water-soluble InsP_3, in irises prelabelled with *myo*-[^{3}H]inositol and stimulated with acetylcholine for 10min (Akhtar & Abdel-Latif, 1980). As can be seen from Fig. 3, carbachol caused a rapid accumulation of InsP_3 and InsP_2, but not of Ins*P*. Thus, at 15s, carbachol caused a 12% increase in the accumulation of InsP_3 and InsP_2 and this stimulation by the agonist was

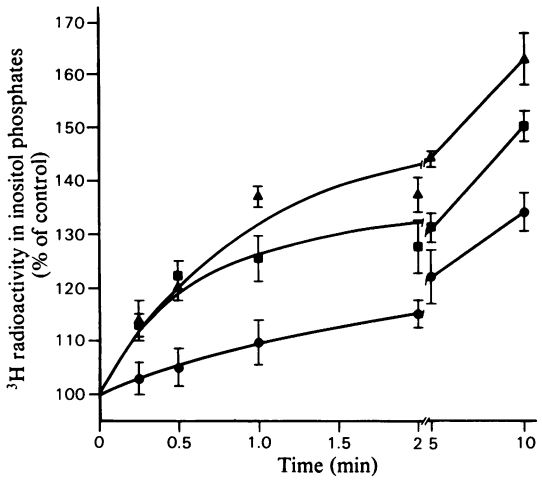

Fig. 3. *Time course for the effect of carbachol on the accumulation of myo-[^3H]inositol phosphates in the iris muscle*

Irises (in pairs) were preincubated for 90min in 1 ml of modified Krebs–Ringer bicarbonate buffer (pH7.4) containing 5μM-*myo*-[^{3}H]inositol (7.8μCi/ml). Carbachol (50μM) was then added to one of each of the pairs and incubations continued for various time intervals as indicated. The radioactivities in inositol phosphates are expressed as percentages of control. Each point is the mean of values from three separate experiments conducted in triplicate. ●, Ins*P*; ■, InsP_2; ▲, InsP_3.

increased with time of incubation. By 30s, the accumulation of InsP_3 was increased by 23% in the presence of the agonist, while the increase in Ins*P* accumulation was only significant between 1 and 2min. By 10min, the carbachol-stimulated increase in Ins*P*, InsP_2 and InsP_3 reached 34, 50 and 63%, respectively. Quantitative comparison of

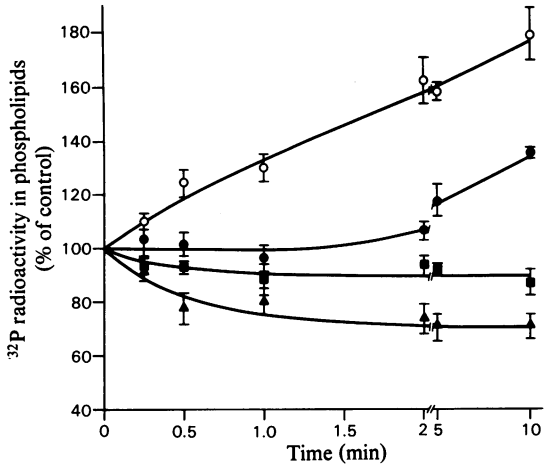

Fig. 4. *Time course for the effect of carbachol on the breakdown of ^{32}P-labelled polyphosphoinositides, and accumulation of ^{32}P-labelled phosphatidic acid and phosphatidylinositol in the iris muscle*

Irises (in pairs) were preincubated for 90min in 1ml of modified Krebs–Ringer bicarbonate buffer (pH7.4) containing 1.5mM-*myo*-inositol and 30μCi of $^{32}P_i$. Carbachol (50μM) was then added to one of each of the pairs and incubations continued for various time intervals as indicated. The incubations were terminated with 10% (w/v) trichloroacetic acid and the phospholipids were extracted and analysed for radioactivity as described in the Materials and methods section. The radioactivities in the phospholipids are expressed as percentages of control. Each point is the mean+S.E.M. of two separate experiments conducted in triplicate. ○, Phosphatidic acid; ●, PtdIns; ■, PtdIns4*P*; ▲, PtdIns(4,5)P_2.

