Stimulation of Hydrolysis of Phosphatidic Acid by Cholinergic Agents in Guinea Pig Synaptosomes*

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SUMMARY

"Light" nerve endings from guinea pig cerebral cortex were incubated with [1-P]orthophosphate, myo-[3H]inositol, [3H]glycerol, or [3H]glucose in the presence or absence of cholinergic agents. Acetylcholine and carbamylcholine increased incorporation of [32P] into phosphatidic acid 100% and into various classes of phosphatidylinositol 20 to 70%. These agents also stimulated the labeling of phosphatidylinositol from myo-[3H]inositol but did not affect the labeling of either phosphatidylinositol or phosphatidic acid from [3H]glycerol or from [3H]glucose. The specific activity of synaptosomal [γ-32P]ATP was unchanged.

In a series of two-step incubations, lipids were first labeled with [32P], then excess unlabeled P, or 2,4-dinitrophenol was added to lower the specific activity or the amount of [γ-32P]-ATP. The addition of acetylcholine stimulated the rate of disappearance of [32P]phosphatidic acid by 80%. This effect of acetylcholine was blocked by atropine. We conclude that cholinergic agents increase phospholipid labeling by increasing the hydrolysis of phosphatidic acid.

The rapid labeling of certain acidic phospholipids, phosphatidate, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine by isotope labeling of phosphorus has been observed in a variety of tissues (2, 3). Although a physiological significance of this rapid lipid metabolism is generally assumed, its precise nature and its biochemical mechanism remain unclear. The turnover of these lipids has been implicated in the mechanism underlying synaptic transmission or the changes in membrane permeability, or both, by a number of observations in sympathetic ganglia and brain slices. Electrical stimulation of these preparations (4, 5) or the addition of neurotransmitters such as acetylcholine to incubations (6) increases [32P] incorporation into phosphatidate or phosphatidylinositol, or both. This effect is blocked by inhibitors of synaptic transmission (7) and was interpreted to be mainly postsynaptic (8, 9) in the ganglion preparation.

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While the stimulation generally requires the presence of intact cells, Hokin and Hokin (10) demonstrated that acetylcholine also stimulated phospholipid labeling in broken cell preparations of guinea pig cortex. Subsequently this effect was localized in the nerve ending fraction (synaptosomes) of the homogenate (11-13). Although questions have been raised whether the action of acetylcholine on synaptosomes is indeed related to the effects observed in vivo and in brain slices or ganglia (14), this system has been demonstrated to show a large and reliable effect of cholinergic agents on phospholipid labeling (12, 15) and it lends itself more readily to biochemical investigations than tissue slices and other whole cell preparations. Continuing our previous studies (12, 15) we have further examined levels of radioactively labeled ATP, phosphatidate, and phosphatidylinositol in guinea pig synaptosomes.

MATERIALS AND METHODS

Preparations and incubations of synaptosomes were performed essentially as described (12). A 10% homogenate of guinea pig cerebral cortex (albino male, Camm Res., Wayne, N. J.) was prepared in 0.32 m sucrose in a Teflon-glass homogenizer. All operations were carried out at 0-4°C. Sucrose solutions contained 1 mM sodium glycylglycinate, pH 6.6. The homogenate was centrifuged at 1,000 × g for 10 min, the pellet was washed once and the combined supernatants were centrifuged for 20 min at 13,000 × g to obtain a crude nerve ending-mitochondria pellet. This pellet was suspended in 30 ml of 0.32 m sucrose by hand homogenization and layered over three gradient tubes each containing 10 ml of 1.1 m sucrose and 10 ml of 0.8 m sucrose. After centrifugation for 150 min at 25,000 rpm in a SW 25 rotor, the interface over 1.1 m sucrose was harvested. After dilution with 0.16 m sucrose, it was pelleted by centrifuging for 30 min at 100,000 × g. The pellet was suspended in 0.32 m sucrose by hand homogenization and designated "Fraction NF 3.1."

Incubations were performed at 37°C in air with shaking in the following medium (final concentrations): 100 mM sodium glycylglycinate (pH 6.6), 160 mM sucrose, 0.8 mM MgSO4, 1 mM sodium pyruvate, 1 mM sodium fumarate, 1 mM cytidine, 1 mM myo-inositol and 0.1 mM NaH2PO4, in a volume of 0.5 ml, and 10 to 30 μCi of [32P] were added per incubation. The [32P] was carrier-free in 0.02 N HCl and was neutralized with an equal amount of 0.02 N NaOH. To remove possible lipid-soluble contaminants, aqueous solutions of [3H]inositol, [3H]glucose, and [3H]glycerol were partitioned three times with chloroform, dried under N2, and dissolved in H2O before addition to the incubations.

