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Acetylcholine Increases the Breakdown of Triphosphoinositide of Rabbit Iris Muscle Prelabelled with [^{32}P]Phosphate

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1. Paired iris smooth muscles from rabbits were incubated for 30min at 37°C in an iso-osmotic salt medium containing glucose, inositol, cytidine and [^{32}P]phosphate. 2. One of the pair was then incubated at 37°C for 10min in unlabelled medium containing 10mM-2-deoxyglucose and the other was incubated in the presence of acetylcholine plus eserine (0.05mM each). 2-Deoxyglucose, which was included in the incubation medium to minimize the biosynthesis of triphosphoinositide from ATP and diphosphoinositide, decreased the amount of labelled ATP by 71% and inhibited further ^{32}P incorporation from ATP into triphosphoinositide by almost 30%. 3. Acetylcholine (0.05mM) increased significantly the loss of ^{32}P from triphosphoinositide (the 'triphosphoinositide effect') in ^{32}P-labelled iris muscle. This effect was measured both chemically and radiochemically. It was also observed when $^{32}P_i$ was replaced by *myo*-[^{3}H]inositol in the incubation medium. 4. The triphosphoinositide effect was blocked by atropine but not by D-tubocurarine. Further, muscarinic but not nicotinic agonists were found to provoke this effect. 5. Acetylcholine decreased by 28% the ^{32}P incorporation into triphosphoinositide, presumably by stimulating its breakdown. This decrement in triphosphoinositide was blocked by atropine, but not by D-tubocurarine. 6. The triphosphoinositide effect was accompanied by a significant increase in ^{32}P labelling, but not tissue concentration, of phosphatidylinositol and phosphatidic acid. The possible relationship between the loss of ^{32}P label from triphosphoinositide in response to acetylcholine and the concomitant increase in that of phosphatidylinositol and phosphatidic acid is discussed. 7. The presence of triphosphoinositide phosphomonoesterase, the enzyme that might be stimulated in the iris smooth muscle by the neurotransmitter, was demonstrated, and, under our methods of homogenization and assay, more than 80% of its activity was localized in the particulate fraction.

Although the 'phosphatidylinositol effect' which may be defined as a change in the rate of metabolism or turnover of this phospholipid when the tissue in which it occurs is stimulated by either neurotransmitters, drugs or other means, was discovered more than 20 years ago (Hokin & Hokin, 1955) and its presence has been confirmed in a number of tissues (for reviews see Hokin, 1968; Hawthorne, 1973; Hawthorne & White, 1975; Michell, 1975), the physiological significance and the molecular mechanism underlying this phenomenon are still unexplained. At least in some tissues, especially nervous tissue, this effect is thought to be associated with synaptic transmission (Hokin & Hokin, 1958; Larrabee *et al.*, 1963). In addition, preliminary evidence was presented by Durell & Garland (1969) which suggested that acetylcholine stimulates the phosphodiesteratic cleavage of phosphoinositides and that this may be the primary effect leading to secondary increases in synthesis of phospholipids, particularly phosphatidic acid and phosphatidylinositol. More recently this concept gained support from the studies by Hokin-Neaverson (1974) and her collaborators (Banschbach *et al.*, 1974*a*) working with pancreas, and Jones & Michell (1974) working with rat parotid fragments, who demonstrated a decrease in phosphatidylinositol in response to acetylcholine.

An effect of acetylcholine on polyphosphoinositide metabolism has also been suggested (Durell *et al.*, 1969) as part of a model on the mechanism of action of acetylcholine. However, the experimental evidence reported by a number of investigators working on the effects of external stimuli on polyphosphoinositides in various tissues has been confusing and in many instances contra-

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dictory. Thus, despite many attempts in the past to alter the labelling of diphosphoinositide and triphosphoinositide by the addition of acetylcholine to media bathing brain slices (Palmer & Rossiter, 1965), sympathetic or vagal ganglia (Hokin, 1965), synaptosomes (Yagihara & Hawthorne, 1972) or by electrical stimulation of brain slices (Pumphrey, 1969), no significant changes have been found. However, in more recent work Birnberger *et al.* (1971) showed an increased turnover of triphosphoinositide in lobster nerves after long incubations and brief electrical stimulation (5min). Schacht & Agranoff (1972*a*) showed a 1.5-fold increase in labelling of diphosphoinositide and triphosphoinositide in goldfish brain after administration of pentylenetetrazol. White *et al.* (1974) reported that stimulation of vagus nerve for 30min increased ^{32}P incorporation into all phospholipids studied, but the increase was significant only for diphosphoinositide and triphosphoinositide. By contrast, Schacht & Agranoff (1972*b*) observed a decreased labelling of polyphosphoinositides with $^{32}P_i$ in guinea-pig brain-cortex subfractions incubated with acetylcholine. White & Larrabee (1973) reported a specific decrease in the labelling of triphosphoinositide in rat vagus nerve after electrical stimulation for 3h.

In previous papers one of us (Abdel-Latif, 1974, 1976; Abdel-Latif *et al.*, 1976) has reported the effects of cholinergic and adrenergic neurotransmitters on ^{32}P incorporation into phospholipids of the rabbit iris, a smooth muscle which is innervated by cholinergic and adrenergic nerve terminals. The phosphatidylinositol effect has also been reported in two other smooth muscles, namely the rat vas deferens (Canessa de Scarnatti & Lapetina, 1974) and the guinea-pig ileum (Jafferji & Michell, 1976). The studies on the iris indicate that acetylcholine and noradrenaline enhance the turnover of phosphatidic acid and phosphatidylinositol; the effects are concentration- and time-dependent and mediated through muscarinic and α-adrenergic receptors. The phosphatidic acid and phosphatidylinositol response to noradrenaline was also found to increase considerably after sympathetic denervation of the iris muscle (Abdel-Latif *et al.*, 1975), which has been shown to lead to complete degeneration of the adrenergic nerve terminals (Roth & Richardson, 1969), thus suggesting the involvement of postsynaptic receptors in this phenomenon.

In studies on the molecular mechanism underlying the phosphatidylinositol effect, we reported a slight decrease in the concentration of phosphatidylinositol and a corresponding increase in that of phosphatidic acid in response to noradrenaline (Abdel-Latif *et al.*, 1976). In further experiments using muscle prelabelled with ^{32}P or *myo*-[^{3}H]-inositol, we found little change in radioactive phosphatidylinositol in response to noradrenaline or acetylcholine (A. A. Abdel-Latif, L. Lakshmanan and J. P. Smith, unpublished work). Further, efforts to demonstrate an increase in diglyceride in response to both of the neurotransmitters, by using the assay method of Banschbach *et al.* (1974*b*) for diglyceride, were unsuccessful (Owen, 1976).

