Acetylcholine and the Exchange of Inositol and Phosphate in Brain Phosphoinositide

LOWELL E. HOKIN† AND MABEL R. HOKIN†

From the Department of Pharmacology, McGill University, Montreal, Canada

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Previous studies showed that acetylcholine, in the presence of eserine, stimulated the incorporation of P\(^{32}\) into the phospholipides in slices of guinea pig brain cortex (2, 3). The major stimulation occurred in phosphoinositide and in phosphatic acid, with a lesser stimulation in phosphatidyl choline (3). The incorporation of glycerol-1-C\(^{14}\) into the total glycerophosphatide fraction was not increased under conditions in which P\(^{32}\) incorporation was stimulated (2). The physiological significance of the phospholipide effect in brain cortex slices is not yet understood, although some suggestions have been advanced (4).

The present study is primarily concerned with the incorporation of inositol-2-P\(^{32}\) into phosphoinositide in slices of guinea pig brain cortex incubated in the presence and absence of acetylcholine. The radioactive phosphoinositide isolated from brain cortex slices after incubation in vitro has been shown to be a monophosphoinositide. Stimulation by acetylcholine of P\(^{32}\) incorporation into this phosphoinositide is accompanied by an equal percentage of stimulation of inositol-2-P\(^{32}\) incorporation; the incorporation of glycerol-1-C\(^{14}\) is not stimulated.

The incorporation of P\(^{32}\), glycerol-1-C\(^{14}\), and inositol-2-P\(^{32}\) into the other phospholipides in the presence and absence of acetylcholine has also been studied.

EXPERIMENTAL

Slices of guinea pig brain cortex were prepared as described previously (2). In the present work, two male guinea pigs that weighed individually between 500 and 600 gm. were used for each experiment. Equal portions of between 80 and 100 mg. of tissue from each brain were placed in each vessel. The slices were incubated in 2 ml. of Krebs-Henseleit bicarbonate saline (5) which also contained 2 mg. of glucose, 1 mg. of inositol, and 125 \(\mu\)g. of glycerol. Drugs were added as indicated. Each vessel contained either Na\(_2\)H\(_2\)PO\(_4\) (50 to 250 \(\mu\)c. per \(\mu\)mole), inositol-2-P\(^{32}\) (1 \(\mu\)c. per \(\mu\)mole), or glycerol-1-C\(^{14}\) (1 \(\mu\)c. per \(\mu\)mole). Inositol-2-P\(^{32}\) was synthesized from myoinosose-2 as described previously (6). The tissues were incubated in stoppered Erlenmeyer flasks (25 ml. capacity) in a Dubnoff metabolic incubator for 2 hours at 38\(^\circ\) with 95 per cent \(O_2\) + 5 per cent \(CO_2\) as the gas phase.

After incubation, the lipides were extracted, separated by chromatography, and assayed for radioactivity as described previously (6, 7).

The total phospholipide-P of brain cortex slices was determined by digestion of samples of the chloroform extracts and estimation of total P by the method of Fiske and SubbaRow (8).

RESULTS

Phospholipide Content of Brain Cortex Slices Incubated in Presence and Absence of Acetylcholine—The quantities of phospholipide-P extracted from separate samples of tissue which had been incubated under identical conditions agreed within 5 per cent. The phospholipide-P content of incubated slices of guinea pig brain was about 125 \(\mu\)g. per 100 mg. of fresh tissue. Addition of acetylcholine (with eserine) to the incubation medium did not result in an increase in the phospholipide-P content of the tissue.

Identification of Phospholipides—After chromatography of the P\(^{32}\)-labeled phospholipides from slices of guinea pig brain cortex, the autoradiograms showed seven clearly defined spots (Fig. 1). Phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, and phosphatic acid were identified by the methods described elsewhere (6, 9).

