Initially the specific binding of the tritiated dopamine analogue was examined in various regions of the rat brain and in kidney. The results obtained are shown in Table 1, and are consistent with the known distribution of dopamine receptors.

Analysis of kinetic characteristics of the binding of tritiated dopamine analogue to striatal synaptic membranes showed a saturable process with an apparent $K_D$ of 1.2 $\mu$m. This is a similar value to the half-maximum value of the dopamine analogue in stimulating adenylate cyclase (3 $\mu$m). In a preliminary study the specific binding of this compound was unaffected by the following: glycine (1 mM), histamine (1 mM) nialamide (10 $\mu$m) and trans-flupenthixol (1 mM). In contrast with this latter result, the potent neuroleptic cis-flupenthixol (1 mM) was 58% and benztpine 24% as active as the dopamine analogue in displacing the specific binding. In all experiments dopamine was more active (160%) than its analogue in displacing the label.

These experiments support the contention that the actions of the dopamine analogue are brought about by specific binding to dopamine receptors.


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Acetylcholine Causes an Increase in the Hydrolysis of Triphosphoinositide Pre-labelled with $^{12}$P Phosphate or $^1$H Myo-Inositol and a Corresponding Increase in the Labelling of Phosphatidylinositol and Phosphatidic Acid in Rabbit Iris Muscle

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We have reported the effects of cholinergic and adrenergic neurotransmitters on the incorporation in vitro of $^{32}$P, into phospholipids of the rabbit iris muscle (Abdel-Latif 1974, 1976; Abdel-Latif et al., 1976). These studies indicate that acetylcholine and noradrenaline enhance the turnover of phosphatidic acid and phosphatidylinositol, which are concentration- and time-dependent and mediated through muscarinic and $\alpha$-adrenergic receptors respectively.

A wide variety of tissues show an enhancement in the incorporation of $^{32}$P into these phospholipids in response to a number of extracellular stimuli, including neurotransmitters, hormones, drugs and electrical impulses [for review see Michell (1975)]. In the past few years significant contributions have been made towards understanding this 'phospholipid effect' and its possible relationship to synaptic neurotransmission and other physiological processes. It must be emphasized, however, that at present the molecular mechanism and physiological significance of this effect are still not clear.

In the present study we report the finding that, in the iris muscle, acetylcholine at concentrations of the order of 0.05 mM appears to bring about the phospholipid effect by stimulating the hydrolysis of triphosphoinositide to diphosphoinositide, which subsequently leads to an increase in phosphatidylinositol and phosphatidic acid. The effect of acetylcholine on the hydrolysis of triphosphoinositide was blocked by atropine. These

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Table 1. Effect of acetylcholine on the distribution of radioactivity in the various phospholipids after the incubation of \( ^{32}P \)-pre-labelled rabbit irises at different time-intervals

To pre-label the iris phospholipids with \( ^{32}P \) (or \([^3H]\)myo-inositol) 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 \( ^{32}P \) (or 10 \( \mu \)Ci of \([^3H]\)myo-inositol) in a final volume of 1 ml. In experiments where the iris muscles were pre-labelled in vivo, young rabbits (3–4 weeks old) were injected intraperitoneally with 1 mCi of \( ^{32}P \), and, after 16 h from the time of administration, the irises were isolated. After pre-labelling, in vitro or in vivo, the irises were washed four times with excess of cold Bradford (1969) medium. For studies on the effect of acetylcholine plus eserine (0.05 mM each) the pre-labelled irises (of the pair, one was used as control and the other as experimental) were incubated in 1 ml of Bradford (1969) medium containing 1.64 mg of 2-deoxyglucose at 37°C for different time-intervals. The reaction was terminated by adding 1 ml of 10% trichloroacetic acid. For extraction of phospholipids inclusive of higher inositides the procedure of Yagihara et al. (1973) was used. The phospholipids were separated, and their radioactive contents determined as previously described (Abdel-Latif et al., 1973). The two values in each column (one in parentheses) are one from each iris of a pair.