Incubations were terminated by the addition of chloroform-methanol-HCl (16). After extraction and washing (16), lipids were separated by two different solvent systems on silica gel plates (Brinkmann Silplate 22). Solvent A, chloroform-methanol-aqueous NH4H2O (45:45:4:11; v/v), separated phosphatidylinositol-
sitol diphosphate from phosphatidylinositol phosphate, but not phosphatidate from phosphatidylinositol. Solvent B, chloroform-methanol-glacial acetic acid-H2O (50:32:11:3; v/v), did not separate the polyphosphoinositides but gave good separation of phosphatidylinositol and phosphatidate. Lipids were identified by co-chromatography with known standards (Supelco, Bellefonte, Pa.) and by high voltage electrophoresis of the water-soluble products after alkaline methanolysis (16). Classes of phosphatidylinositol were separated according to the degree of unsaturation of their fatty acid chains on AgNO3-impregnated thin layer plates (17). 32P-lipids on thin layer chromatography plates were located by autoradiography, scraped, and counted by liquid scintillation spectrometry. 3H-lipids were located from the autoradiographically established RF values of 32P-lipids chromatographed on the same plates. 3H-lipids on silica gel were treated with hydrofluoric acid and counted in a scintillant containing 33% Triton X-100 (18).

ATP, glucose 6-phosphate, and 2-deoxyglucose 6-phosphate were separated from the water-soluble materials by high voltage electrophoresis on Whatman No. 1 paper in pyridine acetate buffer (pH 4.3) at 4000 volts for 30 min. Products were identified by co-electrophoresis with enzymatically prepared 32P-labeled standards. Protein was determined spectrophotometrically (19). Acetylcholine, eserine, and carbamylcholine were purchased from Sigma (St. Louis, MO.), atropine from Pierce Chemical (Rockford, Ill.), and radioactive materials from New England Nuclear (Boston, Mass.).

RESULTS

Effect of Carbamylcholine Concentration—Fig. 1 demonstrates that the effect of carbamylcholine on lipid labeling is similar to that of acetylcholine (12) but requires slightly higher concentrations of the agent. The threshold concentration for stimulation of both phosphatidate and phosphatidylinositol is around 10^-5 M and a maximum is reached at 10^-3 M.

Time Course—Fig. 2 shows the labeling of phospholipids from 32P in the absence and presence of 10^-3 M carbamylcholine. Phosphatidylinositol diphosphate and phosphatidylinositol phosphate equilibrate rapidly with the precursor ATP and their labeling is lower in the presence of carbamylcholine. Phosphatidate labeling reaches a maximum after 2 hours (Figs. 2 and 4) and stimulation by carbamylcholine is apparent at all times. Phosphatidylethanolamine incorporates 32P; at a much slower rate and remains unaffected by the addition of carbamylcholine.

Phosphatidylinositol (Fig. 3) was resolved into three classes depending on the number of double bonds among the two fatty acid chains. Whereas the tetraene and triene species show a similar stimulation by carbamylcholine after an initial lag period, the monoene plus diene fraction appears to be less responsive to carbamylcholine.

During extended incubations (Fig. 4) the nerve endings gradually lose their capability for oxidative phosphorylation. The [32P]phosphatidate level decreases following the decrease in [32P]ATP. Although carbamylcholine does not affect the rate of ATP decrease, phosphatidate, in the presence of carbamylcholine, loses 32P more rapidly than in its absence, resulting in a lower percentage stimulation at later times. The average percentage of
stimulation of phosphatidate labeling by carbamylcholine obtained from three similar experiments was 101% after 1 hour, but only 54% after 4 hours.