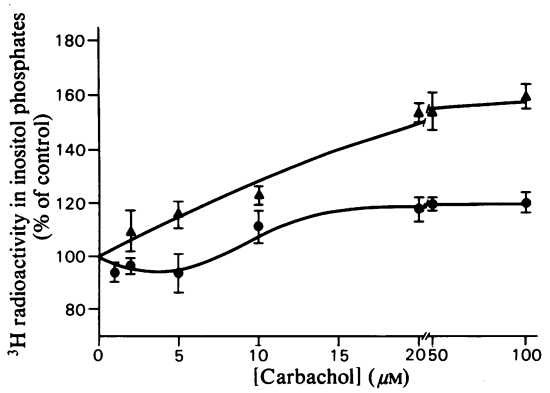

Fig. 5. *Dose–response to carbachol for accumulation of myo-[^{3}H]inositol phosphates in iris muscle*

Irises (in pairs) were preincubated for 90min in 1ml of modified Krebs–Ringer bicarbonate buffer (pH7.4) containing 5μM-*myo*-[^{3}H]inositol (6.9μCi/ml). Various concentrations of carbachol were then added and incubations continued for an additional 2min. Reactions were stopped by addition of 10% (w/v) trichloroacetic acid. Extraction of *myo*-[^{3}H]-inositol phosphates and analysis of radioactivity were as described in the Materials and methods section. The effects of carbachol on *myo*-[^{3}H]-inositol phosphates release are expressed as percentages of their respective controls. Each point is the mean of values from two separate experiments run in triplicate. ●, Ins*P*; ▲, InsP_3.

these data reveal that the net increase in accumulation of Ins*P* at all time intervals is more than that of InsP_2 and InsP_3. This could be due to the fact that the labelling of PtdIns is several times higher than that of PtdIns4*P* and PtdIns(4,5)P_2. On the basis of the data presented in Fig. 3 one can suggest that, in the iris, carbachol-stimulated accumumation of InsP_3 is rapid and it precedes that of Ins*P*.

Time course of carbachol effect on ^{32}P-labelled iris phospholipids

Similarly, time course experiments with ^{32}P also revealed that carbachol-stimulated PtdIns(4,5)P_2 breakdown precedes the increase in PtdIns labelling (Fig. 4). Thus addition of the muscarinic agonist to iris muscle pre-equilibrated with ^{32}P for 90min provoked a 10% loss of radioactivity from PtdIns(4,5)P_2 in 15s and this increased into 26% in 5min. Concomitant with PtdIns(4,5)P_2 breakdown there was an increase (10%) in ^{32}P labelling of phosphatidic acid by the agonist at 15s, and this continued to increase markedly with time of incubation. The loss of ^{32}P from PtdIns4*P* by carbachol was less pronounced, and a significant increase in PtdIns labelling was observed at about 2min. These data indicate that agonist-stimulated PtdIns(4,5)P_2 breakdown is rapid and that it precedes the increased labelling of PtdIns (and probably the breakdown of PtdIns4*P*). In addition, these data show that phosphatidic acid, but not PtdIns, labelling correlates well with the breakdown of PtdIns(4,5)P_2.

Concentration-dependence of carbachol-stimulated accumulation of inositol phosphates

All the data shown in Figs. 3 and 4 were obtained with 50μM-carbachol. When the accumulation of inositol phosphates was measured at different concentrations of carbachol and at short time intervals (2min), a differential effect of the agonist was observed. Thus, a significant increase (16%) in the accumulation of InsP_3 was observed at 5μM of the muscarinic agonist and this was maximal (50% increase) at 20–50μM (Fig. 5). The dose–response curve for InsP_2 was similar to that for InsP_3, except that significant increase in its accumulation was observed at about 10μM-carbachol (results not shown). In contrast, a significant increase in the accumulation of Ins*P* by carbachol

Table 2. *Effects of EGTA and Ca^{2+} on carbachol-stimulated accumulation of myo-[^3H]inositol phosphates in iris muscle*
Irises (of the pair, one was used as control and the other as experimental) were preincubated for 90 min in modified Ca^{2+}-free Krebs–Ringer bicarbonate buffer (pH7.4) that contained 5μM-*myo*-[^{3}H]inositol (6.8μCi/ml). EGTA, Ca^{2+} and/or carbachol were then added to the controls and experimentals as indicated and incubations continued for an additional 10min. The reactions were terminated with 1ml of 10% (w/v) trichloroacetic acid and the inositol phosphates were extracted and analysed for radioactivity as described in the Materials and methods section. The values are means ± S.E.M. obtained from three separate experiments conducted in triplicate. The *P* values for the paired data were calculated by using Student's *t* test; $*P<0.01$.

