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Requirement for calcium ions in acetylcholine-stimulated phosphodiesteratic cleavage of phosphatidyl-*myo*-inositol 4,5-bisphosphate in rabbit iris smooth muscle

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1. The mechanism of acetylcholine-stimulated breakdown of phosphatidyl-*myo*-inositol 4,5-bisphosphate and its dependence on extracellular Ca^{2+} was investigated in the rabbit iris smooth muscle. 2. Acetylcholine (50μM) increased the breakdown of phosphatidylinositol bisphosphate in [^{3}H]inositol-labelled muscle by 28% and the labelling of phosphatidylinositol by 24% of that of the control. Under the same experimental conditions there was a 33 and 48% increase in the production of ^{3}H-labelled inositol trisphosphate and inositol monophosphate respectively. Similarly carbamoylcholine and ionophore A23187 increased the production of these water-soluble inositol phosphates. Little change was observed in the ^{3}H radioactivity of inositol bisphosphate. 3. Both inositol trisphosphatase and inositol monophosphatase were demonstrated in subcellular fractions of this tissue and the specific activity of the former was severalfold higher than that of the latter. 4. The acetylcholine-stimulated production of inositol trisphosphate and inositol monophosphate was inhibited by atropine (20μM), but not tubocurarine (100μM); and it was abolished by depletion of extracellular Ca^{2+} with EGTA, but restored on addition of low concentrations of Ca^{2+} (20μM). 5. Calcium-antagonistic agents, such as verapamil (20μM), dibenamine (20μM) or La^{3+} (2mM), also abolished the production of the water-soluble inositol phosphates in response to acetylcholine. 6. Release of inositol trisphosphate from exogenous phosphatidylinositol bisphosphate by iris muscle microsomal fraction ('microsomes') was stimulated by 43% in the presence of 50μM-Ca^{2+}. 7. The results indicate that increased Ca^{2+} influx into the iris smooth muscle by acetylcholine and ionophore A23187 markedly activates phosphatidylinositol bisphosphate phosphodiesterase and subsequently increases the production of inositol trisphosphate and its hydrolytic product inositol monophosphate. The marked increase observed in the production of inositol monophosphate could also result from Ca^{2+} activation of phosphatidylinositol phosphodiesterase. However, there was no concomitant decrease in the ^{3}H radioactivity of this phospholipid.

In previous work from this laboratory (Abdel-Latif *et al.*, 1977, 1978*a*) we reported that acetylcholine and noradrenaline increased significantly the breakdown of phosphatidyl-*myo*-inositol 4,5-bisphosphate in ^{32}P-labelled rabbit iris smooth muscle and that this effect is mediated through cholinergic muscarinic and α-adrenergic receptors respectively. Furthermore, we reported that the acetylcholine-stimulated breakdown of phosphatidylinositol bisphosphate and labelling of phosphatidic acid is dependent on the presence of Ca^{2+} in the incubation medium (Akhtar & Abdel-Latif, 1978*a*) and the enzyme that might be involved in these effects appears to be phosphatidylinositol bisphosphate phosphodiesterase (Akhtar & Abdel-Latif, 1978*b*). In a more recent study we showed that acetylcholine increases the uptake of $^{45}Ca^{2+}$ in the iris and that this uptake is inhibited by atropine (Akhtar & Abdel-Latif, 1979). These findings led us to suggest that the increased entry of Ca^{2+} into the tissue after activation of cholinergic muscarinic receptors could trigger the breakdown of phosphatidylinositol bisphosphate in the iris muscle (Abdel-Latif *et al.*, 1978*b*).

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To throw more light on the relationship between activation of cholinergic muscarinic receptors and the phosphodiesteratic cleavage of phosphatidylinositol bisphosphate (eqn. 1) in the iris, we have investigated the effects of acetylcholine and other cholinergic agents, Ca^{2+}, ionophore A23187 and Ca^{2+}-antagonistic drugs on the release of water-soluble inositol phosphates.

$$\text{Phosphatidylinositol bisphosphate} \xrightarrow[\text{bisphosphate phosphodiesterase}]{\text{Phosphatidylinositol}} \text{inositol trisphosphate} + \text{diacylglycerol} \quad (1)$$

$$\text{Inositol trisphosphate} \xrightarrow[\text{phosphatases}]{\text{Inositol}} \text{inositol bisphosphate} + \text{inositol monophosphate} \quad (2)$$

The effects of Ca^{2+} and ionophore A23187 on the release of water-soluble inositol phosphates from polyphosphoinositides have been reported in erythrocytes (Allan & Michell, 1978) and synaptosomes (Griffin & Hawthorne, 1978).