Specific Activity of \(^{32}\text{P}\)[ATP]-To investigate whether the stimulation of lipid labeling was mediated by changes in the specific radioactivity of \([\gamma-^{32}\text{P}]\)ATP, we measured the phosphorylation of tritium-labeled 2-deoxyglucose in the presence and absence of carbamylcholine. Table I shows the effect of the presence of increasing amounts of 2-deoxyglucose on the labeling of lipids; the availability of ATP for phosphorylation of lipids is effectively lowered, particularly that required for the increased labeling in the presence of carbamylcholine as seen by the lower percentage of stimulation in the presence of deoxyglucose. Deoxyglucose thus apparently is most efficiently phosphorylated by a pool of ATP relevant to stimulated phospholipid labeling. \(^{32}\text{P}\)-Labeled ATP and doubly labeled 2-deoxy[\(^{3}\text{H}\)]glucose-[\(^{32}\text{P}\)P]-phosphate were separated by high voltage electrophoresis. Results are summarized in Table II. With added carbamylcholine, there is no change in total ATP radioactivity, amount of 2-deoxyglucose phosphorylated or in the ratio, \(^{32}\text{P}:^{3}\text{H}\) (a measure of the specific activity of the \(\gamma\)-phosphate of ATP).

In a total of six experiments under varying conditions the \(^{32}\text{P}:^{3}\text{H}\) value of experimental tubes compared to controls was 1.03 ± 0.07 S.D.

Incorporation of Other Precursors—Increased labeling of phosphatidate can reflect de novo synthesis or increased turnover of the phosphate moiety. We therefore studied the effect of carbamylcholine on the incorporation of radioactivity into phosphatidate from various precursors (Table III). Labeling of phosphatidate and phosphatidylinositol from \([\text{H}]\)glycerol or \([\text{H}]\)glucose was not significantly altered in the presence of carbamylcholine. A stimulation, however, was seen in the incorporation of \(\text{myo}[\text{H}]\)inositol into phosphatidylinositol. The average degree of stimulation from seven experiments was 18% ± 8% S.D.; \(^{32}\text{P}\)-incorporation into phosphatidylinositol was measured parallel in these experiments and it was stimulated 67% ± 12% S.D.

Effects of Preincubation—The apparent lag period in the appearance of stimulated phosphatidylinositol labeling (Fig. 3) was further confirmed in some short time experiments (Table IV) which were designed to investigate the effects of incubation of synaptosomes with acetylcholine prior to the addition of \(^{32}\text{P}\)P, since no significant stimulation of phosphatidylinositol labeling can be detected at 5 min.

If acetylcholine stimulated diglyceride formation, this intermediate should be present at elevated levels after pretreatment of incubations with acetylcholine, and the initial rate of stimulated phosphatidate labeling should be increased by the prior incubation. The results show (Table IV) that 5 min after the addition of \(^{32}\text{P}\)P the stimulated labeling of phosphatidate is indeed higher in those incubations which were treated with acetylcholine 30 min prior to the pulse. Atropine blocks the effect when added initially, but only partially when added after the acetylcholine pretreatment. Fluoride, an inhibitor of phosphohydrolases (20), stimulates phosphatidate labeling dramatically without a concomitant stimulation of acetylcholine-stimulated phosphatidate labeling, and with much less effect on phosphatidylinositol labeling (Table V).

### Table I

**Effect of 2-deoxy-d-glucose on labeling of phosphatidate and phosphatidylinositol**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Phosphatidate</th>
<th>Phosphatidylinositol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>(10^{-4}) (\mu) CCh*</td>
</tr>
<tr>
<td></td>
<td>dpm (^{32}\text{P}) incorporated</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1110</td>
<td>2490 (120%)</td>
</tr>
<tr>
<td>0.01 mM deoxyglucose</td>
<td>1140</td>
<td>2280 (99%)</td>
</tr>
<tr>
<td>1.0 mM deoxyglucose</td>
<td>660</td>
<td>990 (46%)</td>
</tr>
</tbody>
</table>

* CCh, carbamylcholine.

### Table II

**Effect of carbamylcholine on phosphorylation of 2-deoxy-d-[\(^{3}\text{H}\)]glucose**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Recovered radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td></td>
<td>(^{32}\text{P}:^{3}\text{H})</td>
</tr>
<tr>
<td></td>
<td>dpm</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>790</td>
</tr>
<tr>
<td>(10^{-2}) (\mu) CCh</td>
<td>778</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>626</td>
</tr>
<tr>
<td>(10^{-2}) (\mu) CCh</td>
<td>698</td>
</tr>
</tbody>
</table>

* CCh, carbamylcholine.

