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 30 min 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 10 min in unlabelled medium containing 10 mm-2-deoxyglucose and the other was incubated in the presence of acetylcholine plus eserine (0.05 mm 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 (0.05 mm) 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, 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 phosphatic 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 phosphatic 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; Larabee 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., 1974a) 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 diphasphoinositiode and triphosphoinositiode by the addition of acetylcholline 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 triphosphoinositiode in lobster nerves after long incubations and brief electrical stimulation (5 min). Schacht & Agranoff (1972a) showed a 1.5-fold increase in labelling of diphasphoinositiode and triphosphoinositiode in goldfish brain after administration of pentylenetetrazol. White et al. (1974) reported that stimulation of vagus nerve for 30 min increased \(^{32}\)P incorporation into all phospholipids studied, but the increase was significant only for diphasphoinositiode and triphosphoinositiode. By contrast, Schacht & Agranoff (1972b) observed a decreased labelling of polyphosphoinositiodes with \(^{32}\)P, in guinea-pig brain-cortex subfractions incubated with acetylcholine. White & Larrabee (1973) reported a specific decrease in the labelling of triphosphoinositiode in rat vagus nerve after electrical stimulation for 3 h.

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 phosphatidylinositiode 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 phosphatidylinositiode; the effects are concentration- and time-dependent and mediated through muscarinic and \(\alpha\)-adrenergic receptors. The phosphatidic acid and phosphatidylinositiode 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 phosphatidylinositiode effect, we reported a slight decrease in the concentration of phosphatidylinositiode 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-[\(\text{H}\)]inositiode, we found little change in radioactive phosphatidylinositiode 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) for diglyceride, were unsuccessful (Owen, 1976).

In view of the latter observations and of the findings of others on acetylcholine-stimulated breakdown of phosphatidylinositiode 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 polyphosphoinositiodes, of iris muscle labelled with \(^{32}\)P or myo-[\(\text{H}\)]inositiode \textit{in vitro}. In the present study we used 10 mm-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 0.05 mm and at short time-intervals (<10 min): (a) increases significantly the breakdown of triphosphoinositiode (the change in the rate of metabolism of triphosphoinositiode in response to acetylcholine will be referred to hereafter as the 'tripholipidinositiode effect'; this triphosphoinositiode effect is blocked by atropine); (b) increases significantly the labelling of phosphatic acid and phosphatidylinositiode. The possible metabolic relationships between the phosphatidylinositiode effect and the triphosphoinositiode 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 W.6, U.K. CDP-diglyceride, derived from egg phosphatidylcholine, was purchased from Serydary Research Laboratories, London, Ont., Canada; it was purified by means of silicic acid chromatography (Paulus & Kennedy, 1960). Diphasphoinositiode and triphephosphoinositiode were prepared from bovine brain. A crude diphasphoinositiode fraction was prepared by the method of Folch (1949) and diphasphoinositiode and triphosphoinositiode 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-

1977
viously described (Abdel-Latif & Smith, 1970). Sodium [32P]phosphate (129Ci/mg of phosphate) and myo-[3H]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 600-900 g. 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 encased, the irises removed, and the pair of irises from each rabbit was placed in 2 ml 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 animal. 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 32P incorporation into phospholipids of brain synaptosomes. It contained (final concns.) 124 mM-NaCl, 5 mM-KCl, 1.2 mM-KH2PO4, 1.3 mM-MgCl2, 26 mM-Tris/HCl buffer, pH 7.4, 0.75 mM-CaCl2, 1.6 mM-cytidine, 1.6 mM-myoinositol and 10 mM-D-glucose.

To label the iris phospholipids with 32P or myo-[3H]inositol (or myo-[3H]inositol) each pair of irises, obtained from the same rabbit, was preincubated for 30 min at 37°C in the above incubation medium which contained 25 μCi of 32P (or 10-20 μCi of myo-[3H]inositol) in a final volume of 1 ml. After prelabelling, the irises were washed four times with excess of cold nonradioactively labelled medium which contained 10 mM 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 10 min in 1 ml of the unlabelled medium, which contained 2-deoxyglucose (10 mM) and other agents as indicated.