Materials and methods

Materials

Chemicals used in the present study were obtained from the following sources: acetylcholine chloride, atropine sulphate, 2-deoxyglucose, and *myo*-inositol 2-monophosphate from Sigma Chemical Co., St. Louis, MO, U.S.A.; eserine sulphate from Calbiochem, San Diego, CA, U.S.A.; [^{32}P]P_i (carrier-free) and [^{3}H]inositol (sp. radioactivity 12.5 Ci/mmol) from New England Nuclear Corp., Boston, MA, U.S.A.; Dowex 50W (H^+ form) from Bio-Rad Laboratories, Richmond, CA, U.S.A.; dibenamine was from Pfaltz and Baver Inc., Stanford, CT, U.S.A.; and verapamil from Knoll Pharmaceutical Co., Whippany, NJ, U.S.A. Ionophore A23187 was a gift from Eli Lilly and Co., Indianapolis, IN, U.S.A. A crude phosphatidylinositol phosphate fraction was prepared by the method of Folch (1949) and phosphatidylinositol phosphate and phosphatidylinositol bisphosphate were isolated by means of DEAE-cellulose column chromatography (Whatman DE-52, microgranular) as described by Hendrickson & Ballou (1964). Inositol triphosphate was prepared from phosphatidylinositol bisphosphate by the method of Grado & Ballou (1961).

Methods

Preparation and incubation of iris muscle. In the present study albino rabbits of either sex weighing approx. 4 kg were used. The unanaesthetized rabbits were decapitated and eyes were enucleated immediately. The irises were removed and placed, in pairs from the same animal, in tubes containing 2 ml of Bradford medium (Bradford, 1969) of the following composition: 124 mM-NaCl; 5 mM-KCl; 1.2 mM-KH_2PO_4; 1.3 mM-$MgCl_2$; 26 mM-Tris; 0.75 mM-$CaCl_2$; 1.5 mM-cytidine; 10 mM-D-glucose. The pH of the medium was adjusted to 7.4.

To label the tissue phospholipids, the paired irises were incubated in 1 ml of the Bradford medium that contained 10 μCi of [^{3}H]inositol for 60 min. At the end of incubation the irises were washed three times with 5 ml of non-radioactive Bradford medium that contained 10 mM-2-deoxyglucose. In general, for studies on the effects of acetylcholine and other pharmacological agents on the hydrolysis of phosphatidylinositol bisphosphate, the prelabelled irises (of the pair, one was used as control and the other as experimental) were incubated at 37°C for 15 min in 1 ml of the unlabelled medium, which contained 2-deoxyglucose (10 mM) and other agents as indicated. The inclusion of deoxyglucose in the medium, as we have reported previously (Abdel-Latif *et al.*, 1977), lowers the ATP concentration without significantly affecting the metabolism of the tissue. Addition of deoxyglucose thus minimizes the biosynthesis of phosphatidylinositol bisphosphate during the brief exposure of the tissue to the stimulus. Under these experimental conditions, therefore, any loss of label from phosphatidylinositol bisphosphate reflects an increase in breakdown of this lipid. In experiments where the effect of acetylcholine was investigated, equimolar concentration of eserine were added to the incubation medium. In experiments where the effects of atropine, ionophore or other drugs were investigated, the prelabelled irises were preincubated in the presence of these agents for 5 min before the addition of acetylcholine. At the end of incubation 1 ml of 10% (w/v) trichloroacetic acid was added to each tube and the tissue was homogenized in a glass homogenizer. The homogenate was then centrifuged at 3000 *g* for 15 min and the supernatant was removed for analysis of inositol phosphates. In some experiments the pellet was retained for extraction and analysis of phospholipids.

Separation and analysis of inositol phosphates. The supernatant, which contains the water-soluble inositol phosphates, was extracted four times with equal volumes of diethyl ether, then freeze-dried and the residue dissolved in 50 μl of water. The water-soluble inositol phosphates were then separated by means of low-voltage paper electrophoresis in pyridine/acetic acid buffer (pH 3.2) at 40 V/cm for 8 h. The water-soluble fractions from irises incubated in the absence and presence of the various agents

were mixed with an alkaline hydrolysate of Folch fraction I/II. This hydrolysate was obtained from Folch fraction I/II by the method of Grado & Ballou (1961). Briefly 0.5g of the lipids were heated at 100°C for 45min with 20ml of 1M-NaOH. The solution was cooled and Dowex 50 (H^+ form) added batch-wise to make it strongly acidic. The precipitated fatty acids and the resins were filtered off and the filtrate was passed through a column of Dowex 50 (H^+ form) to remove all cations. The eluate was extracted with diethyl ether to remove the remaining fatty acids. The water layer was removed and then made basic by the addition of excess cyclohexylamine. The solution was freeze dried and the residue extracted with hot ethanol to obtain an alkaline hydrolysate of Folch fraction I/II. Any residue that did not dissolve in ethanol was discarded. After electrophoresis the phosphate esters were detected by spraying the paper with Hanes & Isherwood (1949) reagent of the following composition; 5ml of 60% (w/w) $HClO_4$, 25ml of 4% (w/v) ammonium molybdate, 10ml of 1M-HCl and 60ml of water. The paper was air-dried and then exposed to u.v. radiation. All organic phosphate compounds now appear as blue spots, whereas P_i produced a yellow–green colour. The spots were cut out into small pieces and placed in vials containing 1.8ml of water. The vials were left overnight at room temperature and then the samples were neutralized by the addition of 0.2ml of 0.5M-NaOH. Scintillation fluid [8ml; 0.10g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 5.0g of 2,5-diphenyloxazole, 300ml of Triton X-100 and 600ml of toluene] was added to each sample and the radioactivity was determined by liquid-scintillation counting. Each vial was counted for 10min, and corrections for quenching were made by the quench curve based on the external standard ratio method. The counting efficiency for ^3H in the present work was 38%. The migration of various inositol phosphates relative to P_i calculated from the centre of each spot were as follows: inositol monophosphate, 0.594 ± 0.005; inositol diphosphate (inositol bisphosphate), 0.816 ± 0.004; and inositol triphosphate (inositol trisphosphate), 1.183 ± 0.003.