### Table III

**Effect of carbamylcholine on incorporation of labeled glucose, glycerol, and myo-inositol into phosphatidate and phosphatidylinositol**

Experiment I, Fraction NE\(_{1.1}\) (0.8 mg of protein) was incubated for 30 min with 3.5 \(\mu\)Ci of (1 mM) \([\text{H}]\)glucose as described under "Materials and Methods," except that pyruvate and fumarate were omitted from the incubation medium. Experiment II, Fraction NE\(_{1.1}\) (1.8 mg of protein) was incubated for 30 min with 30 \(\mu\)Ci of (1 mM) \([\text{H}]\)glycerol as described under "Materials and Methods." Experiment III, Fraction NE\(_{1.1}\) (1.1 mg of protein) was incubated for 40 min with 20 \(\mu\)Ci of (1 mM) myo-[\(^{2}\text{H}\)]inositol as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Additions</th>
<th>Experiment I (^{32}\text{P}) [([\text{H}])glucose</th>
<th>Experiment II (^{32}\text{P}) [([\text{H}])glycerol</th>
<th>Experiment III (^{32}\text{P}) [([\text{H}])inositol</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpm (^{3}\text{H}) incorporated</td>
<td>1080</td>
<td>27,790</td>
<td>1750</td>
</tr>
<tr>
<td>(10^{-2}) (\mu) CCh</td>
<td>880</td>
<td>22,080</td>
<td>2110</td>
</tr>
<tr>
<td>(10^{-2}) (\mu) CCh + [atropine]</td>
<td>29,160</td>
<td>6740</td>
<td>1750</td>
</tr>
</tbody>
</table>

\(^{a}\) Phosphatidate plus phosphatidylinositol.

\(^{b}\) Phosphatidylinositol.

* CCh, carbamylcholine.
TABLE IV
Effects of preincubation with acetylcholine on labeling of phosphatidate and phosphatidylinositol

Experiment I, Fraction NE1.1 (1 mg of protein) was incubated for 30 min prior to the addition of 30 μCi of 32P1. At 0 min the isotope was added plus the further additions mentioned. Acetylcholine was 10^{-4} M (plus eserine 10^{-8} M) and atropine was 10^{-5} M, final concentrations. Incubations were terminated 5 min after the addition of isotope. See "Materials and Methods."

<table>
<thead>
<tr>
<th>Additions</th>
<th>At -30 min</th>
<th>At 0 min</th>
<th>Phosphatidate</th>
<th>Phosphatidylinositol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>dpm 32P incorporated</td>
<td></td>
</tr>
<tr>
<td>ACh</td>
<td></td>
<td></td>
<td>630</td>
<td>245</td>
</tr>
<tr>
<td>Atropine</td>
<td></td>
<td></td>
<td>1150</td>
<td>266</td>
</tr>
<tr>
<td>ACh + atropine</td>
<td></td>
<td></td>
<td>550</td>
<td>240</td>
</tr>
<tr>
<td>ACh</td>
<td></td>
<td></td>
<td>610</td>
<td>190</td>
</tr>
<tr>
<td>ACh</td>
<td></td>
<td></td>
<td>1520</td>
<td>190</td>
</tr>
<tr>
<td>Atropine</td>
<td></td>
<td></td>
<td>844</td>
<td>192</td>
</tr>
<tr>
<td>ACh + atropine</td>
<td></td>
<td></td>
<td>570</td>
<td>180</td>
</tr>
<tr>
<td>ACh</td>
<td></td>
<td></td>
<td>550</td>
<td>180</td>
</tr>
</tbody>
</table>

* ACh, acetylcholine.

TABLE V
Effect of sodium fluoride on labeling and stimulation by acetylcholine

Fraction NE1.1 (1.2 mg of protein) was incubated for 30 min with 30 μCi of 32P1 as described under "Materials and Methods." Acetylcholine was 10^{-4} M (plus eserine 10^{-8} M), final concentration.

<table>
<thead>
<tr>
<th>NaF</th>
<th>Phosphatidate</th>
<th>Phosphatidylinositol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+ACh</td>
</tr>
<tr>
<td>0</td>
<td>2,810</td>
<td>5,320</td>
</tr>
<tr>
<td>1 mM</td>
<td>3,860</td>
<td>7,180</td>
</tr>
<tr>
<td>10 mM</td>
<td>12,720</td>
<td>13,630</td>
</tr>
</tbody>
</table>

* ACh, acetylcholine.

Two-Step Experiments—To observe degradative reactions in the incubations independent of 32P-lipid synthesis, we designed two-step experiments in which the level or the specific radioactivity of 32P1 was altered after the synaptosomes had been incubated with 32P1.

