EFFECTS OF ACETYICHOLINE ON PHOSPHOLIPIDES IN THE PANCREAS

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We previously reported that acetylcholine or carbamylcholine stimulates enzyme secretion (1) and the incorporation of P32 into the phospholipides of pancreas slices (2). The present report deals with further studies on the effect of acetylcholine and related substances on phospholipide metabolism in pancreas both in vitro and in vivo. It has been found that the increased incorporation of P32 in phospholipides in the presence of acetylcholine is due to an independent turnover of phosphate (and presumably the base attached to it) in preformed phospholipide rather than to an increased synthesis of phospholipide de novo. This increased turnover of phosphate appears to be due to a direct action of acetylcholine on phospholipides or lipoproteins; it does not appear to be closely related to the process of enzyme secretion, as was previously suggested (2).

EXPERIMENTAL

Preparation of Tissue Slices—Pancreas slices from fed pigeons were prepared and incubated and the tissues treated after incubation as described previously (2). The slices were preincubated for 30 minutes to remove amylase from damaged surface cells so that more accurate estimation of amylase secretion could be made. Units of amylase are those defined by Smith and Roe (3).

Mouse Experiments in Vivo—Female albino mice weighing about 25 gm. were injected with approximately 70 µc. of NaH2P32O4 intraperitoneally. Pilocarpine and carbamylcholine were injected intraperitoneally. After a given interval of time the mice were killed by decapitation, and the pancreas was quickly removed and chilled in iced saline. The specific activities of the phospholipides and the acid-soluble phosphate ester fraction were determined as described previously (2), and the amylase content of the tissue was assayed.

Extraction and Fractionation of Phospholipides—The ethanol-ether extracts from the tissues were treated with inorganic phosphate and serial extractions with ether in an atmosphere of nitrogen as described earlier.

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Aliquots of the final ether extracts were taken for determination of the total ether-soluble phospholipides and the determination of specific activity. The remainder of each sample was evaporated to dryness in a conical centrifuge tube and separated into choline-containing and non-choline-containing fractions by adsorption of the acidic lipides on MgO by the method of Taurog, Entenman, Fries, and Chaikoff (4).

**Determination of Specific Activity of Glycerophosphate from Phospholipides**—The ethanol-ether extracts (1) were evaporated to dryness at 70° in a stream of air. 1 ml. of 5 N KOH was added, and the phospholipides were hydrolyzed for 1 hour at 75°. Alkali hydrolyzes lecithins and cephalins to fatty acids, bases, and glycerophosphate (5). After hydrolysis the samples were cooled to 0°, and 6 N perchloric acid was added to precipitate KClO₄ and fatty acids. The pH was adjusted to about 1. After centrifugation the supernatant fluid was heated at 100° for 10 minutes. The pH was adjusted to about 3.5 with KOH, and the mixture was chilled to 0°. After further centrifugation the supernatant fluid was pipetted onto strips of Whatman No. 3 MM filter paper (10 X 57 cm.) (6). About 50 γ of glycerophosphate P were run on each strip. The strips were wetted with buffer and placed in an ionophoresis apparatus previously described (6). 0.02 m potassium lactate buffer, pH 3.6, was used. A potential difference of 1400 volts was applied for 2 to 2½ hours. The strips were removed from the apparatus and dried in a stream of warm air. Phosphorus compounds were detected on paper by the method of Wade and Morgan (7). One major phosphate band which had run about 30 cm. from the base-line had the same mobility as α- and β-glycerophosphates. The glycerophosphate was preceded by two narrower phosphate-containing bands, the furthest from the base-line having the same mobility as orthophosphate.

To test for radiopurity, several samples of the phospholipide hydrolysate were run, and the ionophoresis paper was cut into narrow strips (2 mm. wide) in the neighborhood of the glycerophosphate band. The total radioactivity of each strip was plotted against the distance from the base-line. In experiments with glycerol-1-Cl⁴ the glycerophosphate band corresponded to a sharp peak of radioactivity which was preceded by a much smaller peak. In experiments with P³², peaks of radioactivity corresponded to the glycerophosphate and to the phosphorus-containing bands immediately preceding it.