Extraction and analysis of phospholipids. The pellet obtained from the previous step was washed with water and then extracted once with 2ml of chloroform/methanol/conc. HCl (300:200:1.5, by vol.) and once with 2ml of chloroform/methanol/conc. HCl (400:200:1.5, by vol.). The extracts were combined and processed for analysis of radiolabelled phospholipids as described previously (Abdel-Latif *et al.*, 1977). The radioactivity in the individual phospholipids was corrected for quenching as described in the previous section.

Assay for phosphatidylinositol bisphosphate phosphodiesterase in rabbit iris microsomal fraction ('microsomes'). Release of inositol triphosphate from phosphatidylinositol bisphosphate was taken as a measure of phosphodiesterase activity in the iris microsomal fraction. Preparation of microsomes from the rabbit iris and the assay of phosphatidylinositol bisphosphate phosphodiesterase in this fraction were essentially as described previously (Akhtar & Abdel-Latif, 1978*b*).

In brief, a typical assay mixture contained 40mM-Tris/HCl (pH 7.2), 1.2mM-phosphatidylinositol bisphosphate, 1.5mM-cetyltrimethylammonium bromide and 100μg of microsomal protein in a final volume of 0.5ml. After 15min of incubation the reaction was stopped by addition of 0.1ml of 5% (w/v) bovine serum albumin and 0.5ml of 10% (w/v) trichloroacetic acid. The tubes were placed in ice for 10min and then centrifuged. The clear supernatant was extracted several times with diethyl ether, freeze dried and then electrophoresed. The spots corresponding to the inositol phosphates were cut out and digested with $HClO_4$ for determination of P_i by the method of Bartlett (1959).

Assay for inositol monophosphatase and inositol triphosphatase in the subcellular fractions of the rabbit iris. The assay mixture for inositol monophosphatase and inositol triphosphatase consisted of inositol monophosphate or inositol triphosphate, 1.2mM; Tris/HCl buffer (pH 7.2), 40mM; $MgCl_2$, 1mM; and 200μg of protein in a final volume of 0.5ml. After incubation at 37°C for 15min, the reaction was stopped by addition of 0.5ml of 10% (w/v) trichloroacetic acid and the tubes were centrifuged at 3000*g*. Release of P_i from inositol phosphates was determined in the supernatant by the method of Bartlett (1959).

Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Identification and distribution of [^3H]inositol in the water-soluble inositol phosphates of the rabbit iris

To identify the products of phosphoinositide metabolism in the iris muscle, the tissue was labelled either with $^{32}P_i$ or [^3H]inositol before extraction of the water-soluble inositol phosphates. In preliminary experiments we obtained good separation of the various inositol phosphates by means of descending paper chromatography employing ethanol/13.5M-NH_3 (3:2, v/v) as solvent system. However, better separations of the water-soluble inositol phosphates were obtained with low-voltage paper electrophoresis. High-voltage paper electrophoresis often resulted in streaking of the various spots. Separation of radiolabelled water-soluble inositol phosphates by means of low-voltage paper

electrophoresis revealed the presence of inositol monophosphate, inositol diphosphate, and inositol triphosphate in the tissue extract. This method does not separate inositol 1-phosphate from inositol 1:2-cyclic monophosphate. In a more recent study on the properties of the soluble phosphatidylinositol phosphodiesterase of rabbit iris muscle (Abdel-Latif *et al.*, 1980) the products of phosphatidylinositol hydrolysis were 1,2-diacylglycerol and a mixture of *myo*-inositol 1-phosphate (86%) and *myo*-inositol 1:2-cyclic phosphate (14%). Of the phosphate esters, inositol monophosphate was most radioactively labelled, followed by inositol triphosphate and inositol diphosphate respectively (Table 1). In the [^{3}H]inositol-labelled iris, radioactivity in inositol monophosphate was about five times as high as that in inositol triphosphate. In addition in the ^{32}P-labelled tissue substantial amounts of radioactivity was recovered in spots corresponding to P_i and adenine nucleotides (results not shown). Since the presence of these ^{32}P-labelled nucleotides complicates identification of radioactive inositol phosphates, in the following studies we routinely used [^{3}H]inositol as a more specific precursor for phosphoinositide metabolism in the iris.