In the first series of experiments, 5 mM sodium phosphate buffer (pH 6.6) was added to all vessels after a 60-min incubation in the presence and absence of acetylcholine (Fig. 5). The lowering of the specific radioactivity of the precursor was reflected in [32P] phosphatidate, which lost about 30% of its 32P content in the 45 min following the chase. No such loss of label was observed in phosphatidylinositol. The decline of radioactivity in phosphatidate appeared to be more rapid in those incubations which initially contained acetylcholine.

In other experiments, dinitrophenol was added after 60 min of incubation to block further formation of ATP (Fig. 6). A high dinitrophenol concentration was necessary since lower concentrations (e.g. 10^{-4} M) failed to completely block ATP formation, evidenced by a continued 32P incorporation into phosphatidate (unpublished observations). [32P] Phosphatidate showed an immediate response to dinitrophenol addition, losing about 15% of its radioactivity in the first 15 min. Radioactivity in phosphatidylinositol remained stable. Addition of carbamylcholine or acetylcholine together with dinitrophenol produced a slight increase in [32P] phosphatidate for 2 min which was followed by hydrolysis of phosphatidate at a higher rate than in control incubations. The temporary increase in phosphatidate after acetylcholine addition, which was consistently observed, was interpreted to reflect labeled ATP still available for stimulated phosphatidate labeling. Since this could obscure the more rapid rate of breakdown of phosphatidate if measurements are taken soon after dinitrophenol and acetylcholine addition, we performed a timed series of experiments, and compared the slopes of [32P] phosphatidate hydrolysis with and without acetylcholine from 10 to 30 min after the addition of dinitrophenol. Four such experiments are summarized in Table VI. The decrease of [32P] phosphatidate during this period was about 10% of the amount present 10 min after dinitrophenol and acetylcholine addition. There was an 80% stimulation of [32P] phosphatidate breakdown in the presence of acetylcholine. This
presumably the result of the combined action of kinase and phosphatidate synthase. One of these 10 experiments, a consistent decrease in phosphatidate was measured from 10 to 30 min after the addition of dinitrophenol. The amount of \(^{32}P\) lost is expressed as per cent of label present at 10 min after dinitrophenol. Acetylcholine 10-4 M (plus eserine 10-4 M) and atropine (10-4 M), when indicated, were added with dinitrophenol (2 \times 10^{-4} M).

<table>
<thead>
<tr>
<th>Addition</th>
<th>(^{32}P) lost ± S.D. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11.04 ± 0.61</td>
</tr>
<tr>
<td>ACh</td>
<td>19.12 ± 1.04</td>
</tr>
<tr>
<td>ACh + atropine</td>
<td>11.67 ± 4.38</td>
</tr>
</tbody>
</table>

\(a\) ACh, acetylcholine.

\(b\) Acetylcholine differs from control: \(p < 0.005\); acetylcholine differs from acetylcholine plus atropine: \(p < 0.01\).

Table VI

Effect of acetylcholine on loss of \(^{32}P\) from \([\gamma-{^32}P]ATP\)phosphatidate after addition of dinitrophenol

The values are averages from four experiments (each in duplicate incubations) ± S.D. The experiments were performed as described under "Results." Loss of \(^{32}P\) from \([\gamma-{^32}P]ATP\)phosphatidate was measured from 10 to 30 min after the addition of dinitrophenol. The amount of \(^{32}P\) lost is expressed as per cent of label present at 10 min after dinitrophenol. Acetylcholine 10-4 M (plus eserine 10-4 M) and atropine (10-4 M), when indicated, were added with dinitrophenol (2 \times 10^{-4} M).

The probable reaction sequence for the (basal, unstimulated) labeling of phosphatidate and phosphatidylinositol by \([\gamma-{^32}P]ATP\) is

\[
\begin{align*}
\text{(I) Phosphatidate} + \text{H}_2\text{O} & \rightarrow 1,2\text{-diglyceride} + \text{P}_1 \\
\text{(II) Diglyceride} + [\gamma-{^32}P]\text{ATP} & \rightarrow *\text{phosphatidate} + \text{ADP} \\
\text{(III) *Phosphatidate} + \text{CTP} & \rightarrow \text{CDP}-*\text{diglyceride} + \text{PP}_1 \\
\text{(IV) CDP}-*\text{diglyceride} + \text{myo-inositol} & \rightarrow *\text{phosphatidylinositol} + \text{CMP}
\end{align*}
\]