In view of the latter observations and of the findings of others on acetylcholine-stimulated breakdown of phosphatidylinositol in pancreas (Hokin-Neaverson, 1974) and parotid (Jones & Michell, 1974), we decided to re-investigate the effects of acetylcholine and other cholinergic agents on the breakdown of radioactive phospholipids, including polyphosphoinositides, of iris muscle labelled with ^{32}P or *myo*-[^{32}H]inositol *in vitro*. In the present study we used 10mM-2-deoxyglucose, which we have found to deplete effectively the muscle of its ATP, and thus prevent appreciable turnover of phospholipids in the tissue. It is phosphorylated to 2-deoxyglucose 6-phosphate and is not further metabolized (Sols & Crane, 1954). Further, it has been reported to be most efficiently phosphorylated by a pool of ATP relevant to stimulated phospholipid labelling (Schacht & Agranoff, 1974). In the present paper we report that acetylcholine at 0.05mM and at short time-intervals (<10min): (*a*) increases significantly the breakdown of triphosphoinositide (the change in the rate of metabolism of triphosphoinositide in response to acetylcholine will be referred to hereafter as the 'triphosphoinositide effect'; this triphosphoinositide effect is blocked by atropine); (*b*) increases significantly the labelling of phosphatidic acid and phosphatidylinositol. The possible metabolic relationships between the phosphatidylinositol effect and the triphosphoinositide effect observed in the present work are shown in Scheme 1 in the Discussion section.

Part of the present work has already appeared in preliminary form (Abdel-Latif & Akhtar, 1976).

Materials and Methods

Materials

The neurotransmitters acetylcholine and noradrenaline as well as all the cholinergic agonists and antagonists were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. CDP-diglyceride, derived from egg phosphatidylcholine, was purchased from Serdary Research Laboratories, London, Ont., Canada; it was purified by means of silicic acid chromatography (Paulus & Kennedy, 1960). Diphosphoinositide and triphosphoinositide were prepared from bovine brain. A crude diphosphoinositide fraction was prepared by the method of Folch (1949) and diphosphoinositide and triphosphoinositide were isolated by means of DEAE-cellulose column chromatography (Whatman DE52, microgranular) as described by Hendrickson & Ballou (1964). The remaining phospholipids were from sources pre-

viously described (Abdel-Latif & Smith, 1970). sodium [^{32}P]phosphate (129Ci/mg of phosphate) and *myo*-[^{3}H]inositol (3.5Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of reagent grade.

Methods

Preparation and incubation of iris muscle. In contrast with our previous studies (Abdel-Latif *et al.*, 1976), where we used albino rabbits which were obtained from the slaughterhouse, in the present work we used young New Zealand White rabbits (about 4 weeks old) of either sex, weighing approximately 600–900g. They were bred for us at the Joint Animal Breeding Unit, School of Agriculture, Sutton Bonington, Loughborough, Leics., U.K. They were killed by chloroform exposure, the eyes were immediately enucleated, the irises removed, and the pair of irises from each rabbit was placed in 2ml of a modified Bradford (1969) medium. Because of the reported rapid loss of polyphosphoinositides from animal tissues after death (e.g. see Hayashi *et al.*, 1966; Eichberg & Hauser, 1973; Hawthorne & White, 1975), the muscles were incubated immediately after removal from the animals. The incubation medium used in the present studies is based on that of Bradford (1969) and was used by Yagihara *et al.* (1973) in their studies on ^{32}P incorporation into phospholipids of brain synaptosomes. It contained (final concns.) 124mM-NaCl, 5mM-KCl, 1.2mM-KH_2PO_4, 1.3mM-$MgCl_2$, 26mM-Tris/HCl buffer, pH7.4, 0.75mM-$CaCl_2$, 1.6mM-cytidine, 1.6mM-*myo*-inositol and 10mM-D-glucose.

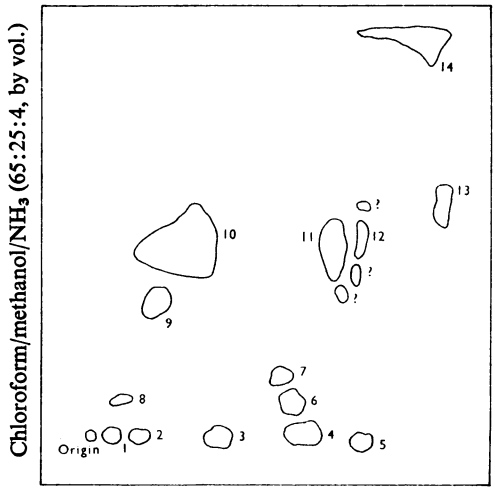
To label the iris phospholipids with $^{32}P_i$ (or *myo*-[^{3}H]inositol) each pair of irises, obtained from the same rabbit, was preincubated for 30min at 37°C in the above incubation medium which contained 25μCi of ^{32}P (or 10–20μCi of *myo*-[^{3}H]inositol) in a final volume of 1ml. After prelabelling, the irises were washed four times with excess of cold non-radioactively labelled medium which contained 10mM of 2-deoxyglucose. In general, for studies on the effects of acetylcholine and other pharmacological agents on the hydrolysis of triphosphoinositide and other phospholipids, the prelabelled irises (of the pair, one was used as control and the other as experimental) were incubated at 37°C for 10min in 1ml of the unlabelled medium, which contained 2-deoxyglucose (10mM) and other agents as indicated.

Determination of ATP in ^{32}P-labelled slices. The nucleotides were extracted from the ^{32}P-labelled slices by homogenizing the tissue twice with 5% (w/v) trichloroacetic acid. The proteins were removed by precipitation. The acid was extracted from the supernatant with diethyl ether, and the aqueous layer was adsorbed on purified activated charcoal; the nucleotides were eluted from the latter by aq. pyridine as described by Burnstock *et al.* (1970). The solutions containing nucleotides were concentrated by freeze-drying and the ATP was separated from the water-soluble materials by high-voltage electrophoresis on Whatman no. 3 paper in pyridine/acetate buffer, pH3.5, at 3kV for 45min. The paper was dried, and the spot, which corresponds to ATP, was marked, cut out and counted for radioactivity in a liquid-scintillation counter (Abdel-Latif & Smith, 1972).