Determination of ATP in 32P-labelled slices. The nucleotides were extracted from the 32P-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 95% 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, pH 3.5, at 3 kV for 45 min. 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 phospholipids (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 3 ml 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 1 ml of chloroform/methanol/HCl (400:200:1.5, by vol.). All the extracts were combined and evaporated under N2. Chloroform (1 ml) was added to each tube and the extract was washed first with 1 ml of 0.1 M-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 N2 and the phospholipids were dissolved in 0.1 ml 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 40 g of silica-gel H with 3 g of magnesium acetate in 95 ml of water. Five glass plates (20×20 cm) were coated with this slurry to a depth of 0.3 mm. The plates were air-dried and activated for 1 h 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 32P-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-[3H]inositol.

Broekhuysen (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-

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lipids were extracted under acidic conditions. Whereas lysophosphatidylcholine was found in very low concentration, lysophosphatidylethanolamine was present in relatively higher concentrations (Fig. 1). Broekhuysen (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 (Broekhuysen, 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 10 ml of a scintillation fluid [6g of 2,5-diphenyloxazole and 120 mg 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 0.7 ml of 72 % (w/v) HClO\(_4\). After cooling and the addition of 4 ml 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 10 ml of the mixture was added to a portion of the aqueous sample (0.5 ml in the present study).

**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 pH 7.4 with Tris/HCl buffer, cut into small pieces with fine scissors, then homogenized thoroughly either in a glass homogenizer (by hand) or in a Tissue-mizer (type X1020; 10 T shaft; International Laboratory Apparatus G.m.b.H., 7801 Dottingen, Germany) at 10000rev./min for 40 s. 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 120000g for 90 min 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, 0.75 \(\mu\)mol of triphosphoinositide (sodium salt) was dispersed in 0.2 ml of water. Then the following constituents were added: 1.5 mm-MgCl\(_2\), 0.75 mm-CaCl\(_2\), 100 mm-KCl, 1 mm-NaF, 42 mm-Tris/HCl buffer, pH 7.4, reduced glutathione (3 mm), pH 7.4, and the enzyme was added as indicated, in a final volume of 0.75 ml. Incubation was carried out for 30 min at 37°C. Appropriate controls accompanied each determination. The reaction was terminated by adding 0.7 ml of HClO\(_4\), and the liberated \(P_1\) 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 10 min at 37°C in the presence and absence of 10 mm-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
A TRIPHOSPHOINOSITIDE EFFECT IN IRIS SMOOTH MUSCLE

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 \([^{32}P]\), each pair of irises, obtained from the same rabbit, was preincubated for 30 min at 37°C in a medium based on that of Bradford (1969) and containing 25 \(\mu\)Ci of \([^{32}P]\) in a final volume of 1 ml. One of the pair was washed four times with excess of unlabelled cold medium and incubated for 10 min in the same medium. The other was washed in Bradford medium that contained 10 mM-2-deoxyglucose and incubated for 10 min 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.

<table>
<thead>
<tr>
<th>Additions</th>
<th>ATP</th>
<th>Phosphatidic acid</th>
<th>Phosphatidylinositol</th>
<th>Triphosphoinositol</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11830</td>
<td>3806</td>
<td>8949</td>
<td>31180</td>
</tr>
<tr>
<td>10mM-2-Deoxyglucose</td>
<td>3410</td>
<td>3439</td>
<td>9394</td>
<td>21890</td>
</tr>
</tbody>
</table>