Phosphatidate synthesized via acylation of glycerophosphate or acyl dihydroxyacetone phosphate (21, 22) can be hydrolyzed to yield diglyceride, which may enter into a number of different synthetic reactions, one of which is the diglyceride kinase reaction (II). The combined reactions of phosphatidate phosphohydrolase (I) and diglyceride kinase (II) constitute a highly active system for labeling phosphatidate from \([\gamma-{^32}P]ATP\). Labeled phosphatidate then can give rise to labeled phosphatidylinositol via Reactions III and IV. Finally, sequential phosphorylation of phosphatidylinositol leads to phosphatidylinositol phosphate and phosphatidylinositol diphosphate. These polyphosphoinositides are also found to be highly labeled following incubation of brain tissue with \(^{32}P\). The radioactivity appears to be located mainly in the phosphomonoesterase positions (12) presumably the result of the combined action of kinase and phosphomonoesterase activities. Figs. 2 and 3 demonstrate the labeling pattern for the nerve ending preparations. Phosphatidate is rapidly and highly labeled as are the polyphosphoinositides, which appear to equilibrate with the precursor ATP after about 30 min. The rate of incorporation of the \(^{32}P\) into phosphatidylinositol is also high, whereas phosphatidylethanolamine incorporates \(^{32}P\) at a much slower rate.

The effect of carbamylcholine on lipid labeling (Figs. 1, 2, and 3) is very similar to the effects exerted by acetylcholine. The threshold concentration for stimulated labeling of both phosphatidate and phosphatidylinositol is around 10-4 M. Redman and Hokin (23) found a considerably lower carbamylcholine threshold concentration (10-5 M) for the stimulation of phosphatidylinositol labeling in a homogenate of guinea pig cortex. Labeling of phosphatidate is stimulated at all times examined, whereas basal and incremental phosphatidylinositol labeling show a slight lag period, probably a reflection of the synthesis of phosphatidylinositol from the increasingly labeled phosphatidate pool. In regard to molecular classes of phosphatidylinositol, the relative degree of labeling of the three classes closely resembles their abundance in brain (24, 25). The classes of phosphatidylinositol do not appear to be equally stimulated by carbamylcholine to incorporate \(^{32}P\), since there seems to be some preferential stimulation of the tetrane and triene fractions.

We can consider several possible mechanisms for the stimulated \(^{32}P\) incorporation into phosphatidate and phosphatidylinositol: (a) increased level of specific activity of \(^{32}P\)-labeled ATP; (b) increased \(de\) novo synthesis of lipid; (c) stimulation of diglyceride kinase; (d) increased availability of diglyceride. The first mechanism was considered unlikely because of the selective stimulation of phosphatidate and phosphatidylinositol labeling observed relative to other lipids (Fig. 2). We measured, nevertheless, the amount of labeled ATP in incubations as well as the specific activity of the \(\gamma-{^32}P\) ATP by another approach. Deoxyglucose is known to be transported into cells and to be phosphorylated via the hexokinase reaction, as is glucose (26). The transport of 2-deoxyglucose into synaptosomes is unaffected by acetylcholine and the hexokinase reaction is not rate-limiting in the phosphorylation of deoxyglucose added to synaptosomal incubations (27). Since the phosphate ester is not further metabolized (26), the amount of deoxyglucose 6-phosphate which accumulates reflects the level of ATP in the cell during the labeling pulse, and the specific radioactivity of the deoxyglucose-6-\(^{32}P\)ATP reflects the specific radioactivity of the \(\gamma-{^32}P\)ATP. None of these approaches indicated any influence of cholinergic agents on the radioactivity of ATP (Table II).

The second possibility, \(de\) novo synthesis of phosphatidate and phosphatidylinositol, cannot be studied by chemical analysis because the predicted incremental phospholipid formed due to the action of cholinergic agents is small compared to the total amount present (12). In order to evaluate \(de\) novo phospholipid synthesis, \([\gamma-{^32}P]\)glycerol or \([\gamma-{^32}P]\)glucose was added to incubations, and incorporation into phosphatidate and phosphatidylinositol was measured (Table III). Their incorporation was not increased by the presence of acetylcholine. This confirms and extends previous findings (28) that the incorporation of \([\gamma-{^32}P]\)glycerol into the glycerophosphate fraction of guinea pig cortex slices was not stimulated by the addition of acetylcholine. It therefore must be assumed that the glycerol moiety need not turn over in response to acetylcholine in order to produce increased \(^{32}P\) incorporation.