Extraction and isolation of phospholipids. For extraction of phopholipids (inclusive of higher inositides) the procedure of Yagihara *et al.* (1973) was essentially used. The muscle was washed twice with 5% (w/v) trichloroacetic acid and once with water. To each muscle 3ml of chloroform/methanol/HCl (200:200:1, by vol.) was added, and the lipids were extracted by homogenizing in a glass homogenizer. The residue from the first extraction was extracted twice with 1ml of chloroform/methanol/HCl (400:200:1.5, by vol.). All the extracts were combined and evaporated under N_2. Chloroform (1ml) was added to each tube and the extract was washed first with 1ml of 0.1M-HCl, and then three times with its 'synthetic upper phase'. After discarding the upper phase, the chloroform layer was evaporated to dryness under a stream of N_2 and the phospholipids were dissolved in 0.1ml of chloroform, and separated into the individual phospholipids by means of two-dimensional t.l.c. with silica-gel H. In the present work the silica-gel slurry was prepared by mixing 40g of silica-gel H with 3g of magnesium acetate in 95ml of water. Five glass plates (20×20cm) were coated with this slurry to a depth of 0.3mm. The plates were air-dried and activated for 1h at 110°C just before use. The solvent systems used were the same as previously described (Abdel-Latif *et al.*, 1974). Since in our previous work with the iris muscle we extracted the lipids with neutral solvents (Abdel-Latif, 1974) we did not observe the polyphosphoinositides. In the present work we extracted the lipids with acidified solvents, and, as can be seen from Fig. 1, this method separated all the phospholipids including the polyphosphoinositides. Identification of diphosphoinositide and triphosphoinositide was based also on standard samples developed under the same conditions. Further, standard ^{32}P-labelled diphosphoinositide and triphosphoinositide were prepared from rabbit brain slices and isolated as described by Gonzalez-Sastre *et al.* (1971). In the present studies both phospholipids were also labelled with *myo*-[^{3}H]inositol.

Broekhuyse (1968) reported that, when iris phospholipids were extracted under neutral conditions, lysophospholipids were present in very low concentrations. He also observed five unidentified phospholipids in his t.l.c. which were present in small amounts. In the present study phospho-

lipids were extracted under acidic conditions. Whereas lysophosphatidylcholine was found in very low concentration, lysophosphatidylethanolamine was present in relatively higher concentrations (Fig. 1). Broekhuyse (1968) reported a high percentage of ethanolamine plasmalogen in the calf iris muscle. Since the formation of lysophospholipids from the corresponding plasmalogens is known to proceed rapidly under acidic conditions (Broekhuyse, 1968), it is possible that lysophosphatidylethanolamine was formed from ethanolamine plasmalogen during extraction of the phospholipids. The unidentified lipids were found to contain negligible amounts of ^{32}P radioactivity. Lipids were detected by means of I_2 vapour, and the phospholipid spots were scraped from the plates and counted for radioactivity in a liquid-scintillation spectrometer with 10ml of a scintillation fluid [6g of 2,5-diphenyloxazole and 120mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of xylene]. For determination of the radioactivity and phosphate content of the phospholipids, the spots were scraped off the plate and digested in 0.7ml of 72% (w/v) $HClO_4$. After cooling and the addition of 4ml of water, the sample was centrifuged and the clear supernatant was analysed for phosphate (Bartlett, 1959) and radioactivity. For determination of the latter, the scintillation fluid was mixed with one-half of its volume (the weights of the scintillators were adjusted accordingly) of Triton X-100 and 10ml of the mixture was added to a portion of the aqueous sample (0.5ml in the present study).



Butan-1-ol/acetic acid/water (6:1:1, by vol.)

Fig. 1. *Separation of iris muscle phospholipids by means of two-dimensional t.l.c.*

Numbers in the Figure refer to the following compounds: 1, triphosphoinositide; 2, diphosphoinositide; 3, CDP-diglyceride; 4, phosphatidylserine; 5, phosphatidic acid; 6, phosphatidylinositol; 7, lysophosphatidylethanolamine; 8, lysophosphatidylcholine; 9, sphingomyelin; 10, phosphatidylcholine; 11, phosphatidylethanolamine; 12, cardiolipin; 13, unidentified; 14, cholesterol and neutral lipids.

Assay for triphosphoinositide phosphomonoesterase in the soluble and particulate fractions of the iris muscle. To prepare the soluble and particulate fractions from the iris muscle, the following procedure was followed (Abdel-Latif & Smith, 1976). The irises were placed in cold 0.25M-sucrose, adjusted to pH7.4 with Tris/HCl buffer, cut into small pieces with fine scissors, then homogenized thoroughly either in a glass homogenizer (by hand) or in a Tissuemizer (type X1020; 10 T shaft; International Laboratory Apparatus G.m.b.H., 7801 Dottingen, Germany) at 10000rev./min for 40s. The homogenate was quickly passed through two layers of cheesecloth under mild suction to remove most of the connective tissue and debris. The filtrate was then centrifuged at 120000*g* for 90min to give a sediment (the particulate fraction) and a clear supernatant (the soluble fraction). The method used for assay of triphosphoinositide phosphomonoesterase was a modification of the method described by Sheltawy *et al.* (1972). To assay for the enzyme, 0.75μmol of triphosphoinositide (sodium salt) was dispersed in 0.2ml of water. Then the following constituents were added: 1.5mM-$MgCl_2$, 0.75mM-$CaCl_2$, 100mM-KCl, 1mM-NaF, 42mM-Tris/HCl buffer, pH7.4, reduced glutathione (3mM), pH7.4, and the enzyme was added as indicated, in a final volume of 0.75ml. Incubation was carried out for 30min at 37°C. Appropriate controls accompanied each determination. The reaction was terminated by adding 0.7ml of $HClO_4$, and the liberated P_i was determined in the supernatant as described by Bartlett (1959). Protein was determined as described by Lowry *et al.* (1951), with crystalline bovine serum albumin as a standard.