Additions		^{3}H radioactivity in inositol phosphates (% of control)		
Control	Experimental	Ins*P*	InsP_2	InsP_3
–	Carbachol (50μM)	178 ± 21*	184 ± 30*	158 ± 15*
EGTA (0.25mM)	EGTA (0.25mM) + carbachol (50μM)	99 ± 7	96 ± 7	90 ± 15
EGTA (0.25mM) + Ca^{2+} (1.5mM)	EGTA (0.25mM) + Ca^{2+} (1.5mM) + carbachol (50μM)	210 ± 24*	172 ± 34*	177 ± 24*

was observed only at concentrations >10 μM of the agonist. These data suggest that the agonist-stimulated release of InsP_3 precedes that of Ins*P* (and probably that of InsP_2). The reason for the differential effect of the agonist concentration on inositol phosphates accumulation is not clear.

Effects of Ca^{2+} and EGTA on carbachol-stimulated accumulation of inositol phosphates

When irises were pre-incubated with *myo*-[^{3}H]-inositol for 90min in a Ca^{2+}-free modified Krebs–Ringer buffer and then stimulated with carbachol for 10min in the same medium, the accumulation of Ins*P*, InsP_2 and InsP_3 was increased by 78, 84 and 58%, respectively (Table 2). The stimulatory effect of carbachol on inositol phosphates accumulation was inhibited by 0.25mM-EGTA, and this inhibition was reversed by excess Ca^{2+} (1.5mM). These data suggest some requirement for Ca^{2+} in the agonist-stimulated breakdown of phosphoinositides in this tissue.

Effect of prazosin on noradrenaline- and ionophore A23187-stimulated accumulation of inositol phosphates

In previous communications from this laboratory we have reported that ionophore A23187 provokes a loss of ^{32}P from PtdIns(4,5)P_2 in iris muscle prelabelled with ^{32}P (Akhtar & Abdel-Latif, 1978) and increased the accumulation of InsP_3 in iris muscle prelabelled with *myo*-[^{3}H]-inositol (Akhtar & Abdel-Latif, 1980). The ionophore also induces contraction in intestinal smooth muscle (Rosenberger & Triggle, 1979) and recently Warenycia & Vohra (1983), working with vas deferens, reported that the ionophore A23187-induced muscle contraction was blocked by the α-blocker phentolamine. The latter finding prompted us to re-investigate the effect of this ionophore on PtdIns(4,5)P_2 breakdown in the iris. Both noradrenaline and the ionophore stimulated the accumulation of inositol phosphates, and prazosin, an α_1-blocker, blocked this stimulated accumulation of inositol phosphates (Table 3). This suggests that the ionophore-induced accumulation of inositol phosphates is probably secondary to the release of noradrenaline by the ionophore.

Effect of high extracellular K^+ concentration on the accumulation of inositol phosphates

K^+ depolarization of smooth muscle causes influx of extracellular Ca^{2+} and leads to muscle contraction (Hurwitz & Suria, 1971; Berridge, 1975; Triggle, 1982). When iris muscle was pre-incubated in modified Krebs–Ringer bicarbonate buffer containing *myo*-[^{3}H]inositol for 90min and then incubated in the absence and presence of high K^+ concentration (80mM) for 10min, the release of inositol phosphates was unaffected by K^+ depolarization (Table 4). Under the same experimental conditions high K^+ had no effect on the incorporation of *myo*-[^{3}H]inositol and ^{32}P into the phosphoinositides of the iris (results not shown). These data indicate that PtdIns(4,5)P_2 breakdown is not associated with the potential-dependent Ca^{2+} channel and furthermore that this phenomenon is not secondary to the increase in cytosolic Ca^{2+} concentration.