Folch (10) showed that calf brain contains a diphosphoinositide which, on hydrolysis in 6 \(N\) HCl at 100\(^\circ\) for 10 minutes, yielded 84 per cent of the P of the mother substance as inositol medadiphosphate and 4 per cent as orthophosphate. Hydrolysis under the same conditions of a sample of the potassium salt of diphosphoinositide kindly given to us by Dr. Folch, followed by electrophoresis on paper in 0.02 \(N\) lactate buffer, pH 3.6 (11), revealed four phosphate-containing spots (located by the method of Wade and Morgan, (12)) which had the same mobilities as authentic samples of inositol medadiphosphate, orthophosphate, glycercophosphate, and inositol monophosphate; the relative mobilities of these compounds are indicated by arrows in Fig. 2. Hutchison et al. (13) have previously shown that inositol medadiphosphate has a higher electrophoretic mobility than orthophosphate. To identify the P\(^{32}\)-labeled phospholipide of Spot 2 on the chromatograms (Fig. 1), the material was eluted in chloroform-methanol-water (75:25:2) (6) and hydrolyzed in 6 \(N\) HCl at 100\(^\circ\) for 10 minutes. Electrophoresis on paper of the hydrolysate with authentic samples of inositol monophosphate, glycercophosphate, orthophosphate, and inositol medadiphosphate (6), followed by spraying and autoradiography, showed that the main radioactive spot coincided with the inositol monophosphate spot; there was also a labeled glycercophosphate spot, a labeled orthophosphate spot, and a
labeled spot with very low mobility which was not identified (Fig. 2). The radioactivity of the inositol monophosphate accounted for 67 per cent of the total radioactivity of the hydrolysate; 18 per cent of the total radioactivity was recovered as glycerophosphate, 2 per cent as orthophosphate, and 1 per cent as the unidentified spot near the baseline. There was no evidence of a P32-labeled inositol metadiphosphate spot. It therefore appears that the radioactive material of Spot 2 (Fig. 1) is a monophosphoinositide. This labeled monophosphoinositide is not an artifact in vivo in brain slices. When phospholipides labeled with P32 in vivo in mouse brain (9) were examined, the same highly labeled phosphoinositide spot was seen. Hydrolysis of an eluate of this material with 6 N HCl at 100° for 10 minutes yielded 80 per cent of the radioactivity as inositol monophosphate, 14 per cent as glycerophosphate, and 3 per cent as orthophosphate; there was no evidence of an inositol metadiphosphate spot on the autoradiogram of the electrophogram.

Chromatography of a sample of diphosphoinositide under the same conditions as those used for the separation of phospholipides from brain extracts, followed by staining with Rhodamine G and visualization in ultraviolet light (14), revealed spots with an Rf which ranged from 0 to 0.03. On the chromatograms of P32-labeled lipides from brain cortex slices the baseline was usually only faintly labeled, and no spot with appreciable radioactivity was seen between the baseline and Spot 1 (Rf, 0.13) (Fig. 1). It seemed, therefore, either that very little P32 was incorporated into diphosphoinositide, or that most of the diphosphoinositide was not present in the lipid extracts as prepared here. Further evidence for this was obtained. Carrier diphosphoinositide (100 µg.) was added to a P32-labeled phospholipide extract which contained 22.5 µg. of phospholipide-P, and the mixture was hydrolyzed in 6 N HCl at 100° for 10 minutes. After electrophoresis of the hydrolysate, spraying revealed phosphate-containing spots with mobilities identical with those of authentic samples of inositol metadiphosphate, orthophosphate, glycerophosphate, and inositol monophosphate; there was also a fifth spot of very low mobility. These five spots were also seen on the autoradiogram; they showed the following percentage composition with respect to P32: inositol metadiphosphate, 1.5; orthophosphate, 18; glycerophosphate, 51.4; inositol monophosphate, 27.5; the unidentified spot, 1.6.

Several groups of workers have reported that a highly labeled inositol-containing substance, presumably glycerophosphorylinositol, was obtained after hydrolysis with 0.2 M methanolic NaOH of P32-labeled phospholipides from brain “dispersions” (15), brain mitochondria (16), and brain cortex slices (3). It was assumed that this hydrolysate product was derived from diphosphoinositide. Since the methods of extraction of the lipides were essentially the same as used here, the highly labeled inositol-containing hydrolysate product was in all likelihood derived from brain monophosphoinositide rather than from diphosphoinositide.

Effect of Acetylcholine on Incorporation of Inositol-2-H3, P32, and Glycerol-1-C14 into Monophosphoinositide in Slices of Guinea Pig Brain Cortex—After incubation of slices of guinea pig brain cortex with inositol-2-H3, the only lipide which contained any significant radioactivity on the chromatograms was the monophosphoinositide of Spot 2; the H3 radioactivity of the monophosphoinositide agreed within experimental error with the H3 radioactivity of the inositol monophosphate which was not identified (Fig. 2). The radioactivity of the inositol monophosphate accounted for 67 per cent of the total radioactivity of the hydrolysate; 18 per cent of the total radioactivity was recovered as glycerophosphate, 2 per cent as orthophosphate, and 1 per cent as the unidentified spot near the baseline. There was no evidence of a P32-labeled inositol metadiphosphate spot. It therefore appears that the radioactive material of Spot 2 (Fig. 1) is a monophosphoinositide. This labeled monophosphoinositide is not an artifact in vivo in brain slices. When phospholipides labeled with P32 in vivo in mouse brain (9) were examined, the same highly labeled phosphoinositide spot was seen. Hydrolysis of an eluate of this material with 6 N HCl at 100° for 10 minutes yielded 80 per cent of the radioactivity as inositol monophosphate, 14 per cent as glycerophosphate, and 3 per cent as orthophosphate; there was no evidence of an inositol metadiphosphate spot on the autoradiogram of the electrophogram.
Acetylcholine Effects in Brain Slices

TABLE I

Effect of acetylcholine on incorporation of $^{32}$P, glycerol-1-Cl4, and inositol-2-H3 into individual phospholipides in slices of guinea pig brain cortex

All slices were from the same animals and pooled as described in the text. The experimental vessels contained $10^{-3}$ M acetylcholine plus $3 \times 10^{-4}$ M eserine, NaH$_2$P$_2$O$_4$, glycerol-1-Cl4, or inositol-2-H3 were added as indicated. The counts per minute are corrected for initial specific activities in the medium of 25 c.p.m. of $^{32}$P per pmole of phosphate, 1 c.p.m. of Cl4 per pmole of glycerol, and 1 c.p.m. of H3 per pmole of inositol.