Results

Effect of 2-deoxyglucose on the amount of radioactivity in ATP and on the phospholipids of iris muscle which were labelled with [^{32}P]phosphate

To measure the effectiveness of 2-deoxyglucose in depleting the muscle of its ATP and thus inhibiting further ^{32}P incorporation into phospholipids, the ^{32}P-labelled slices were incubated for 10min at 37°C in the presence and absence of 10mM-deoxyglucose. As can be seen from Table 1, in the presence of deoxyglucose, the ATP radioactivity in the tissue decreased by 71% compared with that of the control, and that of triphosphoinositide by 30%. This

Table 1. *Effect of 2-deoxy-D-glucose on the amount of radioactivity in ATP and phospholipids of rabbit iris muscle which were labelled with [^{32}P]phosphate*

To label the iris phospholipids with $^{32}P_i$, each pair of irises, obtained from the same rabbit, was preincubated for 30min at 37°C in a medium based on that of Bradford (1969) and containing 25μCi of $^{32}P_i$ in a final volume of 1ml. One of the pair was washed four times with excess of unlabelled cold medium and incubated for 10min in the same medium. The other was washed in Bradford medium that contained 10mM-2-deoxyglucose and incubated for 10min in the same medium. The phospholipids and ATP were isolated, and their radioactive contents determined as described in the Materials and Methods section. The results are the means of two different experiments.

Additions	^{32}P radioactivity (c.p.m.) in ATP	Phosphatidic acid	Phosphatidylinositol	Triphosphoinositide
None	11830	3806	8949	31180
10mM-2-Deoxyglucose	3410	3439	9394	21890

Table 2. *Effects of acetylcholine on the breakdown of triphosphoinositide and other phospholipids of rabbit iris muscle prelabelled with [^{32}P]phosphate in vitro*

To prelabel the iris phospholipids with $^{32}P_i$, each pair of irises obtained from the same rabbit was preincubated for 30min at 37°C in a medium based on that of Bradford (1969) and containing 25μCi of $^{32}P_i$ in a final volume of 1ml. After prelabelling the irises, they were washed four times with excess of unlabelled cold medium that contained 10mM-2-deoxyglucose. For studies on the effect of acetycholine+eserine (0.05mM each) the prelabelled irises (of the pair, one was used as control and the other as experimental) were incubated at 37°C for 10min in 1ml of unlabelled medium that contained 10mM-2-deoxyglucose. The reaction was terminated by adding 1ml of 10% (w/v) trichloroacetic acid. Extraction of phospholipids inclusive of higher inositides, their separation by means of two-dimensional t.l.c. and determination of their radioactive contents are described in the Materials and Methods section. The results reported here are the means±S.E.M. of four different experiments run in duplicate.

Additions	^{32}P radioactivity (c.p.m.) in Phosphatidic acid	Phosphatidyl-inositol	Diphospho-inositide	Triphospho-inositide	Phosphatidyl-choline
None (zero time)	5324±373	9549±630	5026±186	23538±953	3246±292
None (control)	2501±175*	9818±635	3076±108*	16160±640*	3272±287
Acetylcholine+eserine (0.05mM each)	3600±252† (144)‡	12380±720† (126)	3233±113 (105)	11800±250† (73)	3341±311 (102)

* Significant difference from zero time ($P<0.001$).
† Significant difference from control ($P<0.001$).
‡ Values expressed as % of control.

suggests that the availability of ATP for phosphorylation of lipids is effectively decreased when deoxyglucose is added to the incubation medium.

Breakdown, in response to acetylcholine, of radioactive triphosphoinositide and other phospholipids of muscle prelabelled in vitro with [^{32}P]phosphate

When irises which had been labelled with ^{32}P *in vitro* (Table 2, zero time) were incubated for 10min in unlabelled medium that contained 10mM-2-deoxyglucose, there was a significant loss of radioactivity from phosphatidic acid, diphosphoinositide and triphosphoinositide, but not from phosphatidylinositol or phosphatidylcholine (Table 2, control). The loss of radioactivity from triphosphoinositide was more than 50% higher than the combined loss from phosphatidic acid and diphosphoinositide. Further, addition of acetylcholine plus eserine (0.05mM each) to the incubation medium enhanced the loss of radioactivity from triphosphoinositide by 27% after 10min of incubation (Table 2). Simultaneously the radioactivity of phosphatidic acid and phosphatidylinositol was increased by 44 and 26% respectively. No significant changes were observed in the labelling of phosphatidylserine, phosphatidylethanolamine, sphingomyelin, cardiolipin or CDP-diglyceride.

Time-course of breakdown of radioactive triphosphoinositide and other phospholipids in the absence of acetylcholine

Rapid turnover of polyphosphoinositides has been demonstrated in a variety of tissues both *in vivo* and *in vitro*. Garrett *et al.* (1976) observed that the poly-

phosphoinositides in rabbit erythrocyte membranes can break down with a half-life of approx. 1 min. Since most of the studies on the phospholipid effect have been reported from experiments in which the incubations were carried out for long time-intervals (30 min–2 h), it is possible that the rapid breakdown of triphosphoinositide could have prevented previous workers from observing consistent changes in the labelling of this phospholipid in response to acetylcholine. In the present studies, when the irises which had been prelabelled with ^{32}P were incubated for longer time-intervals, there was a loss of radioactivity from triphosphoinositide and phosphatidic acid and an increase in that of phosphatidylinositol with time (Fig. 2). In general we observed an increase in phosphatidylcholine labelling. After 30 min of incubation, the losses of radioactivity (based on zero-time labelling) from phosphatidic acid and triphosphoinositide were 84 and 60% respectively. By contrast, the labelling in phosphatidylinositol increased by 20%.

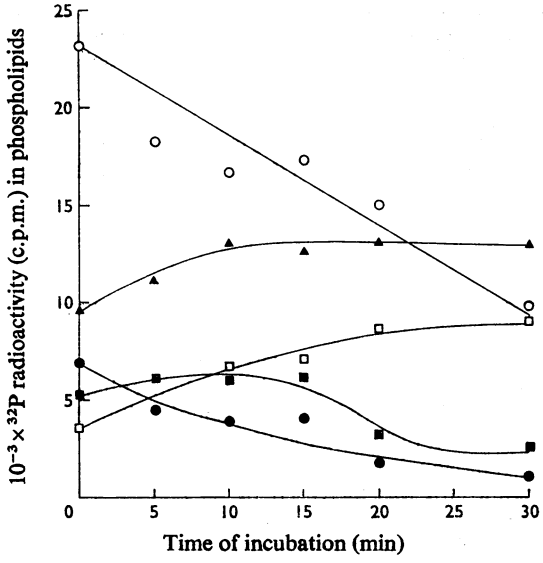


Fig. 2. *Time-course, in the absence of acetylcholine, of loss of radioactivity from triphosphoinositide and other phospholipids of rabbit iris muscle labelled with [^{32}P]phosphate*
Conditions for incubation of ^{32}P-prelabelled irises in an unlabelled medium for various time-intervals were the same as described under Table 2. Each point represents the mean of two different experiments. ●, Phosphatidic acid; ▲, phosphatidylinositol; ■, diphosphoinositide; ○, triphosphoinositide; □, phosphatidylcholine.