To determine the specific activity of the glycerophosphate, the band containing it was cut out and eluted with 1 m NH₄OH, as described previously for ribonucleotides (6). Aliquots were taken for counts and total phosphorus.

**Expression of Specific Activity**—In experiments in vitro all P³² counts have been corrected to relate them to a specific activity of 100,000 c.p.m. per γ of
P for the inorganic P of the medium. The C\textsuperscript{14} counts of the glycerophosphate isolated from the phospholipides are expressed as counts per minute per microgram of glycerophosphate P. In mouse experiments the specific activities of the phospholipides are expressed as a percentage of the specific activity of the acid-soluble phosphate ester fraction.

\section*{Results}

\textit{Incorporation of P\textsuperscript{32} into Choline-Containing and Non-Choline-Containing Phospholipide Fractions}—In unstimulated slices the specific activity of the non-choline-containing phospholipide fraction was about twice that of the choline-containing fraction. When acetylcholine or carbamylcholine was added, the incorporation of P\textsuperscript{32} into the non-choline-containing phospholipides was stimulated 1000 to 2500 per cent; the incorporation of P\textsuperscript{32} into the choline-containing phospholipides was stimulated 400 to 600 per cent. These effects were abolished by atropine. A representative experiment is shown in Table I. The stimulation in the choline-containing fraction was not due to contamination with non-choline phospholipides, since it remained constant after successive treatments of the choline-containing phospholipides with MgO.

The phospholipide P content was the same in slices incubated with and without carbamylcholine. The ratio of the choline-containing to the non-choline phospholipides was about 3:1 and was not affected by carbamylcholine.

\textit{Effect of Acetylcholine on Incorporation of Glycerol-1-C\textsuperscript{14} and P\textsuperscript{32} into Glycerophosphatides}—Studies reported in a preliminary communication (8) indicated that the incorporation of glycerol-1-C\textsuperscript{14} into the total phospho-

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
\textbf{Addition} & \textbf{Specific activity of phospholipides, c.p.m. per \( \gamma P \)} & \multicolumn{2}{c|}{\textbf{Phospholipide \( \gamma P \)}} \\
 & & \textbf{Choline-} & \textbf{Non-choline-} \\
 & & \textbf{containing} & \textbf{containing} \\
\hline
None & 148 & 108 & 287 \\
Carbamylcholine (5 \( \times \) 10\textsuperscript{-5} M) & 1108 & 660 & 4470 \\
Acetylcholine (5 \( \times \) 10\textsuperscript{-6} M) & 1096 & 800 & 3600 \\
Carbamylcholine (5 \( \times \) 10\textsuperscript{-4} M) + atropine (3 \( \times \) 10\textsuperscript{-5} M) & 136 & 127 & 388 \\
\hline
\end{tabular}
\caption{Incorporation of P\textsuperscript{32} into Choline- and Non-Choline-Containing Phospholipides in Slices of Pigeon Pancreas}
\end{table}


Medium, bicarbonate saline; duration of incubation, 2 hours.
lipide fraction was not appreciably stimulated by cholinergic substances; this result suggests that the synthesis of phospholipides \textit{de novo} was not stimulated. But in order to achieve unequivocal results it was necessary to isolate from the phospholipide fraction a chemically defined substance containing both glycerol and phosphate. This was achieved by alkaline hydrolysis of the total phospholipides and isolation of glycerophosphate by ionophoresis on paper, as described above.