Effect of acetylcholine on the radioactivity of phosphoinositides and water-soluble inositol phosphates in rabbit iris labelled with [^{3}H]inositol in vitro

Acetylcholine, at a concentration of 50μM, increased the breakdown of phosphatidylinositol bisphosphate in [^{3}H]inositol-labelled iris by 28% and increased the labelling of phosphatidylinositol by 24% of that of the control (Table 1). There was little change in the labelling of phosphatidylinositol phosphate. Under the same experimental conditions there was a 48 and 33% increase in the ^{3}H radioactivity of inositol monophosphate and inositol triphosphate respectively. In general the radioactivity recovered as free [^{3}H]inositol, which was almost 10–15 times higher than that of total inositol phosphates, remained essentially unchanged under the present experimental conditions. Since there did not appear to be a good correlation between the amount of radioactivity lost from [^{3}H]phosphatidylinositol bisphosphate and the amount of radioactivity recovered in the water-soluble inositol phosphates, it was of interest to investigate if this could be due to a stepwise degradation of inositol triphosphate to inositol monophosphate. Inositol monophosphatase and inositol triphosphatase were found to be present in the soluble as well as the particulate fractions of the iris. The specific activities of inositol monophosphatase and inositol triphosphatase were highest in the microsomal and soluble fractions respectively. In all the fractions studied the specific activity of inositol triphosphatase was about ten times as high as that of inositol monophosphatase. This could explain the poor correlation observed between the loss of ^{3}H radioactivity from [^{3}H]phosphatidylinositol bisphosphate and gain in that of [^{3}H]inositol triphosphate (Table 1). Atropine (20μM), a muscarinic blocker, inhibited the release of the water-soluble [^{3}H]inositol phosphates from the ^{3}H-labelled iris muscle (Table 2). In contrast tubocurarine (100μM), a nicotinic blocker, had no effect on the release of the inositol phosphates.

Effects of Ca^{2+} and acetylcholine

To demonstrate a requirement for Ca^{2+}, the ^{3}H-labelled irises were extensively washed with EGTA-containing buffer before the addition of acetylcholine and/or Ca^{2+} in the incubation medium.

Table 1. *Effect of acetylcholine on the radioactivity of phosphoinositides and water-soluble inositol phosphates in iris smooth muscle prelabelled with [^{3}H]inositol in vitro*

Paired irises were incubated in 1 ml of Bradford medium containing 10μCi of [^{3}H]inositol for 60 min. At the end of incubation the irises were washed three times with 5 ml of non-radioactive Bradford medium containing 10 mM-2-deoxyglucose. The labelled irises were then incubated singly (of the pair, one was used as a control and the other as experimental) in 1 ml of Bradford medium for 15 min in the presence or absence of acetylcholine. At the end of incubation 1 ml of 10% (w/v) trichloroacetic acid was added to each tube. Then the phosphoinositides and their soluble metabolic products were isolated from the tissue and analysed for radioactivity as described under 'Methods'. The control incubations were conducted in the absence of acetylcholine. The values given are means ± S.E.M. for three separate experiments conducted in duplicate. *P* values, as compared with the control, were calculated by Student's *t* test for paired data. *, not significant ($P > 0.05$).

	^{3}H incorporated (d.p.m.)					
	Phosphatidylinositol	Phosphatidylinositol phosphate	Phosphatidylinositol bisphosphate	Inositol monophosphate	Inositol diphosphate	Inositol triphosphate
Control	24315 ± 1140	3899 ± 342	10207 ± 495	1560 ± 123	141 ± 22	287 ± 12
Acetylcholine (50μM)	30201 ± 1837 ($P < 0.05$)	3502 ± 375*	7353 ± 315 ($P < 0.01$)	2315 ± 174 ($P < 0.02$)	142 ± 23*	382 ± 35 ($P < 0.05$)

Table 2. *Effect of atropine and tubocurarine on acetylcholine-stimulated labelling of inositol phosphates in iris smooth muscle prelabelled with [^3H]inositol in vitro*

The experimental conditions were the same as described in the legend of Table 1. The ^3H-labelled irises were incubated with atropine or tubocurarine as indicated in the table for 5 min before the addition of acetylcholine. The incubation time with the neurotransmitter was 15 min. The inositol phosphates were isolated from the tissue and analysed for radioactivity as described under 'Methods'. The values given are means ± S.E.M. for three separate experiments consisting of duplicate samples. *P* values, compared with the control, were calculated by Student's *t* test for paired data. *, not significant ($P > 0.05$).

	^3H incorporated (d.p.m.)		
Additions	Inositol monophosphate	Inositol diphosphate	Inositol triphosphate
Control	1565 ± 61	179 ± 18	296 ± 25
Acetylcholine (50 μM)	2533 ± 370 ($P < 0.05$)	169 ± 36*	420 ± 34 ($P < 0.05$)
Atropine (20 μM)	1833 ± 151*	156 ± 10*	299 ± 21*
Acetylcholine (50 μM) + atropine (20 μM)	1720 ± 89*	144 ± 14*	326 ± 22*
Tubocurarine (100 μM)	1665 ± 110*	195 ± 7*	324 ± 50*
Acetylcholine (50 μM) + tubocurarine (100 μM)	2112 ± 109 ($P < 0.01$)	201 ± 24*	476 ± 40 ($P < 0.01$)

Table 3. *Effects of Ca^{2+} and acetylcholine on the radioactivity of phosphoinositides and inositol phosphates in iris muscle labelled with [^3H]inositol in vitro*

Experimental conditions were exactly the same as described in Table 1, except that the ^3H-labelled irises were washed three times in EGTA containing Bradford medium before the addition of acetylcholine and/or $CaCl_2$. The control incubations were conducted in the absence of acetylcholine and $CaCl_2$. The phosphoinositides and inositol phosphates were isolated from the tissue and analysed for radioactivity as described under 'Methods'. The values given are means ± S.E.M. for three separate experiments conducted in duplicate. *P* values, as compared with the control, were calculated by Student's *t* test for paired data. *, not significant ($P > 0.05$).