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 \([^{32}P]\), each pair of irises obtained from the same rabbit was preincubated for 30 min at 37°C in a medium based on that of Bradford (1969) and containing 25 \(\mu\)Ci of \([^{32}P]\) in a final volume of 1 ml. After prelabelling the irises, they were washed four times with excess of unlabelled cold medium that contained 10 mM-2-deoxyglucose. For studies on the effect of acetylcholine+eserine (0.05 mM each) the prelabelled irises (of the pair, one was used as control and the other as experimental) were incubated at 37°C for 10 min in 1 ml of unlabelled medium that contained 10 mM-2-deoxyglucose. The reaction was terminated by adding 1 ml 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 \(\pm\) S.E.M. of four different experiments run in duplicate.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Phosphatidic acid</th>
<th>Phosphatidylinositol</th>
<th>Diphosphoinositide</th>
<th>Triphosphoinositol</th>
<th>Phosphatidylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (zero time)</td>
<td>5324 (\pm) 373</td>
<td>9549 (\pm) 630</td>
<td>5026 (\pm) 186</td>
<td>23538 (\pm) 953</td>
<td>3246 (\pm) 292</td>
</tr>
<tr>
<td>None (control)</td>
<td>2501 (\pm) 175*</td>
<td>9818 (\pm) 635</td>
<td>3076 (\pm) 108*</td>
<td>16160 (\pm) 640*</td>
<td>3272 (\pm) 287</td>
</tr>
<tr>
<td>Acetylcholine+eserine (0.05 mM each)</td>
<td>3600 (\pm) 252†</td>
<td>12380 (\pm) 720†</td>
<td>3233 (\pm) 113</td>
<td>11800 (\pm) 250†</td>
<td>3341 (\pm) 311</td>
</tr>
</tbody>
</table>

* 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 10 min in unlabelled medium that contained 10 mM-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 (0.05 mM each) to the incubation medium enhanced the loss of radioactivity from triphosphoinositide by 27% after 10 min 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 vitro and in vivo. 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 (30min–2h), 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%.

![Graph](image)

**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.

**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 50 nm. 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 32P incorporation into triphosphoinositide and other phospholipids**

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

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**Table 3. Effects of atropine and D-tubocurarine on acetylcholine-stimulated breakdown of triphosphoinositide and other phospholipids of rabbit iris muscle prelabelled with [32P]phosphate**

Conditions of incubation were the same as described for Table 1, except that the [32P]-prelabelled muscle was first incubated in the presence or absence of the antagonist for 5 min, then acetylcholine and eserine were added as indicated and the incubation was continued for another 10 min. The results reported here are means (±S.E.M.) for four different experiments run in duplicate.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration (nm)</th>
<th>Phospholipid...</th>
<th>Phosphatidic Phosphatidyl-</th>
<th>Diphospho-</th>
<th>Triphospho- Phosphatidyl-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>acid inositol</td>
<td>inositol</td>
<td>choline</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>0.05</td>
<td></td>
<td>135±7</td>
<td>138±7</td>
<td>102±4</td>
</tr>
<tr>
<td>Acetylcholine+</td>
<td>0.05 and 0.1</td>
<td></td>
<td>104±3*</td>
<td>99±5*</td>
<td>121±11*</td>
</tr>
<tr>
<td>atropine respectively</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylcholine+</td>
<td>0.05 and 0.1</td>
<td></td>
<td>152±10†</td>
<td>143±7†</td>
<td>95±5†</td>
</tr>
<tr>
<td>D-tubocurarine</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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

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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.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration (mM)</th>
<th>Phosphatidic acid</th>
<th>Phosphatidyl-inositol</th>
<th>Diphosphoinositide</th>
<th>Triphosphoinositide</th>
<th>Phosphatidylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>0.05</td>
<td>5514*; 9797†</td>
<td>16310; 20470</td>
<td>5882; 5383</td>
<td>22390; 16180</td>
<td>8209; 8271</td>
</tr>
<tr>
<td>Acetylcholine+ atropine</td>
<td>0.05 and 0.1</td>
<td>4867; 5595</td>
<td>15830; 16630</td>
<td>5875; 6022</td>
<td>15920; 16620</td>
<td>8661; 9410</td>
</tr>
<tr>
<td>Acetylcholine+ D-tubocurarine</td>
<td>0.05 and 0.1</td>
<td>4691; 8961</td>
<td>14090; 16830</td>
<td>5319; 4832</td>
<td>17380; 13400</td>
<td>8835; 8413</td>
</tr>
</tbody>
</table>