Acetylcholine stimulated the incorporation of P$^{32}$ into the glycerophosphatides about 850 per cent (Table II). Glycerol-1-C$^{14}$ was incorporated into the glycerophosphatides, but this incorporation was increased no more than 20 per cent in the presence of acetylcholine. This indicates that the

\begin{table}
\begin{tabular}{|l|c|c|}
\hline
Addition & Specific activity of glycerophosphate from phospholipides, c.p.m. per \gamma \text{ glycerophosphate P} \\
\hline
None & 19.5 & 114 \\
Acetylcholine (3 \times 10^{-4} M) + eserine (3 \times 10^{-4} M) & 23.8 & 979 \\
\hline
\end{tabular}
\end{table}

Incubation with either glycerol-1-C$^{14}$ or P$^{32}$ carried out in separate vessels; glycerol-1-C$^{14}$, 1 \mu c. per \mu M, 2 \mu M per ml.; approximately 175 mg. of pigeon pancreas per vessel; slices incubated in 3 ml. of Mixture III without added organic constituents (1); temperature, 40°; duration of incubation, 2 hours; alkali in center well.

stimulated incorporation of P$^{32}$ into phospholipides is due to an increased turnover of the phosphate-base moiety in preformed phospholipides rather than to an increased synthesis of phospholipides \textit{de novo}.

The data presented above suggest that the incorporation of glycerophosphate into phospholipides observed by Kornberg and Pricer (9, 10) and Kennedy (11) may represent the normal pathway of phospholipide synthesis, whereas the incorporation of phosphorylcholine (10) may represent an independent turnover of this moiety in preformed phospholipide. Artom (12) has provided evidence that choline may, under certain circumstances, turn over in phospholipides independently of phosphate.

\textit{Effects of Various Substrates on Incorporation of P$^{32}$ into Phospholipides of Pancreas Slices}—If acetylcholine stimulates phosphate turnover rather than total phospholipide synthesis, substances known to be present in phospholipides should not appreciably increase the stimulated incorporation of P$^{32}$. But these substances might increase the unstimulated incor-
poration of P³², since this probably represents, at least to some extent, total phospholipide synthesis. A mixture of phospholipide constituents did increase the unstimulated rate of P³² incorporation from 50 to 100 per cent, but had no measurable effect on the stimulated rate of P³² incorporation (Table III). Kennedy (11) has shown that unlabeled glycerol approximately doubles the incorporation of P³² into phospholipides in rat liver mitochondria. Artom and Cornatzer (13) and Platt and Porter (14) have shown that ethanolamine injection increases the incorporation of P³² into

**Table III**

*Effect of Various Substrates on Incorporation of P³² into Phospholipides in Slices of Pigeon Pancreas*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Addition</th>
<th>Specific activity of phospholipides, c.p.m. per γ P²³⁰⁰</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without carbamylcholine</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Inositol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Ethanolamine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DL-Serine</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Inositol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanolamine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DL-Serine</td>
<td></td>
</tr>
</tbody>
</table>

Substrates added to give 2 µM per ml. of medium, except for DL-serine which was 4 µM per ml. of medium. Medium, bicarbonate saline; duration of incubation, 2 hours.

choline-containing and non-choline-containing phospholipides. Choline was not included in the mixture used here, because in high concentrations it simulates the actions of acetylcholine to some extent (see below).

*Disproportionate Effects of Cholinergic Substances on Amylase Secretion and on Incorporation of P³² into Phospholipides in Pancreas Slices—*The effects of increasing concentrations of acetylcholine on enzyme secretion and on the incorporation of P³² into phospholipides do not parallel each other (Table IV). Maximal enzyme secretion was obtained when the concentration of acetylcholine was 10⁻⁶ M; the maximal effect on phospholipides was not reached until the acetylcholine concentration was 10⁻⁵ M. Similar results were obtained with carbamylcholine which stimulated enzyme secretion half maximally before the concentration was high enough to
give any significant effect on the incorporation of $P^{32}$ into the phospholipides. Table IV also shows that $3 \times 10^{-4}$ M eserine alone stimulated enzyme secretion about 40 per cent as much as $10^{-6}$ M acetylcholine (with eserine), but stimulated the incorporation of $P^{32}$ into phospholipides only 5 per cent as much as the acetylcholine. The fact that eserine can