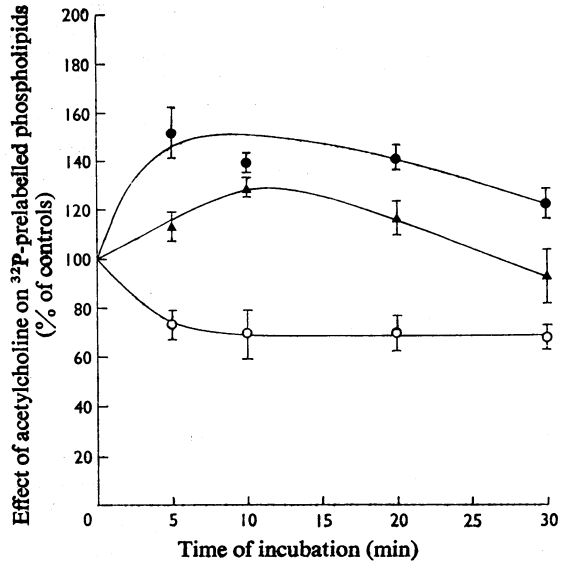


Fig. 3. *Effect of acetylcholine on the breakdown of triphosphoinositide and other phospholipids of rabbit iris muscle prelabelled with [^{32}P]phosphate with time*
Conditions for incubation of ^{32}P-prelabelled irises in an unlabelled medium in the presence and absence of acetylcholine plus eserine (0.05 mM each) for various time-intervals were the same as described for Table 2. The effects of acetylcholine are expressed as a percentage of control value and are means±S.E.M. for three different experiments. Each experiment was run in duplicate. ●, Phosphatidic acid; ▲, phosphatidylinositol; ○, triphosphoinositide.

Time-course of breakdown of radioactive triphosphoinositide and other phospholipids in response to acetylcholine

To investigate the optimum conditions for the triphosphoinositide effect, the loss of radioactivity from triphosphoinositide in response to acetylcholine was investigated at various time-intervals (Fig. 3), and at different concentrations of acetylcholine (Fig. 4, below). Fig. 3 shows that the triphosphoinositide effect increased with time and reached a maximum value (about 25% hydrolysis) after 10 min of incubation. At the same time the labelling in phosphatidic acid and phosphatidylinositol increased by almost 40 and 30% respectively. However, the stimulation of phosphatidylinositol labelling decreased after 20 min of incubation, and after 30 min there was a slight net breakdown of phosphatidylinositol in response to the neurotransmitter. This effect on the breakdown of phosphatidylinositol at longer time-intervals (30 min) could be due to (*a*) depletion with time of precursor

pools with high specific radioactivity; (*b*) decrease in conversion of triphosphoinositide into phosphatidylinositol.

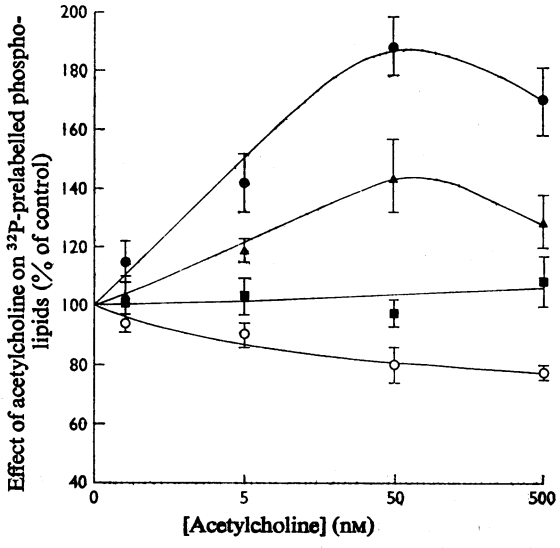


Fig. 4. *Effects of different concentrations of acetylcholine on the breakdown of triphosphoinositide and other phospholipids of muscle prelabelled with [^{32}P]phosphate*
Conditions for incubation of ^{32}P-prelabelled irises in an unlabelled medium in the presence and absence of different concentrations of acetylcholine plus eserine (0.05mM each) for 10min were the same as described for Table 2. The effects of acetylcholine are presented as a percentage of the control value, and are means ±S.E.M. for three different experiments. Each experiment was run in duplicate. ●, Phosphatidic acid; ▲, phosphatidylinositol; ■, diphosphoinositide; ○, triphosphoinositide.

Effect of different concentrations of acetylcholine on the triphosphoinositide effect

An increase in the triphosphoinositide effect can be seen at all concentrations of acetylcholine (Fig. 4). Also, there was a corresponding increase in the labelling of phosphatidic acid and phosphatidylinositol. The optimum concentration for this stimulation is about 50nm. Diphosphoinositide appears to behave as a transient intermediate in this tissue.

Effects of muscarinic and nicotinic antagonists on the triphosphoinositide effect

To show whether the receptor which triggered this triphosphoinositide effect is of muscarinic or nicotinic type, we investigated the effects of (*a*) atropine, which blocks muscarinic receptors and the acetylcholine stimulation of phosphatidic acid and phosphatidylinositol labelling in most tissues investigated (Michell, 1975), and (*b*) D-tubocurarine, which blocks the action of acetylcholine at nicotinic receptors, on the breakdown of triphosphoinositide and other phospholipids. As can be seen from Table 3, atropine, but not D-tubocurarine, blocked the acetylcholine-stimulated loss of label from triphosphoinositide and the increase in phosphatidic acid and phosphatidylinositol labelling. This suggests that both the triphosphoinositide and phosphatidylinositol effects are mediated by muscarinic receptors.

Effects of muscarinic and nicotinic antagonists on ^{32}P incorporation into triphosphoinositide and other phospholipids

In previous studies on the iris muscle we have shown that atropine inhibited the acetylcholine-stimulated ^{32}P incorporation into phosphatidic acid and phosphatidylinositol (Abdel-Latif, 1974; Abdel-

Table 3. *Effects of atropine and D-tubocurarine on acetylcholine-stimulated breakdown of triphosphoinositide and other phospholipids of rabbit iris muscle prelabelled with [^{32}P]phosphate*
Conditions of incubation were the same as described for Table 1, except that the ^{32}P-prelabelled muscle was first incubated in the presence or absence of the antagonist for 5min, then acetylcholine and eserine were added as indicated and the incubation was continued for another 10min. The results reported here are means (±S.E.M.) for four different experiments run in duplicate.