<table>
<thead>
<tr>
<th>Time of incubation (min)</th>
<th>No. of experiments</th>
<th>Phosphatidic acid (c.p.m.)</th>
<th>Phosphatidylinositol (c.p.m.)</th>
<th>Diphosphoinositide (c.p.m.)</th>
<th>Triphosphoinositide (c.p.m.)</th>
<th>Phosphatidylycholine (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>7550</td>
<td>6343</td>
<td>5100</td>
<td>50824</td>
<td>3886</td>
</tr>
<tr>
<td>2.5</td>
<td>2</td>
<td>7198 (10066)*</td>
<td>7067 (7694)</td>
<td>5600 (5950)</td>
<td>49745 (47702)</td>
<td>3973 (4010)</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>5428 (11050)</td>
<td>8661 (10113)</td>
<td>5780 (6100)</td>
<td>49200 (40241)</td>
<td>3890 (4008)</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>4317 (7548)</td>
<td>11690 (14174)</td>
<td>5902 (6000)</td>
<td>25908 (15859)</td>
<td>4711 (4888)</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>4226 (5749)</td>
<td>10548 (12554)</td>
<td>4955 (5402)</td>
<td>18017 (12251)</td>
<td>5772 (5646)</td>
</tr>
<tr>
<td>10†</td>
<td>2</td>
<td>300 (370)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Acetylcholine plus eserine (0.05 mM each) were added.
† The irises were pre-labelled in vivo.
Table 2. Effects of acetylcholine and atropine on the distribution of radioactivity in the various phospholipids of rabbit irises pre-labelled with $^{32}$P or $[^3H]$-myoinositol

Conditions of incubation were the same as described under Table 1 except that the pre-labelled slices were preincubated for 5 min in the presence or absence of atropine (0.27 mM) before the addition of acetylcholine plus eserine (0.05 mM each). After the addition of the neurotransmitter the slices were incubated for 10 min, and phospholipids extracted as in Table 1. The numbers of experiments are given in parentheses. Each experiment was an average of two to three irises incubated and analysed individually. Results are given as means ± S.E.M.

<table>
<thead>
<tr>
<th>Isotope used in the pre-labelling in vitro of muscle</th>
<th>Additions</th>
<th>Phosphatidic acid</th>
<th>Phosphatidylinositol</th>
<th>Diphosphoinositide</th>
<th>Triphosphoinositide</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{32}$P</td>
<td>—</td>
<td>$151 \pm 13 (16)$</td>
<td>$142 \pm 11 (13)$</td>
<td>$104 \pm 6 (7)$</td>
<td>$71 \pm 5 (9)$</td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>$61 (2)$</td>
<td>$100 (2)$</td>
<td>$97 (2)$</td>
<td>$150 (2)$</td>
</tr>
<tr>
<td>$[^3H]$myo-Insitol</td>
<td>—</td>
<td>$124 \pm 5 (7)$</td>
<td>$95 \pm 7 (7)$</td>
<td>$68 \pm 4 (7)$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>$96 (2)$</td>
<td>$95 (2)$</td>
<td></td>
<td>$215 (2)$</td>
</tr>
</tbody>
</table>
conclusions are supported by the data presented in Tables 1 and 2. At zero time, the radioactivity in triphosphoinositide was about 69% of the total radioactivity incorporated into phosphatidic acid, phosphatidylinositol, di- and tri-phosphoinositide and phosphatidylcholine (Table 1). At the same time the radioactivity in phosphatidylinositol and phosphatidic acid was 9 and 10%, respectively. After 2.5 min of incubation there was little change in radioactivity in control samples. However, in samples containing acetylcholine (0.05 mM) the radioactivity in triphosphoinositide fell to 63%, whereas that in phosphatidylinositol and phosphatidic acid was increased to 10 and 13%, respectively. When the incubation was continued for 10 min in the presence of acetylcholine the percentage radioactivity in triphosphoinositide, phosphatidylinositol and phosphatidic acid was 33, 29 and 16 respectively. Under the same conditions these values in the control samples were 49, 22 and 8 respectively. Interestingly, there was little change in total radioactivity in control and experimental samples. However, the decrease in radioactivity observed in triphosphoinositide in the presence of acetylcholine and the increases in radioactivity in phosphatidylinositol and phosphatidic acid were statistically significant. The amount of labelling in diphosphoinositide did not change appreciably, but that in phosphatidylcholine increased with time, and the neurotransmitter exerted a negligible effect on the $^{32}$P$_1$ labelling of these phospholipids (Table 1). Similar conclusions can be drawn from the data presented in Table 2 where $[^3H]$myo-inositol was used in addition to $^{32}$P, as precursor. Atropine, a drug that is known to block muscarinic receptors, inhibited the hydrolysis of triphosphoinositide pre-labelled with $^{32}$P, and $[^3H]$myo-inositol (Table 2). This drug probably acts by inhibiting the enzyme triphosphoinositide phosphomonoesterase, and acetylcholine could act by stimulating the same enzyme. The conclusions drawn from the data presented can be summarized as shown below.

```
Membrane phosphatidate  →  Membrane 1,2-diglyceride
                 ↑                  ↓
Membrane phosphatidylinositol  Membrane phosphatidylcholine
                 ↓                  ↓
Membrane diphosphoinositide
                 ↑
Membrane triphosphoinositide
```