Concerning the third hypothesis, a stimulation of diglyceride kinase by acetylcholine has been proposed (29), but this appears
unlikely since acetylcholine does not exert a direct effect on the enzyme (28, 30). Also, this mechanism does not offer a simple explanation to the decrease of phosphatidate labeling seen in long term incubations (Fig. 4) and in the prior incubation experiments (Figs. 5 and 6).

With regard to the last possibility, it has been suggested that diglyceride limits the reaction sequence (II to IV) (28, 31) and thus the incorporation of **P into phosphatidate and phosphatidylinositol. Increased diglyceride formation mediated by acetylcholine would increase labeling of phosphatidate and phosphatidylinositol. Since de novo synthesis can be ruled out as a prerequisite for increased phosphatidate labeling, another source of diglyceride must be assumed. The formation of a precursor in the stimulated pathway can indeed be deduced from the studies in which acetylcholine is added before the addition of **P (Table IV). Prior presence of acetylcholine increases **P-labeling above the amount of stimulation achieved when acetylcholine is added together with the radioactive phosphate. Atropine, which completely blocks the acetylcholine effect when added together with the neurotransmitter, only partly blocks cholinergic stimulation when added after a preincubation with acetylcholine. This suggests the presence of a higher amount of diglyceride resulting from the acetylcholine addition. Such diglyceride has been speculated to be derived from phosphatidylinositol or phosphatidylinositol phosphate and phosphatidylinositol diphosphate (31, 32). In the case of the purported stimulated reaction: Phosphatidylinositol + H2O → diglyceride + d-myoinositol 1:2-cyclic phosphate (33), the acetylcholine-stimulated pathway would in this way complete a closed reaction sequence (31). Stimulation by acetylcholine of the phosphodiesteric cleavage of phosphatidylinositol has been reported (34), but this claim is in contrast to prior (31) and subsequent (15, 35) investigations. In addition, we do not find prelabeled **P phosphatidylinositol responsive to the presence of cholinergic agents (Figs. 5 and 6). Hokin and Hokin (36) concluded for the acetylcholine effect on avian salt gland that the incremental labeling of phosphatidylinositol is cumulative and remains stable when the tissue reverts to the unstimulated state. The polyphosphoinositides have also been considered as possible sources of diglyceride (37). Although acetylcholine can be demonstrated to decrease labeled phosphatidylinositol phosphate and phosphatidylinositol diphosphate (15), these lipids are not likely donors of diglyceride for the acetylcholine effect, for a number of reasons relating to the nonspecificity of the decrease (38).

We have previously speculated (12) that phosphatidate cleavage to diglyceride might be enhanced by acetylcholine. Several indirect indications led us to investigate this possibility further. The stimulation of **P3 incorporation into phosphatidate decreased with time (Fig. 4). There was however, no apparent change in the **P3ATP level in the presence of acetylcholine. The breakdown of **P phosphatidate in the presence of acetylcholine, after **P phosphatidate had apparently reached an equilibrium with **P3ATP, therefore appears to be more rapid than in its absence. Fluoride, an inhibitor of phosphatidate phosphohydrolase (39), may block the acetylcholine effect at a concentration which apparently does not block oxidative phosphorylation (Table V). In experiments designed to directly observe possible catabolic processes that might be stimulated by acetylcholine, nerve endings were incubated with **P, for 60 min with and without acetylcholine, at which time the specific activity of ATP was lowered drastically by the addition of 5 mM sodium phosphate (Fig. 5). **P-Labeled phosphatidate decreased, whereas labeled phosphatidylinositol remained stable. In addition, this decrease of **P phosphatidate appeared to be accelerated in the presence of acetylcholine. When we labeled nerve endings for 60 min, and then added dinitrophenol to prevent ATP formation during further incubations in the presence or absence of acetylcholine (Fig. 6), radioactivity in phosphatidylinositol again remained stable, but **P phosphatidate rapidly decreased. This decrease was again more rapid in the presence of acetylcholine than in its absence. In experiments in which we compared the rates of **P phosphatidate hydrolysis in the presence and absence of acetylcholine between 10 and 30 min after the addition of dinitrophenol (Table VI), we observed an 80% stimulation of **P phosphatidate breakdown in the presence of acetylcholine. Atropine, 10^-5 M, effectively blocked this effect. Since stimulated conversion of phosphatidate to phosphatidylinositol or to lyso phosphatidate was deemed unlikely by the experimental findings, the results are best explained by increased phosphatidate hydrolysis.