### Table IV

**Effect of Increasing Concentration of Acetylcholine on Amylase Secretion and Incorporation of $P^{32}$ into Phospholipides in Slices of Pigeon Pancreas**

<table>
<thead>
<tr>
<th>Concentration of acetylcholine</th>
<th>Other additions</th>
<th>Amylase secretion*</th>
<th>Specific activity of phospholipides</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td></td>
<td>units per mg. dry weight</td>
<td>c.p.m. per $\gamma P$</td>
</tr>
<tr>
<td>0</td>
<td>None</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>Eserine ($3 \times 10^{-4}$ M)</td>
<td>6.1</td>
<td>98</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>Same</td>
<td>13.5</td>
<td>120</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>&quot;</td>
<td>17.0</td>
<td>524</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>&quot;</td>
<td>16.2</td>
<td>830</td>
</tr>
<tr>
<td>&quot;</td>
<td></td>
<td>16.9</td>
<td>770</td>
</tr>
</tbody>
</table>

Medium, bicarbonate saline; duration of incubation, 2 hours.

* Expressed as the difference between the amylase in the medium of slices with added eserine and acetylcholine and the amylase in the medium of the control slice.

### Table V

**Effect of Choline on Amylase Secretion and Incorporation of $P^{32}$ into Phospholipides in Slices of Pigeon Pancreas**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Amylase secretion*</th>
<th>Specific activity of phospholipides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units per mg. dry weight</td>
<td>c.p.m. per $\gamma P$</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>Choline ($2 \times 10^{-3}$ M)</td>
<td>19.6</td>
<td>99</td>
</tr>
<tr>
<td>&quot; ($2 \times 10^{-3}$ M) + eserine ($3 \times 10^{-4}$ M)</td>
<td>35.2</td>
<td>120</td>
</tr>
<tr>
<td>Carbamylcholine ($5 \times 10^{-5}$ M)</td>
<td>35.2</td>
<td>360</td>
</tr>
</tbody>
</table>

Medium, bicarbonate saline; duration of incubation, 2 hours.

* Expressed as in Table IV.

stimulate enzyme secretion in slices of pigeon pancreas suggests that small amounts of acetylcholine are present in the tissue.

Choline ($2 \times 10^{-3}$ M) stimulated enzyme secretion about half maximally and increased the incorporation of $P^{32}$ into phospholipides about 150 per cent (Table V). Choline with eserine ($3 \times 10^{-4}$ M) was as effective as $5 \times 10^{-6}$ M carbamylcholine in stimulating amylase secretion, but was only about 25 per cent as effective as carbamylcholine in stimulating the incorporation of $P^{32}$ into phospholipides.
The above results show that the phospholipide effect, rather than being secondary to the secretion of enzymes, as was suggested previously (2), seems to be a direct response to cholinergic substances.

Amylase Secretion and Incorporation of $^{32}$P into Phospholipides in Mouse Pancreas in Vivo—Since feeding is the normal physiological stimulus to pancreatic secretion, the specific activities of the phospholipides of pancreas from fed and fasted mice were compared 1 hour after injection of NaH$_2$P$_{32}$O$_4$. The specific activities of the phospholipides were the same in fed mice which were given food ad libitum and of mice fasted for 24 hours (Table VI). On the other hand, the administration of pilocarpine or carbamylcholine to either fed or fasted mice increased the incorporation of $^{32}$P into the phospholipides of the pancreas over 2-fold. These effects are much less than those observed with pilocarpine or carbamylcholine in pigeon or mouse pancreas in vitro, but this is probably due to the much lower concentration of the drug attained in vivo. The injection of pilocarpine or carbamylcholine to fed or fasted mice appreciably lowered the amylase content of the pancreas, indicating a good stimulation of enzyme secretion. It should be noted that the amylase content of the pancreas of the fasted mice was less than half that of the fed animals. The 24 hour fasting may so deplete the animal of substrates for enzyme synth-
sis that the enzyme content of the pancreas falls, in spite of the fact that the fasted mouse secretes less enzyme.