Additions	Concentration (mM)	Effects of atropine and D-tubocurarine on acetylcholine-stimulated breakdown of triphosphoinositide and other phospholipids (% of control)				
		Phosphatidic acid	Phosphatidylinositol	Diphosphoinositide	Triphosphoinositide	Phosphatidylcholine
Acetylcholine	0.05	135±7	138±7	102±4	72±2	99±9
Acetylcholine+atropine	0.05 and 0.1 respectively	104±3*	99±5*	121±11*	119±6*	108±8†
Acetylcholine+D-tubocurarine	0.05 and 0.1 respectively	152±10†	143±7†	95±5†	77±4†	93±4†

* Significant difference from the control, which contained acetylcholine ($P<0.005$).
† No significant difference from that of the control, which contained acetylcholine.

Table 4. *Effects of atropine and* D-*tubocurarine on acetylcholine-stimulated* ^{32}P *incorporation into triphosphoinositide and other phospholipids of rabbit iris muscle*

In this experiment the irises were first incubated for 5 min in ^{32}P-containing medium in the presence or absence of the blockers as shown in Table 3. Acetylcholine plus serine (0.05 mM each) were then added, and the incubation was continued at 37°C for another 30 min. The reaction was stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid. Extraction and analysis of the lipids were carried out as described in the Materials and Methods section. The results are the mean of two experiments.

Additions	Concentration (mM)	^{32}P radioactivity (c.p.m.) in Phosphatidic acid	Phosphatidylinositol	Diphosphoinositide	Triphosphoinositide	Phosphatidylcholine
Acetylcholine	0.05	5514*; 9797† (178)‡	16310; 20470 (125)	5882; 5383 (92)	22390; 16180 (72)	8209; 8271 (101)
Acetylcholine+ atropine	0.05 and 0.1 respectively	4867; 5595 (115)	15830; 16630 (105)	5875; 6022 (103)	15920; 16620 (104)	8661; 9410 (109)
Acetylcholine+ D-tubocurarine	0.05 and 0.1 respectively	4691; 8961 (191)	14090; 16830 (119)	5319; 4832 (91)	17380; 13400 (77)	8835; 8413 (95)

* Control.
† Plus acetylcholine (or acetylcholine plus antagonist).
‡ Values expressed as % of control.

Table 5. *Effects of muscarinic and nicotinic agonists on breakdown of triphosphoinositide and other phospholipids of rabbit iris muscle prelabelled with* [^{32}P]*phosphate*

Conditions of incubation were the same as described for Table 1, except that the muscarinic or nicotinic agonists (0.1 mM) were added instead of acetylcholine. The results reported here are the means for two different experiments.

Additions	Type of agonist	Effect of agonists on breakdown of triphosphoinositide and other phospholipids of iris muscle prelabelled with [^{32}P]phosphate (% of control) Phosphatidic acid	Phosphatidylinositol	Diphosphoinositide	Triphosphoinositide	Phosphatidylcholine
Carbamoylcholine	Muscarinic	170	113	109	89	108
Philocarpine	Muscarinic	130	119	112	89	97
Choline	Muscarinic	108	104	96	93	107
Lobeline	Nicotinic	105	101	108	107	112

Latif *et al.*, 1976). In the present studies this drug was also found to block the triphosphoinositide effect, in addition to blocking the phosphatidylinositol effect (Table 4). The decrease in the triphosphoinositide labelling observed in the presence of the neurotransmitter is presumably caused by an increase in its breakdown. By contrast, D-tubocurarine exerted no effect. This again suggests that the triphosphoinositide effect is mediated by muscarinic receptors.

Effects of muscarinic and nicotinic agonists on the triphosphoinositide effect

Carbamoylcholine and pilocarpine are muscarinic-receptor agonists. Both drugs provoked an increase in the breakdown of triphosphoinositide and a simultaneous increase in the labelling of phosphatidic acid and phosphatidylinositol (Table 5). The base choline exerted a slight effect on the former, but had no effect on the labelling of the latter lipids. By contrast, lobeline, a nicotinic agonist (Carrier, 1972) had no effect.

Effect of acetylcholine on breakdown of phosphoinositides of muscle prelabelled with myo-[^3H]*inositol*

In further experiments designed to throw more light on the molecular mechanism underlying the triphosphoinositide effect, two types of experiments were carried out: (*a*) the muscle was prelabelled with *myo*-[^{3}H]inositol and the effect of acetylcholine on the breakdown of the phosphoinositides was investigated (Table 6). (*b*) The muscle was prelabelled with $^{32}P_i$, and loss of radioactivity and phosphate from the phosphoinositides was investigated (Table 7).

From the average of 11 observations, phosphatidylinositol, diphosphoinositide and triphospho-

Table 6. *Effects of acetylcholine on breakdown of triphosphoinositide and other phospholipids of muscle prelabelled with myo-[^{3}H]inositol in vitro*

Conditions of incubation were the same as described for Table 1, except that [^{32}P]phosphate was replaced by *myo*-[^{3}H]inositol (10–20μCi/ml). Results are means for two to four determinations.

Expt.	Incubation time (min)	No. of observations	^{3}H radioactivity (c.p.m.) in phospholipids in the presence and absence of acetylcholine		
			Phosphatidylinositol	Diphosphoinositide	Triphosphoinositide
1	10	3	4460*; 4346† (97)‡	391; 420 (108)	1144; 867 (76)
2	0	4	6852	233	2729
	30	2	8197; 7850 (96)	325; 319 (98)	3064; 2656 (87)
	60	2	7351; 7177 (98)	271; 223 (82)	2571; 2088 (81)

* Control.
† Acetylcholine plus eserine (0.05mM each) added.
‡ Values expressed as % of control.

Table 7. *Loss of radioactivity and phosphate from triphosphoinositide and other phospholipids in response to acetylcholine*

Conditions of incubation were exactly as described for Table 1. In this experiment 16 rabbits were used. Each pair of irises was prelabelled separately. One of the pair was used as control and the other as experimental (acetylcholine plus eserine, 0.05mM each). After incubation for 10min at 37°C, each of the 32 incubations was processed separately. For determination of phosphates in triphosphoinositide, phosphatidylinositol and phosphatidic acid the corresponding spots from eight incubations were pooled to give us two control and two experimental values. For determination of phosphatidylcholine and phosphatidylethanolamine, the spots from four incubations were pooled to give us four control and four experimental values. For determination of radioactivity, a portion of the $HClO_4$-digested material was taken for radioactivity counting and the rest was used for phosphate determination. The values given are the means from two to four determinations. Results are means of two determinations.