Cholinergic agents therefore appear to increase phosphatidate labeling by increasing its hydrolysis, coupled with the action of diglyceride kinase, which is substrate-limited. This hypothesis predicts that phosphatidate labeling is increased in the presence of acetylcholine or carbamylcholine only as long as the available phosphatidate pool is largely unlabeled so that the rate of labeling from the increased diglyceride levels determines the **P incorporation into phosphatidate. After equilibration of **P phosphatidate with (γ-**P)ATP, however, labeling of phosphatidate in the presence of acetylcholine should be lowered since stimulation of phosphatidate hydrolysis lowers the amount of phosphatidate. Although we cannot measure the latter even after prolonged incubations (Fig. 4), the decrease in stimulation of phosphatidate labeling with time is in good agreement with this hypothesis.

In studies on guinea pig synaptosomal fractions, Yagihara et al. (40) report a decrease in phosphatidate following acetylcholine addition. A high degree of synaptosomal phosphatidate phosphohydrolase activity has been localized to the plasma membrane fraction (41). In our hands a plasma membrane fraction obtained by synaptosomal fractionations following incubation has the greatest amount of stimulated lipid (14).

Whether phosphatidate phosphohydrolase is specifically stimulated by the presence of cholinergic agents or whether the neurotransmitter-membrane interaction changes membrane conformation to expose phosphatidate to the enzyme cannot be determined from the available data. The latter suggests that physiological processes leading to membrane motion, e.g. pinocytosis, secretion, etc. would lead to increased lipid labeling (2).

Our hypothesis predicts that stimulation of phosphatidate labeling is due to an increase in its specific radioactivity rather than in its level. Thus, labeling of phosphatidylinositol from **P3, since **P phosphatidylinositol is synthesized from **P phosphatidate, is expected to be increased. Labeling of phosphatidylinositol from [H]inositol, however, should not be stimulated. Yet we see a small stimulation (18% ± 8% S.D.) of [H]inositol incorporation. An essentially parallel stimulation of incorporation of **P, and [H]inositol into phosphatidylinositol in the presence of acetylcholine has been reported for brain slices (42), whereas in ganglia, [H]inositol incorporation into phosphatidylinositol is stimulated significantly less by preganglionic nerve pulses than the incorporation of **P (43). In the present study the small increase of [H]inositol incorporation in the presence of cholinergic agents may be interpreted as a secondary effect, due for instance to an increase in permeability of the synaptosomal membranes to inositol, or to a stimulation of
lipid metabolism at a site different from that of the phosphatidate effect (44, 45). It is indeed likely that there is more than one acetylcholine-lipid labeling effect. Hokin (8, 9) and Larabee (7) have presented evidence for a specific stimulation of phosphatidylinositol labeling in postsynaptic neurons by various agents. Since the synaptosomal fragments should not contain the intact postsynaptic cell, it is clear that our preparation will not reflect such labeling.

Increased phosphatidylinositol labeling in the absence of stimulated phosphatidate labeling has been observed in various tissue preparations (5, 7). Such findings cannot be explained by hypotheses relating the action of cholinergic agents to increased diglyceride formation from phosphatidylinositol or from any lipid other than phosphatidate unless further assumptions about compartmentation, pool-size changes, etc. are made. In terms of our suggestion that phosphatidate hydrolysis mediates the effect of cholinergic agents, such results might reflect the dependence of the observed stimulation of phosphatidate labeling upon the time chosen for the experiment. Acetylcholine effects therefore depend upon the tissue preparation examined, on the labeled precursor used, and on the experimental conditions. We have demonstrated an acetylcholine-effect acting primarily on 32P incorporation into phosphatidate and phosphatidylinositol, and to a much lesser degree on [3H]inositol incorporation into or loss from (18) phosphatidylinositol. It may be that much of the confusion in understanding the action of cholinergic agents on lipid labeling can be attributed to their multiple effects in intact tissues.

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REFERENCES

22. Manning, R., and Brindley, D. N. (1972) Biochem. J. 130, 1003-1012