<table>
<thead>
<tr>
<th>Phospholipide</th>
<th>NaH$_2$P$_2$O$_4$</th>
<th>Glycerol-1-Cl4</th>
<th>Inositol-2-H3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Stimulated</td>
<td>Increase</td>
</tr>
<tr>
<td>Unidentified Spot 1</td>
<td>3,300</td>
<td>4,102</td>
<td>94%</td>
</tr>
<tr>
<td>Phosphoinositide</td>
<td>10,800</td>
<td>25,700</td>
<td>136%</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>24,400</td>
<td>31,000</td>
<td>27%</td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td>3,500</td>
<td>3,880</td>
<td>8%</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>3,880</td>
<td>4,850</td>
<td>25%</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>4,180</td>
<td>16,000</td>
<td>385%</td>
</tr>
<tr>
<td>Nonphosphorus-containing lipids</td>
<td>68,100</td>
<td>108,000</td>
<td>58%</td>
</tr>
</tbody>
</table>

* —, counts not significantly above background.
† Not corrected for retention on paper.

radioactivity of the total lipide fraction (Table I). This provided good evidence that the H3 was incorporated as inositol-2-H3 under these conditions, and also established that estimation of the H3 radioactivity of the total lipid extract was a valid procedure for the estimation of inositol-2-H3 incorporation into monophosphoinositide.

As reported previously (3), acetylcholine stimulated the incorporation of $^{32}$P into a phosphoinositide in slices of guinea pig brain cortex. Chromatography of the lipides showed that this stimulation occurred in the monophosphoinositide of Spot 2. In slices from the same animals, which were pooled as described above, acetylcholine increased the incorporation of P32 and inositol-2-H3 into this monophosphoinositide to approximately the same extent (Table I). In eight experiments, the average stimulation of P32 incorporation into monophosphoinositide was 74 per cent (range, 31 to 139 per cent); in five separate experiments with different animals the average stimulation of inositol-2-H3 incorporation into monophosphoinositide was 81 per cent (range, 34 to 144 per cent). These results further substantiate the fact that acetylcholine stimulates the turnover of phosphate and inositol in monophosphoinositide to the same extent.

Glycerol-1-Cl4 incorporation into the monophosphoinositide was not stimulated in the presence of acetylcholine (Table I).

The major finding of this work is that a highly labeled monophosphoinositide is present in slices of guinea pig brain cortex which have been incubated with either $^{32}$P or inositol-2-H3, and that the stimulation by acetylcholine of P32 incorporation into this phosphoinositide is accompanied by an equal stimulation of the incorporation of inositol-2-H3. This suggests that in the presence of acetylcholine there is an accelerated rate of formation of monophosphoinositide from labeled inositol monophosphate.

DISCUSSION

In view of previous work with other tissues (4, 6, 17-20), it seems likely that the phospholipide effect in brain slices is related to a stimulation by acetylcholine of the active transport of some material in brain tissue.

In pancreas slices, the stimulation of the incorporation of P32 and inositol-2-H3 into phosphoinositide A is accompanied by a stimulation of the incorporation of glycerol-1-Cl4 (6). In slices of guinea pig brain cortex, glycerol-1-Cl4 incorporation into the monophosphoinositide is not stimulated by acetylcholine, although the incorporation of inositol-2-H3 and the incorporation of P32 are both stimulated. It is hoped that work with cell-free preparations may help to elucidate the mechanisms involved.
in the incorporation of these three labeled precursors in the unstimulated and the stimulated system.

**SUMMARY**

1. After incubation of guinea pig brain cortex slices with NaH$_2$P$_4$O$_4$, the lipides were separated by paper chromatography and five different radioactive phospholipides were identified. These were phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidic acid, and a monophosphoinositide. Two additional unidentified radioactive spots were seen.

2. When the slices were incubated with inositol-2-$^3$H, the only $^3$H-labeled phospholipide in the lipid extracts was a monophosphoinositide.

3. In the presence of acetylcholine, the incorporation of inositol-2-$^3$H and of $^{32}$P into monophosphoinositide were both increased to approximately the same extent. This suggests that acetylcholine stimulates the incorporation of inositol phosphate as a unit into monophosphoinositide. The average stimulation was about 80 per cent. Glycerol-1-$^{14}$C incorporation into monophosphoinositide was not increased in the presence of acetylcholine.

4. Acetylcholine stimulated the incorporation of $^{32}$P into phosphatidic acid about 100 per cent. There was no equivalent increase in the incorporation of glycerol-1-$^{14}$C into phosphatidic acid.

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**REFERENCES**

Acetylcholine and the Exchange of Inositol and Phosphate in Brain Phosphoinositide
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