* 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.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Type of agonist</th>
<th>Phosphatidic acid</th>
<th>Phosphatidyl-inositol</th>
<th>Diphosphoinositide</th>
<th>Triphosphoinositide</th>
<th>Phosphatidylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamoylcholine</td>
<td>Muscarinic</td>
<td>170</td>
<td>113</td>
<td>109</td>
<td>89</td>
<td>108</td>
</tr>
<tr>
<td>Philocarpine</td>
<td>Muscarinic</td>
<td>130</td>
<td>119</td>
<td>112</td>
<td>89</td>
<td>97</td>
</tr>
<tr>
<td>Choline</td>
<td>Muscarinic</td>
<td>108</td>
<td>104</td>
<td>96</td>
<td>93</td>
<td>107</td>
</tr>
<tr>
<td>Lobeline</td>
<td>Nicotinic</td>
<td>105</td>
<td>101</td>
<td>108</td>
<td>107</td>
<td>112</td>
</tr>
</tbody>
</table>

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-$^{3}H$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-[³²P]inositol in vitro

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

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Incubation time (min)</th>
<th>No. of observations</th>
<th>³H radioactivity (c.p.m.) in phospholipids in the presence and absence of acetylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>3</td>
<td>4460*; 4346†</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>4</td>
<td>6852</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2</td>
<td>8197; 7850</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2</td>
<td>7351; 7177</td>
</tr>
</tbody>
</table>

* Control.
† Acetylcholine plus eserine (0.05 mM 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, 0.05 mM each). After incubation for 10 min 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 HClO₄-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.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Radioactivity (% of that in total lipids)</th>
<th>Change (%)</th>
<th>Phosphate content (% of total lipid phosphorus)</th>
<th>Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Acetylcholine plus eserine</td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Triphosphoinositide</td>
<td>39.4*</td>
<td>29.9</td>
<td>−24</td>
<td>4.1</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>32.5*</td>
<td>42.5</td>
<td>+30.8</td>
<td>7.4</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>5.1*</td>
<td>6.8</td>
<td>+33</td>
<td>1.8</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>7.8†</td>
<td>8.3</td>
<td>+6.4</td>
<td>45.2</td>
</tr>
<tr>
<td>Phosphatidylethanol-amine</td>
<td>1.6†</td>
<td>1.6</td>
<td>0</td>
<td>22.0</td>
</tr>
</tbody>
</table>

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

Loss of radioactivity and phosphate from phosphoinositides in response to acetylcholine

When irises prelabelled with ³²P 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 concentration.
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.

<table>
<thead>
<tr>
<th>Age</th>
<th>No. of rabbits</th>
<th>Fresh weight of muscle (g)</th>
<th>(nmol of P1 liberated from triphosphoinositide/h per g of tissue)</th>
<th>(nmol of P1 liberated from triphosphoinositide/h per mg of protein)</th>
<th>Distribution of the enzyme (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 weeks</td>
<td>5</td>
<td>0.39</td>
<td>4970</td>
<td>300</td>
<td>Particulate: 82 Soluble: 18</td>
</tr>
<tr>
<td>Adult</td>
<td>3</td>
<td>0.56</td>
<td>5000</td>
<td>400</td>
<td>Particulate: 84 Soluble: 16</td>
</tr>
</tbody>
</table>

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, 1964a) 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 P1 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 (1964a,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, 1962a,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 ~10⁻⁶ M and for short time-intervals (<10 min), 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 [³²P] or myo-[³H]inositol and labelling experiments with [³²P] (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 phosophatic 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 0.1 mM-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.
A TRIPHOSPHOINOSITIDE EFFECT IN IRIS SMOOTH MUSCLE

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

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, p-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 phosphatidlyesterase.

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


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