	^3H incorporated (d.p.m.)					
Additions	Phosphatidyl-inositol	Phosphatidyl-inositol phosphate	Phosphatidyl-inositol bisphosphate	Inositol monophosphate	Inositol diphosphate	Inositol triphosphate
Control	26382 ± 1760	4114 ± 232	11560 ± 263	987 ± 113	136 ± 15	198 ± 7
Acetylcholine (0.05 mM)	33722 ± 1790 ($P < 0.05$)	4454 ± 216*	11055 ± 619*	1079 ± 190*	123 ± 12*	213 ± 21*
Ca^{2+} (0.75 mM)	27411 ± 1976*	3970 ± 321*	10586 ± 256 ($P < 0.05$)	1547 ± 66 ($P < 0.01$)	131 ± 18*	277 ± 18 ($P < 0.02$)
Acetylcholine (0.05 mM) + Ca^{2+} (0.75 mM)	34951 ± 922 ($P < 0.02$)	4130 ± 230*	8144 ± 263 ($P < 0.01$)	2498 ± 115) ($P < 0.01$)	124 ± 23*	389 ± 21 ($P < 0.01$)

As shown in Table 3 acetylcholine alone increased the labelling of phosphatidylinositol but had little effect on the labelling of phosphatidylinositol bisphosphate and the water-soluble inositol phosphates. In contrast, Ca^{2+} alone had no effect on the labelling of phosphatidylinositol but increased the breakdown of phosphatidylinositol bisphosphate by 8% and increased the labelling of inositol triphosphate and inositol monophosphate by 40 and 57% respectively. When both acetylcholine and Ca^{2+} were added there was a 32% increase in phosphatidylinositol labelling and 30% increase in the breakdown of phosphatidylinositol bisphosphate and a concomitant increase of 96 and 153% in those of inositol triphosphate and inositol monophosphate respectively (Table 3). The radioactivities of phosphatidylinositol phosphate and inositol diphosphate were unchanged under all experimental conditions.

A dose–response curve of Ca^{2+}, in the presence of acetylcholine, showed that this cation in concentrations as low as 20 μM was effective in bringing about a significant increase in the radioactivities of both inositol monophosphate and triphosphate (Fig. 1). Maximal increase in radioactivity of the water-

soluble inositol phosphates was observed at 100 μM-Ca^{2+}, which was followed by a decrease in that of inositol monophosphate.

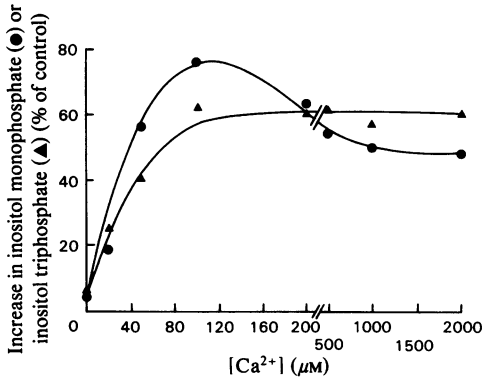

Fig. 1. *Effects of different concentrations of Ca^{2+} on acetylcholine-stimulated release of water-soluble inositol phosphates from [^{3}H]inositol-labelled iris muscle*

Paired irises were labelled with [^{3}H]inositol for 60 min as described in the legend to Table 1. At the end of incubation the irises were washed three times with excess non-radioactive medium that contained 10 mM-2-deoxyglucose and 0.25 mM-EGTA. The prelabelled irises (of the pair, one was used as control and the other as experimental) were then incubated in the absence and presence of acetylcholine plus eserine (50 μM each) for 15 min in unlabelled Bradford medium that contained 10 mM-2-deoxyglucose and various concentrations of $CaCl_2$. At the end of incubation, the inositol phosphates were isolated and analysed as described under 'Methods'. Points on the curves represent means for three determinations, and are taken from one of two similar experiments.

Effects of carbamoylcholine and ionophore A23187

Carbamoylcholine, a cholinergic agonist, also increased the release of [^{3}H]inositol monophosphate and [^{3}H]inositol triphosphate from [^{3}H]-inositol-labelled irises by 31 and 25% respectively (Table 4). This increase was completely inhibited by 20 μM-atropine. In the absence of Ca^{2+}, ionophore A23187 had no effect on the release of the water-soluble inositol phosphates (results not shown). However, in the presence of this cation the ionophore increased the release of inositol monophosphate and inositol triphosphate by 26 and 21% respectively (Table 4). In contrast with the effects of acetylcholine and carbamoylcholine on the release of [^{3}H]inositol phosphates, that of ionophore A23187 was not inhibited by atropine.