Phospholipid	Radioactivity (% of that in total lipids)			Phosphate content (% of total lipid phosphorus)		
	Control	Acetylcholine plus eserine	Change (%)	Control	Acetylcholine plus eserine	Change (%)
Triphosphoinositide	39.4*	29.9	−24	4.1	2.8	−32.0
Phosphatidylinositol	32.5*	42.5	+30.8	7.4	6.8	−8.1
Phosphatidic acid	5.1*	6.8	+33	1.8	1.9	+5.5
Phosphatidylcholine	7.8†	8.3	+6.4	45.2	46.3	+2.4
Phosphatidylethanolamine	1.6†	1.6	0	22.0	23	+4.5

* Average of two determinations, and each was from eight incubations.
† Average of four determinations and each was from four incubations.

inositide were found to contain 72.5, 3.5 and 24% of the total ^{3}H radioactivity respectively (Table 6). When muscle was prelabelled with *myo*-[^{3}H]inositol, addition of acetylcholine increased the breakdown of triphosphoinositide and phosphatidylinositol by 24 and 3% respectively after 10min of incubation and by 19 and 2% respectively after 1 h of incubation. There was a slight increase in the labelling of phosphatidylinositol and a slight decrease in that of triphosphoinositide after 1 h of incubation (based on the zero-time values).

Loss of radioactivity and phosphate from phosphoinositides in response to acetylcholine

When irises prelabelled with ^{32}P were exposed to acetylcholine there was a 24% loss of radioactivity from triphosphoinositide and a simultaneous increase, of about 30%, in that of phosphatidic acid and phosphatidylinositol (Table 7). Moreover, when the tissue phospholipids were measured chemically, there was a marked decrease (about 32%) in the tissue triphosphoinositide concen-

Table 8. *Distribution of triphosphoinositide phosphomonoesterase activity in soluble and particulate fractions of the rabbit iris muscle*

Preparation of the soluble and particulate fractions and assay of the enzyme were as described in the Materials and Methods section. The results reported here are the means of two different experiments.

			Triphosphoinositide phosphomonoesterase activity				
			(nmol of P_i liberated from triphosphoinositide/h per g of tissue)	(nmol of P_i liberated from triphosphoinositide/h per mg of protein)		Distribution of the enzyme (%)	
Age	No. of rabbits	Fresh weight of muscle (g)		Particulate	Soluble	Particulate	Soluble
4 weeks	5	0.39	4970	300	75	82	18
Adult	3	0.56	5000	400	69	84	16

tration and an 8% decrease in phosphatidylinositol concentration.

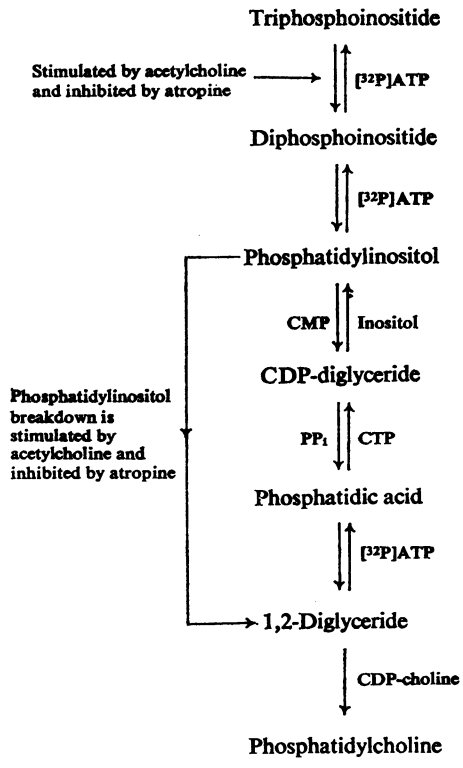
Distribution of triphosphoinositide phosphomonoesterase in the soluble and particulate fractions of the iris muscle

Since the enzyme triphosphoinositide phosphomonoesterase, which is responsible for the breakdown of triphosphoinositide into diphosphoinositide and subsequently phosphatidylinositol (Dawson & Thompson, 1964; Thompson & Dawson, 1964*a*) has not previously been reported for smooth muscle, its activity and subcellular distribution in the iris were studied. In both young rabbits, which we used in the present work, and adult rabbits, the reaction rate (nmol of P_i liberated/h per g fresh muscle) was found to be about 5000, and about 84% of this activity was found to be localized in the particulate fraction (Table 8). Thus the activity of this enzyme is considerably lower than that of the brain tissue, and in contrast with brain, where most of the activity is localized in the soluble fraction (Salway *et al.*, 1967), in muscle it is mostly particulate. Sheltawy *et al.* (1972), working with guinea-pig brain, also concluded that this enzyme might be localized in the cell-surface membrane.

Discussion

Several years ago, Thompson & Dawson (1964*a*,*b*) and Dawson & Thompson (1964) described the partial purification of brain phosphatases that act on the polyphosphoinositides. One of these enzymes was a phosphomonoesterase (Dawson & Thompson, 1964) which dephosphorylated triphosphoinositide to diphosphoinositide. These authors concluded that one of the pathways for triphosphoinositide catabolism involves the reaction triphosphoinositide→diphosphoinositide→monophosphoinositide. Earlier, Brockerhoff & Ballou (1961, 1962*a*,*b*) suggested from their studies on brain slices that the general metabolism of the polyphosphoinositides follows a reversible sequence, monophosphoinositide⇌diphosphoinositide⇌triphosphoinositide. The present data on the iris smooth muscle are in accord with this conclusion, and the metabolic pathways that may be affected by acetylcholine are shown in Scheme 1.