Discussion

The data presented in the present paper confirm and extend our previous findings that, in the iris muscle, activation of Ca^{2+}-mobilizing receptors, such as muscarinic cholinergic and α_1-adrenergic receptors, leads to the rapid loss of ^{32}P from

Table 3. *Effect of prazosin on noradrenaline- and ionophore A23187-stimulated accumulation of myo-[^3H]inositol phosphates in the iris muscle*

Irises (of the pair, one was used as control and the other as experimental) were preincubated for 90min in 1ml of modified Krebs–Ringer bicarbonate buffer (pH7.4) that contained 5μM-*myo*-[^{3}H]inositol (6.8μCi/ml). In the last 5min of preincubation prazosin was added as indicated. Noradrenaline and ionophore A23187 were added as indicated and incubation continued for an additional 10min. Incubations were terminated by the addition of 1ml of 10% (w/v) trichloroacetic acid and *myo*-[^{3}H]inositol phosphates were extracted and analysed for radioactivity as described in the Materials and methods section. The values are means ± S.E.M. obtained from two separate experiments carried out in triplicate. The *P* values for the paired data were calculated by using Student's *t* test; $*P<0.01$.

Additions	^{3}H radioactivity in inositol phosphates (% of control)		
	Ins*P*	InsP_2	InsP_3
Noradrenaline (50μM)	173 ± 2*	149 ± 19*	144 ± 17*
Noradrenaline (50μM) + prazosin (10μM)	85 ± 7	96 ± 10	102 ± 2
Ionophore A23187 (20μM)	147 ± 7*	143 ± 10*	141 ± 9*
Ionophore A23187 (20μM) + prazosin (10μM)	108 ± 16	89 ± 12	91 ± 6

Table 4. *Effect of high extracellular K^+ concentration on the accumulation of myo-[^3H]inositol phosphates in the iris muscle*

Irises were preincubated for 90min in 1ml of modified Krebs–Ringer buffer (pH7.4) that contained 5μM-[^{3}H] inositol (6.8μCi/ml). One of the pair (control) was transferred to 1ml of the modified Krebs–Ringer containing *myo*-[^{3}H]inositol and the other (experimental) was transferred to 1ml of Krebs–Ringer containing 80mM-K^+, 38mM-Na^+ and *myo*-[^{3}H]inositol. After incubation for 10min, the reaction was stopped with 10% (w/v) trichloroacetic acid and the inositol phosphates were analysed as described in the Materials and methods section. The data are means ± S.E.M. of three determinations.

	$10^{-2} \times {}^3$H radioactivity (d.p.m./iris)		
	Ins*P*	InsP_2	InsP_3
Control	378 ± 36	65 ± 9	42 ± 5
80mM-K^+	365 ± 26	64 ± 3	39 ± 6

PtdIns(4,5)P_2 (Abdel-Latif *et al.*, 1977) and to the phosphodiesteratic cleavage of PtdIns(4,5)P_2 into diacylglycerol and InsP_3 (Akhtar & Abdel-Latif, 1980). In the present work carbachol, a muscarinic agonist, stimulated the accumulation of the inositol phosphates by about 50–60% in 10min, and this was blocked by the muscarinic antagonist, atropine (Table 1). The time-course studies, both with *myo*-[^{3}H]inositol (Fig. 3) and with ^{32}P (Fig. 4), and the dose–response studies (Fig. 5) clearly suggest that in the iris the agonist-stimulated breakdown of PtdIns(4,5)P_2 into InsP_3 and diacylglycerol precedes that of PtdIns depletion. Furthermore, these data suggest that the phosphodiesteratic cleavage of PtdIns(4,5)P_2 is an early (initial) event in the pathway which leads from activation of the Ca^{2+}-mobilizing receptors to muscle response. This conclusion is based on the following observations. (*a*) The time course experiments with *myo*-[^{3}H]inositol revealed that the carbachol-stimulated accumulation of InsP_3 occurs within 15s; in contrast, a significant accumulation of Ins*P* by the agonist was not observed until about 2min (Fig. 3). (*b*) The time course experiments with ^{32}P revealed a 10% loss of radioactivity from PtdIns(4,5)P_2 and a corresponding 10% increase in phosphatidic acid labelling by carbachol in 15s; in contrast, a significant increase in PtdIns labelling occurred in 5min (Fig. 4). This simply suggests an agonist-stimulated phosphodiesteratic cleavage of PtdIns(4,5)P_2 into InsP_3 and diacylglycerol; the latter is then phosphorylated into phosphatidic acid, via diacylglycerol kinase. (*c*) The dose–response experiments showed that 5μM-carbachol increased the accumulation of InsP_3, but not that of Ins*P* (Fig. 5). Significant increase in Ins*P* accumulation was observed at relatively higher concentrations of the agonist (>10μM). These data are in accord with our previous studies on the effects of different noradrenaline concentrations on PtdIns(4,5)P_2 breakdown in the sympathetically denervated iris, in which we reported that PtdIns(4,5)P_2 breakdown and phosphatidic acid, but not PtdIns, labelling are associated with denervation supersensitivity (Abdel-Latif *et al.*, 1979). These kinetic data suggest that the phosphodiesteratic cleavage of PtdIns(4,5)P_2, rather than PtdIns, constitutes the initial reaction following activation of muscarinic cholinergic receptors in the iris, a conclusion also reached by others