The incorporation of P32 into the phospholipides in the pancreas of the fed and fasted mice was also followed with time. After an initial lag period the specific activities of the phospholipides (expressed directly as counts per minute per microgram of P) increased at a constant rate up to about 60 minutes and then began falling off. The rates of incorporation of P32 into phospholipides of fed and fasted mice were not significantly different. These findings show that a pancreas which is secreting under physiological conditions does not have a higher rate of incorporation of P32 into phospholipides than has a non-secreting pancreas. The quantities of acetylcholine liberated in the pancreas by the cholinergic nerves during normal secretion are probably too small to have any appreciable effect on phospholipides.

DISCUSSION

Acetylcholine may stimulate the independent turnover of phosphate (and presumably the base attached to it) in phospholipides by activating enzyme systems responsible for this independent turnover or by rendering the phospholipides more accessible to these enzyme systems. The latter alternative is preferred for the following reasons. In the cell, phospholipides are chiefly combined with proteins, and one of the major types of combination is very probably a salt-like linkage between the phosphate group of the phospholipide and basic groups of the protein (15, 16). It is likely that in this combined state an independent turnover of the phosphate-base moiety would be prevented. If acetylcholine split certain susceptible lipoproteins into free phospholipides and proteins, or if it prevented their combination, the phosphate-base moiety in the phospholipides would become accessible to enzymes which could catalyze its independent turnover. A chemical basis for this interpretation is provided by our recent observation that acetylcholine can prevent the formation of an insoluble complex between phospholipides and albumin.1

Acetylcholine has properties which might make it particularly capable of splitting lipoproteins. It is structurally similar to cationic detergents, which are known to split lipoproteins (15), and it is active at aqueous-non-aqueous interfaces (17). The high biological specificity of acetylcholine would of course depend upon the presence of specific receptor lipoproteins. Assuming that the above interpretation is correct, the pancreas would appear to be particularly rich in this type of lipoprotein. However, we

1 Unpublished observations.
have found that cholinergic drugs also stimulate the incorporation of P^32 into the phospholipides of slices of guinea pig brain cortex (2, 18), rabbit parotid,1 and rabbit submaxillary gland.1

The mechanism of action of acetylcholine postulated here, i.e. the dissociation of lipoproteins, could be its mechanism of action at the molecular level in all types of cells responsive to this substance, although in many instances the changes may be too subtle to be detected by the technique used here. For instance, small changes in the physical state of the lipoproteins of the cell membrane, by affecting permeability, could lead to marked changes in physiological function.

SUMMARY

1. Acetylcholine stimulates the incorporation of P^32 into the choline-containing phospholipides of pancreas slices by 400 to 600 per cent and into the non-choline-containing phospholipides by 1000 to 2500 per cent.

2. Acetylcholine stimulates the incorporation of P^32 into the glycerophosphatides by 850 per cent. Glycerol-1-C^14 is incorporated into the glycerophosphatides, but this incorporation is not stimulated by acetylcholine, indicating that acetylcholine stimulates the independent turnover of the phosphate-base moiety in preformed phospholipides.

3. A mixture of inositol, glycerol, ethanolamine, and DL-serine increases the unstimulated rate of incorporation of P^32 into phospholipides by 50 to 100 per cent, but has no appreciable effect on the stimulated rate of P^32 incorporation.

4. Enzyme secretion and the incorporation of P^32 into phospholipides in vitro do not parallel each other as the concentration of acetylcholine is increased or when choline or eserine is added. The incorporation of P^32 in vivo into phospholipides is the same in the pancreas of fed and fasted mice. Injection of pilocarpine or carbamylcholine stimulates the incorporation of P^32 into the phospholipides of the pancreases of both fed and fasted mice. These findings indicate that enzyme secretion and the incorporation of P^32 into phospholipides are not directly related.

5. It is suggested that acetylcholine acts at the molecular level by splitting phospholipide-protein complexes (lipoproteins), rendering the phosphate-base moiety of the phospholipides accessible to enzymes which catalyze its rapid turnover.

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