Effects of Ca^{2+} antagonists

Studies on the effects of Ca^{2+}-antagonistic drugs on neurotransmitter-stimulated phosphoinositide turnover in the ileal smooth muscle has already been reported (Jafferji & Michell, 1976). Thus it was of interest to investigate the effects of these drugs on the acetylcholine-stimulated release of inositol phosphates in the iris. The concentrations of the drugs were selected such that when added alone they had no effect on the release of labelled water-soluble inositol phosphates from [^{3}H]inositol-labelled irises (Table 5). Acetylcholine-stimulated release of the water-soluble inositol phosphates from [^{3}H]-inositol-labelled muscle was inhibited by verapamil (20 μM), dibenamine (20 μM) and $LaCl_3$ (2 mM).

Mechanism of Ca^{2+}-dependent hydrolysis of phosphatidylinositol bisphosphate by iris muscle microsomes

In previous studies it was shown that microsomal

Table 4. *Effects of carbamoylcholine and ionophore A23187 on the radioactivity of inositol phosphates in iris smooth muscle prelabelled with [^{3}H]inositol in vitro*

The paired irises were labelled with [^{3}H]inositol as described in the legend to Table 1. The prelabelled irises were preincubated with atropine for 5 min before the addition of carbamoylcholine or ionophore A23187, and the incubation was continued for 15 min. The inositol phosphates were isolated from the tissue and analysed for radioactivity as described under 'Methods'. The control incubations were conducted in the absence of the added drugs. The values given are means ± S.E.M. for three experiments conducted in duplicate. *P* values, as compared with the control, were calculated by Student's *t* test for paired data. *, not significant ($P > 0.05$).

Additions	^{3}H incorporated (d.p.m.) Inositol monophosphate	Inositol diphosphate	Inositol triphosphate
Control	1490 ± 93	199 ± 8	292 ± 18
Carbamylcholine (0.1 mM)	1951 ± 178 ($P < 0.05$)	185 ± 27*	365 ± 26 ($P < 0.05$)
Carbamylcholine (0.1 mM) + atropine (0.02 mM)	1564 ± 67*	196 ± 22*	311 ± 6*
Ionophore A23187 (0.02 mM)	1879 ± 65 ($P < 0.02$)	195 ± 22*	355 ± 8 ($P < 0.01$)
Ionophore A23187 (0.02 mM) + atropine (0.02 mM)	2014 ± 121 ($P < 0.01$)	188 ± 8*	368 ± 21 ($P < 0.02$)

Table 5. *Effects of Ca^{2+} antagonists on acetylcholine-stimulated release of inositol phosphates in iris smooth muscle prelabelled with [^3H]inositol in vitro*

The paired irises were labelled with [^{3}H]inositol and washed in non-radioactive Bradford medium as described in the legend to Table 1. The prelabelled irises were incubated with the drugs, as indicated, for 5 min before the addition of acetylcholine. This was followed by incubation for 15 min. The inositol phosphates were then isolated from the tissue and analysed for radioactivity as described under 'Methods'. The values given are means ± S.E.M. for three separate experiments conducted in duplicate. *P* values, compared with the control, were calculated by Student's *t* test for paired data. *, not significant ($P > 0.05$).

Additions	^{3}H incorporated (d.p.m.) Inositol monophosphate	Inositol diphosphate	Inositol triphosphate
Control	1684 ± 139	163 ± 26	289 ± 13
Acetylcholine (50 μM)	2560 ± 283 ($P < 0.05$)	171 ± 20*	460 ± 41 ($P < 0.02$)
Verapamil (20 μM)	1670 ± 218*	129 ± 18*	294 ± 34*
Verapamil (20 μM) + acetylcholine (50 μM)	1808 ± 301*	131 ± 8*	325 ± 25*
Dibenamine (20 μM)	1882 ± 292*	193 ± 40*	321 ± 28*
Dibenamine (20 μM) + acetylcholine (50 μM)	2021 ± 39*	264 ± 36*	294 ± 10*
$LaCl_3$ (2 mM)	1747 ± 311	203 ± 52	333 ± 46*
$LaCl_3$ (2 mM) + acetylcholine (50 μM)	1692 ± 23*	241 ± 13*	300 ± 53*

Table 6. *Effects of Ca^{2+}, Mg^{2+} and bivalent cation chelators on the activity of phosphatidylinositol bisphosphate phosphodiesterase*

The assay mixture consisted of 40 mM-Tris/HCl (pH 7.2), 1.2 mM-phosphatidylinositol bisphosphate, 1.5 mM-cetyltrimethylammonium bromide and 100 μg of microsomal protein. After 15 min of incubation, the reaction was stopped by the addition of 0.1 ml of 5% (w/v) bovine serum albumin and 0.5 ml of 10% (w/v) trichloroacetic acid. The water-soluble products released during the reaction were separated by low-voltage paper electrophoresis. The spots corresponding to inositol triphosphate were cut out and digested with $HClO_4$ for determination of phosphate. The control incubations were conducted in the absence of $CaCl_2$, $MgCl_2$ or bivalent cation chelators. The values given are means for three separate experiments conducted in duplicate. *P* values, compared with the control, were calculated by Student's *t* test. *, $P < 0.01$; **, μmol of inositol triphosphate released/mg of protein per h.