working with other tissues (for reviews see Downes & Michell, 1982; Fisher *et al.*, 1984; Berridge, 1984).

The increase in Ins*P* by carbachol could arise from the following. (*a*) The stepwise dephosphorylation of InsP_3 to InsP_2, and InsP_2 to Ins*P*, by the specific phosphatases. In the iris the specific activity of InsP_3 phosphatase was found to be several-fold higher than that of Ins*P* phosphatase (Akhtar & Abdel-Latif, 1980). (*b*) Phosphodiesteratic cleavage of PtdIns into Ins*P* and diacylglycerol. While the data presented indicate that the accumulation of InsP_3 precedes that of Ins*P*, and that some of the Ins*P* is derived from InsP_3, we cannot exclude the possibility that some of the Ins*P* could have come from the phosphodiesteratic cleavage of PtdIns. This is complicated by the fact that, in this tissue, the accumulation of Ins*P* is several times as high as that of InsP_3 (Table 1). It has recently been suggested that the disappearance of PtdIns in response to agonists may be secondary to the initial breakdown of PtdIns(4,5)P_2 and subsequent conversion of PtdIns into PtdIns(4,5)P_2 by respective kinases (Michell *et al.*, 1981; Berridge, 1983; Creba *et al.*, 1983).

The other question which we sought to answer in the present study was whether the agonist-stimulated PtdIns(4,5)P_2 breakdown in the iris is regulated by intracellular Ca^{2+}. In previous communications from this laboratory we have reported a requirement for Ca^{2+} in this phenomenon in the iris, derived mainly from studies on the inhibitory effects of EGTA and the stimulation of PtdIns(4,5)P_2 breakdown by ionophore A23187 (Akhtar & Abdel-Latif, 1978, 1980). In the past year, Warenycia & Vohra (1983) reported that ionophore A23187-induced muscle contraction in vas deferens is blocked by phentolamine. This led us to re-investigate the role of Ca^{2+} and the ionophore in PtdIns(4,5)P_2 breakdown in the iris. The marked stimulation (58–78%) of inositol phosphates accumulation by carbachol in the absence of Ca^{2+}, after the tissue had been preincubated for 90min in Ca^{2+}-free medium, suggests that this phenomenon is not entirely dependent on extracellular Ca^{2+} in this tissue (Table 2). However, this carbachol stimulation of inositol phosphates accumulation was inhibited by EGTA, and the inhibitory effect of this Ca^{2+}-chelating agent was reversed by addition of excess Ca^{2+}. The inhibition of carbachol-stimulated PtdIns(4,5)P_2 breakdown by EGTA does not appear to be caused by a generalized metabolic disturbance in the tissue, since this inhibition was reversed by addition of excess Ca^{2+} (Table 2). Whereas the precise mechanism for the action of EGTA in Ca^{2+} metabolism is still unclear, it is possible that the treatment of the tissue with EGTA depletes the intracellular Ca^{2+} to the extent that the activity of the Ca^{2+}-requiring polyphosphoinositide phosphodiesterase is inhibited. This suggests to us that some Ca^{2+} is needed for this phenomenon. In addition to the iris, Ca^{2+} requirement for the agonist-stimulated breakdown of PtdIns(4,5)P_2 has been reported in synaptosomes, hepatocytes, pancreatic islets and several other tissues (for reviews see Abdel-Latif, 1983; Hawthorne, 1983; Fisher *et al.*, 1984). On the other hand, in parotid acinar cells and rat pituitary cells this phenomenon does not seem to require extracellular Ca^{2+} (for review see Fisher *et al.