Attempts to show an increase in liberated $[^3H]$myo-inositol derivatives in the trichloroacetic acid extract were unsuccessful. Jungalwala et al. (1971) working on the metabolism of phosphatidylinositol in the pig thyroid gland suggested that the inositol moiety may be turning over and leaving the phosphate moiety attached to some lipid residue, which remains in the membrane and is resynthesized into phosphatidylinositol.

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) or by electrical stimulation of brain slices (Pumphrey, 1969), no significant changes have been found. More recent evidence indicates that such changes may occur. Birnberger et al. (1971) showed an increased turnover of triphosphoinositide in lobster nerves after long incubations and brief electrical stimulation (5 min). Schacht & Agranoff (1972) observed a decreased labelling of polyphosphoinositides with $^{32}$P$_1$ 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 3 h. Later it was reported (White et al., 1974) that stimulation of vagus nerve for 30 min increased phosphate incorporation into all the phospholipids studied, but the increase was significant only for tri- and di-phosphoinositide. The present findings with the iris muscle should throw more light on these observations.
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Torda (1974) proposed a model of a depolarization–hyperpolarization cycle, in which she suggested that the regulatory subunit of triphosphoinositide phosphomonoesterase constitutes a part of the post-synaptic nicotinic acetylcholine receptor.

We are grateful to Professor J. N. Hawthorne for fruitful discussions during the course of these studies. This work was supported, in part, by United States Public Health Service Grant NS-07876 from the Institute of Neurological Diseases and Stroke.


The Long-Chain Fatty Acyl-Coenzyme A Hydrolase Activity of Rat Cerebral Cortex

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Unesterified fatty acids constitute a small but very labile pool of the brain lipids (Rowe, 1964; Lunt & Rowe, 1968, 1969, 1971; Bazan & Joel, 1968; Bazan, 1971). Electroshock and certain convulsive drugs increase the concentration of unesterified fatty acids in brain (Bazan, 1970, 1971), and it has been suggested that they may be involved in the changes in membrane permeability that accompany synaptic transmission (Lunt & Rowe, 1971; Vyvoda & Rowe, 1973). The source of the acids is not yet known. Vyvoda and Rowe (1972) showed that guinea-pig brain contained mono-, di- and tri-glyceride lipase activities, whose action on exogenous substrates is modulated by neurotransmitters, and Gullis & Rowe (1975a) have reported the presence of a phospholipase A2 in guinea-pig synaptic membranes, which is also sensitive to neurotransmitters. It is probable that, under conditions of increased transmitter release in vitro or in vivo, the hydrolysis of neutral glycerides and phospholipids contributes markedly to the unesterified fatty acid pool. We have previously shown, however, that, under non-stimulated conditions in rat cerebral cortex in vivo, fatty acids appear to be incorporated into neutral glycerides and phospholipids, rather than being derived from them (Bonsen & Lunt, 1976). We suggested that an alternative source of the unesterified fatty acids may be a long-chain fatty acyl-CoA hydrolase activity. The presence of such an activity (palmitoyl-CoA hydrolase; EC 3.1.2.2) in brain has been known for some time (Srere et al., 1958; Vignais & Zabin, 1958). If, as suggested previously (Bonsen & Lunt, 1976), a long-chain fatty acyl-CoA hydrolase activity contributes to the maintenance of the brain pool of unesterified fatty acids it may be that the enzyme activity is sensitive to neurotransmitters or to cyclic nucleotides, as is the case of the phospholipase A2 described by Gullis & Rowe (1975a, b).