Additions	Concentrations (mM)	Inositol triphosphate**	% of control
Control	—	4.68 ± 0.14	—
EDTA	1.0	1.17 ± 0.12*	25
EGTA	1.0	2.53 ± 0.11*	54
$CaCl_2$	0.05	6.95 ± 0.24*	148
$MgCl_2$	1.0	3.77 ± 0.06*	80

fraction prepared from rabbit iris contains phosphatidylinositol bisphosphate phosphodiesterase and phosphatidylinositol bisphosphate phosphomonoesterase (Akhtar & Abdel-Latif, 1978*b*). In an attempt to identify the nature of the organic phosphates released in this reaction, an electrophoretic analysis of the water-soluble products revealed the presence of inositol triphosphate and P_i and little inositol monophosphate. Quantitatively, the release of inositol triphosphate was much higher than that of P_i. This observation is consistent with our previous finding that in the microsomal fraction of iris muscle the specific activity of phosphatidylinositol bisphosphate phosphodiesterase is about four to six times higher than that of phosphatidylinositol bisphosphate phosphomonoesterase (Akhtar & Abdel-Latif, 1978*b*). Under the present experimental conditions release of inositol triphosphate from phosphatidylinositol bisphosphate was linear to up to 15 min, then decreased thereafter, thus indicating that the substrate is rate limiting (results not shown). Addition of either EDTA or EGTA inhibited the release of inositol triphosphate (Table 6). Ca^{2+} (50 μM) increased the phosphodiesterase activity by 43% and Mg^{2+} (1 mM) inhibited it by 20%. Dose–response studies revealed that Ca^{2+} in concentrations as low as 10 μM can stimulate the phosphodiesterase activity. However, maximal stimulation (48%) occurred at a concentration of 40 μM of this cation (Fig. 2). Higher concentrations of Ca^{2+} (>60 μM) inhibited the phosphodiesterase activity.

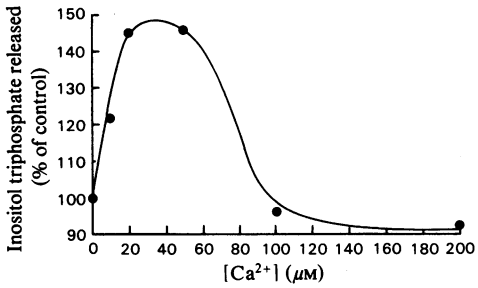

Fig. 2. *Effects of different concentrations of Ca^{2+} on the activity of phosphatidylinositol bisphosphate phosphodiesterase*

Conditions of assay were the same as described in the legend to Table 6, except that various concentrations of $CaCl_2$ were added in the assay mixture. Points on the curve are means for two determinations, and are taken from one of three similar experiments.

Discussion

We postulated previously that in the rabbit iris smooth muscle Ca^{2+} mediates acetylcholine-stimulated breakdown of phosphatidylinositol bisphosphate probably through activation of phosphatidylinositol bisphosphate phosphodiesterase (Abdel-Latif *et al.*, 1978*b*). The results reported in the present paper add further support to this hypothesis. Thus, when [^{3}H]inositol-labelled muscle was incubated in the presence of acetylcholine, there was a loss of radioactivity from phosphatidylinositol bisphosphate and a concomitant rise in that of inositol triphosphate and inositol monophosphate (Table 1). This finding suggests the possible involvement of phosphatidylinositol bisphosphate phosphodiesterase (eqn. 1). No significant changes were observed in either phosphatidylinositol phosphate or inositol diphosphate. Under the same experimental conditions, however, the radioactivities of both phosphatidylinositol and its water-soluble hydrolytic product, inositol monophosphate, were significantly increased. The increase in phosphatidylinositol labelling occurs despite the fact that the tissue no longer has access to [^{3}H]inositol from the outside. This could suggest two possibilities. (*a*) In the presence of acetylcholine, there is an increased hydrolysis of phosphatidylinositol followed by its resynthesis by using an internal pool of [^{3}H]inositol that is not diluted out by the washing procedure. Furthermore, there could be a transient increase in [^{3}H]inositol pool by the label derived from the hydrolysis of inositol triphosphate. (*b*) Acetylcholine actually inhibits the loss of label that has been incorporated into phosphatidylinositol. Since there is no convincing evidence to support the latter possibility (for review, see Michell, 1975), it is reasonable to conclude that acetylcholine increases the labelling of phosphatidylinositol by enhancing the turnover of this phospholipid. The observed increase in the ^{3}H radioactivity of inositol monophosphate could also be due to: (*a*) stimulated hydrolysis of phosphatidylinositol; (*b*) enzymic degradation of inositol triphosphate (eq. 2). The latter possibility is supported by the demonstration in the present work of inositol triphosphatase in all the subcellular fractions of the iris muscle.