*, 1984). Two other pieces of evidence which indicate that agonist-stimulated PtdIns(4,5)P_2 breakdown in the iris is not regulated by Ca^{2+} are the findings that: (*a*) like that of noradrenaline, the ionophore A23187-stimulated PtdIns(4,5)P_2 breakdown is inhibited by prazosin (Table 3), suggesting that the observed effect of the ionophore on PtdIns(4,5)P_2 breakdown is probably due to the release of noradrenaline from the tissue, and (*b*) that depolarization of the iris with high K^+ has no effect on PtdIns(4,5)P_2 breakdown (Table 4). The ionophore has also been shown to cause release of noradrenaline from adrenergic nerve terminals in smooth muscle (Cohen *et al.*, 1981; Triggle, 1979), and from synaptosomes (Bradford *et al.*, 1983). The finding that K^+ depolarization does not stimulate PtdIns(4,5)P_2 breakdown suggests that in the iris the breakdown of this polyphosphoinositide is not controlled by the potential-dependent Ca^{2+} channel and that it is not a consequence of an increase in intracellular Ca^{2+} concentration.

In conclusion, the data presented demonstrate that, in the iris, carbachol stimulates the rapid breakdown of PtdIns(4,5)P_2 into InsP_3 and diacylglycerol, measured as phosphatidic acid, and that the accumulation of InsP_3 precedes that of InsP_2 and Ins*P*. In addition, these data suggest that the stimulated breakdown of PtdIns(4,5)P_2 could be involved in the mechanism of both the phasic (fast) and tonic (slow) components of the contractile response in the smooth muscle. The Ca^{2+} studies indicate that, whereas polyphosphoinositide phosphodiesterase requires some Ca^{2+} for its activity, the carbachol-stimulated PtdIns(4,5)P_2 breakdown is not regulated by Ca^{2+} in this tissue. The latter conclusion is based on the following findings in the present study: (*a*) carbachol stimulated the accumulation of InsP_3 in a Ca^{2+}-free medium; (*b*) the stimulation of PtdIns(4,5)P_2 breakdown by ionophore A23187 is secondary to the release of noradrenaline by the ionophore; (*c*) K^+ depolarization did not change the level of InsP_3.

As to the functions of the agonist-stimulated breakdown of PtdIns(4,5)P_2 in the iris we can offer

the following possibilities. (*a*) The stimulated breakdown of PtdIns(4,5)P_2 mediates the neurotransmitter-induced depolarization of the smooth muscle plasma membrane (Akhtar & Abdel-Latif, 1978; Abdel-Latif, 1983). The structural and conformational changes which may result from such a breakdown could lead to permeability changes in the plasma membrane. In smooth muscle the signal responsible for activation of the contractions is depolarization of the plasma membrane, which can release Ca^{2+} from intracellular sites even in a Ca^{2+}-free medium (Mangel *et al.*, 1982). (*b*) InsP_3, the water-soluble product of PtdIns(4,5)P_2 breakdown, could function to mobilize Ca^{2+} from the endoplasmic reticulum, as has recently been reported (Berridge, 1983; Streb *et al.*, 1983; Joseph *et al.*, 1984). (*c*) Diacylglycerol, the water-insoluble product of PtdIns(4,5)P_2 breakdown, could either activate a protein kinase C phospholipid complex (Nishizuka, 1984) or it could be metabolized by diacylglycerol- and monoacylglycerol-lipases to liberate arachidonic acid for prostaglandin biosynthesis.

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