In the present study we have demonstrated that acetylcholine, at concentrations ~10nM and for short time-intervals (<10min), significantly stimulates the breakdown of triphosphoinositide (Figs. 3 and 4). This breakdown in response to the neurotransmitter has been measured both chemically and radiochemically (Tables 2 and 7) in prelabelling experiments with ^{32}P or *myo*-[^{3}H]inositol and labelling experiments with ^{32}P (Table 4). The finding that the data from the radiochemical and chemical determinations of phosphatidylinositol are not equivalent suggests the existence of more than one pool responsive to acetylcholine stimulation. In the present work no attempt was made to determine the distribution of label among the three phosphates of triphosphoinositide. In our previous studies with the iris muscle (Abdel-Latif *et al.*, 1976) we reported a slight decrease in phosphatidylinositol concentration and a corresponding increase in that of phosphatidic acid in response to noradrenaline. Jafferji & Michell (1976), working with the smooth muscle of guinea-pig ileum, also found a 13% decrease in phosphatidylinositol concentration in response to 0.1mM-carbamoylcholine. However, these authors observed that, in a number of experiments where the phosphatidylinositol concentration of control and carbamoylcholine-treated tissue were compared, there appeared to be a slight but often statistically insignificant decrease caused by stimulation. Our present findings are in accord with their observations. It is possible that smooth muscle possesses a greater ability than other tissues, e.g. pancreas or parotid, to resynthesize the phosphatidylinositol lost in response to cholinergic stimulation. By contrast, the resynthesis of triphosphoinositide from ATP and phosphatidylinositol is decreased appreciably in the presence of 2-deoxyglucose



Scheme 1. *Inter-relationships between phosphoinositides and other phospholipids in the iris muscle, and the metabolic pathways that may be affected by acetylcholine*

(Table 1). In view of this latter finding and the fact that the hydrolysis of triphosphoinositide proceeds at a much faster rate than that of its formation (Kai & Hawthorne, 1969), it can be concluded that the resynthesis of triphosphoinositide from ATP and diphosphoinositide is limited under our conditions of incubation. Atropine, a muscarinic-receptor blocker, inhibited the acetylcholine-stimulated breakdown of triphosphoinositide (Tables 3 and 4). By contrast, D-tubocurarine, a nicotinic-receptor blocker, had no effect. Further, only muscarinic agonists, but not nicotinic agonists, were found to induce an acetylcholine-stimulated breakdown of triphosphoinositide (Table 5). This suggests that the triphosphoinositide effect is mediated through muscarinic receptors. So far all tissues which have exhibited a phospholipid effect have been found to be of the muscarinic type (Michell, 1975), except the electric organs of the *Torpedo* (Rosenberg, 1973; Bleasdale *et al.*, 1976), which are considered to have receptors of the nicotinic type. De Robertis (1971) envisages phosphatidylinositol as a part of the nicotinic cholinergic receptor. Torda (1973, 1974) proposed a model of a depolarization–hyperpolarization cycle in which she claims to have identified one of the postsynaptic nicotinic acetylcholine receptors as the regulatory subunit of triphosphoinositide phosphomonoesterase.

In addition to demonstrating a triphosphoinositide effect in the iris smooth muscle, the data presented reveal that this effect is accompanied by a significant increase in the labelling of phosphatidic acid and phosphatidylinositol (Tables 2–7 and Figs. 3 and 4). Although the loss of radioactivity from triphosphoinositide is about 20% higher than the combined gain in the labelling of phosphatidic acid plus phosphatidylinositol, only part of this gain can be derived from the radioactive polyphosphoinositide (Table 2). This conclusion is based on the fact that the diester phosphate constitutes only one-third of the total triphosphoinositide phosphorus and may have a lower specific radioactivity than the monoester phosphates. The question arises as to how this triphosphoinositide effect is related to the acetylcholine stimulation of phosphatidic acid and phosphatidylinositol labelling. A number of metabolic pathways can contribute to this increase (Scheme 1), including the following. (*a*) From triphosphoinositide breakdown. An enhance-

ment in triphosphoinositide catabolism can lead to an increase in phosphatidylinositol labelling and subsequently into phosphatidic acid and phosphatidylcholine (Scheme 1). In our preliminary communication (Abdel-Latif & Akhtar, 1976) we postulated the breakdown of phosphatidylinositol into phosphatidic acid and free inositol. More recently Hokin-Neaverson *et al.* (1975) showed the products of the acetylcholine-stimulated breakdown of phosphatidylinositol in mouse pancreas to be phosphatidic acid and free inositol. Jungalwala *et al.* (1971) reported that all the enzymes required for phosphatidylinositol synthesis in pig thyroid are microsomal, and demonstrated the incorporation of [^{3}H]inositol into phosphatidylinositol in the presence of CDP-diglyceride or CTP+ATP. Incubation of a microsomal fraction, prelabelled with ^{32}P- and [^{3}H]-inositol, with unlabelled inositol in the presence of nucleotide coenzymes resulted in loss of ^{3}H, but not ^{32}P, from phosphatidylinositol. Such a phospholipase D activity, which would leave membrane-bound phosphatidic acid for recycling, has not been demonstrated in mammalian tissues, and the authors suggested that the appearance of [^{3}H]inositol might be due to a reversal of the CDP-diglyceride–inositol phosphoryltransferase activity. The increase in the amount of diglyceride [which appears to be a transient intermediate in this tissue (Owen, 1976)] resulting from phosphatidic acid and/or phosphatidylinositol hydrolysis could, in the presence of ^{32}P-labelled CDP-choline, stimulate phosphatidylcholine synthesis (Table 7). (*b*) Through the re-incorporation of ^{32}P (liberated as a result of the triphosphoinositide effect) into phosphatidic acid via diglyceride kinase and subsequently into phosphatidylinositol. (*c*) From resynthesis of phosphatidylinositol through the CDP-diglyceride pathway (Scheme 1). Since acetylcholine also stimulates the breakdown of phosphatidylinositol (Scheme 1), this synthetic pathway could operate to replenish the cell's lost phosphatidylinositol. In spite of our thorough washing procedure, the presence of high-specific-radioactivity [^{32}P]CDP-diglyceride and *myo*-[^{3}H]inositol precursors could indeed contribute to the observed increase in phosphatidic acid and phosphatidylinositol labelling. The above-mentioned possibilities (*a–c*) could also be responsible for the small changes in the phosphatidylinositol concentrations recorded in response to acetylcholine in the present work (Table 7) and those of Jafferji & Michell (1976).

Although the triphosphoinositide effect can be related to the phosphatidylinositol effect metabolically (Scheme 1), the functional inter-relationship between the two phenomena remains to be determined. In the present study we have shown the triphosphoinositide phosphomonoesterase to be localized in the particulate fraction. Sheltawy *et al.* (1972), working with guinea-pig brain, developed an assay system for this enzyme which we used in the present work, found it to possess a distribution similar to that of 5′-nucleotidase and concluded that it might be localized, along with its substrate, in the cell surface membrane. Michell (1975) has suggested that the majority of the phosphoinositides could be localized at the inner face of the plasma membrane. The present demonstration of a triphosphoinositide effect, presumably located in the vicinity of the muscarinic receptor, would tend to support these observations.

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