In accord with our previous studies with ^{32}P-labelled iris muscle (Abdel-Latif *et al.*, 1977; Akhtar & Abdel-Latif, 1978*a*), the acetylcholine-stimulated breakdown of phosphatidylinositol bisphosphate as measured by the release of water-soluble ^{3}H-labelled inositol triphosphate is mediated through cholinergic muscarinic receptors (Table 2) and is dependent on the presence of Ca^{2+} in the incubation medium (Table 3). In contrast, the acetylcholine-stimulated labelling of phosphatidylinositol in iris muscle prelabelled with [^{3}H]inositol (Table 3) or [^{32}P]P_i (Akhtar & Abdel-Latif, 1978*a*) is not dependent on the presence of Ca^{2+} in the incubation medium. This observation is in accord with that of Jafferji & Michell (1976), who reported that acetylcholine-stimulated hydrolysis of phosphatidylinositol in guinea-pig ileum is independent of extracellular Ca^{2+}. These investigators have proposed a hypothesis according to which the agonist-stimulated breakdown of phosphatidylinositol in target tissue leads to the opening of Ca^{2+} gates, and increased intracellular Ca^{2+} concentration, which in turn could trigger the observed cellular responses. In this connection it has recently been reported that the acetylcholine-stimulated increase in the labelling of phosphatidylinositol in synaptosomes is inhibited by EGTA and restored by the addition of Ca^{2+} (Griffin *et al.*, 1979; Fisher & Agranoff, 1979). It should be pointed out that although the acetylcholine-stimulated labelling of phosphatidylinositol in iris smooth muscle is not dependent on the presence of Ca^{2+} in the incubation medium, the release of inositol monophosphate does appear to be dependent on the presence of this cation (Table 3). As mentioned above, this increase in the release of inositol monophosphate could result from the degradation of inositol triphosphate. In addition the iris muscle contains phosphatidylinositol phosphodiesterase, which is activated by low concentrations of Ca^{2+} (Abdel-Latif *et al.*, 1980).

Activation of cholinergic muscarinic receptors in the iris muscle (Akhtar & Abdel-Latif, 1979) as well as in a variety of tissues (Triggle, 1972; Rubin, 1974; Berridge, 1975) leads to an increase in Ca^{2+} uptake. Thus it is not unreasonable to assume that the observed increase in acetylcholine-stimulated release of inositol triphosphate from [^{3}H]inositol-

labelled muscle could be a consequence of the increase in Ca^{2+} influx (eq. 3).

containing lipids and/or water-soluble inositol phosphates (Hawthorne & White, 1975).

$$\begin{array}{c}\text{Acetylcholine} + \text{receptor} \rightarrow \text{Acetylcholine–receptor complex} \rightarrow \\ Ca^{2+}\ \text{influx} \uparrow \rightarrow \text{phosphatidylinositol bisphosphate breakdown} \rightarrow \\ \text{inositol triphosphate} + \text{diacylglycerol}\end{array} \quad (3)$$

This conclusion is supported by the following observations. (i) Release of inositol triphosphate in response to acetylcholine was abolished when extracellular Ca^{2+} was depleted by EGTA, and restored when the cation was added back to the incubation medium. (ii) Release of inositol triphosphate was increased by Ca^{2+} that was introduced into the muscle by ionophore A23187. The effect of ionophore was not blocked by atropine, thus indicating that the ionophore bypasses the muscarinic receptors. (iii) Verapamil, dibenamine and La^{3+}, which prevent Ca^{2+} movement through plasma membrane, also inhibited the acetylcholine-stimulated release of inositol triphosphate. (iv) Release of inositol triphosphate from phosphatidylinositol bisphosphate by iris muscle microsomal fraction is increased *in vitro* by low concentrations of Ca^{2+}.

Ca^{2+}-dependent loss of phosphatidylinositol phosphate and phosphatidylinositol bisphcsphate with concomitant increase in inositol diphosphate and inositol triphosphate have been reported in human erythrocytes (Allan & Michell, 1978), suggesting Ca^{2+} activation of a phosphodiesterase, which hydrolyses both phosphatidylinositol phosphate and phosphatidylinositol bisphosphate. Working with guinea-pig brain synaptosomes, Griffin & Hawthorne (1978) have reported that in the presence of extracellular Ca^{2+} inositol diphosphate was the major water-soluble product of polyphosphoinositide metabolism. Based on the relative loss of radioactivity from phosphatidylinositol phosphate and phosphatidylinositol bisphosphate these authors concluded that phosphatidylinositol bisphosphate phosphomonoesterase and phosphatidylinositol phosphate phosphodiesterase are activated by Ca^{2+}.

It is interesting to note that increased Ca^{2+} influx into ^{32}P-labelled erythrocytes (Allan & Michell, 1978), into [^{3}H]inositol-labelled synaptosomes (Griffin & Hawthorne, 1978) and into [^{3}H]inositol-labelled iris muscle markedly increases the production of inositol triphosphate and inositol diphosphate, inositol diphosphate and small amounts of inositol monophosphate and inositol triphosphate and inositol monophosphate respectively. The variations observed in production of the water-soluble inositol phosphates in these tissues could be due in part to differences in concentrations of the enzymes involved in the degradation of the inositol-

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