Stimulation by Acetylcholine of Phosphatidylinositol Labelling

SUBCELLULAR DISTRIBUTION IN RAT CEREBRAL-CORTEX SLICES

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1. Rat cerebral-cortex slices were incubated with $^{32}\text{P}_i$, acetylcholine and eserine for periods of 10 min and 2 h. The specific radioactivity of phosphatidylinositol was elevated during these treatments by 36 and 106% respectively. 2. The specific radioactivities of the phosphatidylinositol in different cell structures were determined after subcellular fractionation. They were highest in the nuclear, microsomal and synaptic-vesicle fractions and lowest in myelin, both in the controls and in the acetylcholine-treated slices. 3. The stimulated labelling of phosphatidylinositol was relatively evenly distributed: no subcellular fraction showed a stimulation markedly higher than that in the homogenate. 4. Studies of the distributions and activities of marker enzymes indicated that the subcellular fractionation achieved was similar to that with fresh tissue. 5. The results are discussed in relation to the previous report that the stimulation is observed throughout the neuronal cell-bodies and in relation to the hypothesis that the labelled phosphatidylinositol produced by stimulation is a component of an acetylcholine-receptor proteolipid localized in the synaptic junction.

Several types of cell and tissue, including nervous tissue, various exocrine and endocrine glands, lymphocytes and polymorphonuclear leucocytes, show an increase in the labelling of phosphatidylinositol, but not of the bulk of their phospholipids, as one of their biochemical responses to external stimuli. This has been extensively studied in various types of nervous tissue, including cerebral cortex and sympathetic ganglia, both during electrical stimulation (Pumphrey, 1969; Larrabee *et al.*, 1963) and in the presence of acetylcholine (Hokin & Hokin, 1955, 1959; Hokin *et al.*, 1960). In the nervous system the response occurs only in tissues that contain synapses, and is localized in the postsynaptic neurone (Larrabee & Leicht, 1965). Hokin (1969) used radioautography of acetylcholine-treated sympathetic ganglia and concluded that the inositol lipid that was labelled after stimulation was distributed throughout the postsynaptic neurone.

De Robertis (1971) has described a proteolipid that appears to have the properties of an acetylcholine receptor molecule, which is, as expected, localized within the structure of the synaptic junction. Lunt *et al.* (1971) have presented results that indicate that the phosphatidylinositol labelled after stimulation is a component only of this receptor proteolipid. If this is so, then the responsive lipid should be localized within the synaptic structure. We have therefore investigated the distribution of the phosphatidylinositol labelled with ^{32}P in subcellular fractions from rat cerebral-cortex slices incubated in the presence and absence of added acetylcholine.

Methods

Assays

Protein. This was measured by the method of Lowry *et al.* (1951).

Total phosphate. This was determined by the method of Galliard *et al.* (1965).

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7). This was assayed at 37°C by the method by Ellman *et al.* (1961).

Cytochrome oxidase (cytochrome c-oxygen oxidoreductase, EC 1.9.3.1). This was determined by the method of Wharton & Tzagoloff (1967) except that 0.1% (w/v) Triton X-100 was included in the incubation medium (P. H. Cooper, personal communication).

Preparation and incubation of slices

For each experiment 20 male albino rats (300 g) were used. Slices from the cerebral cortices were prepared by using a guide and cutter (McIlwain, 1961). For each incubation the cortical slices from five brains were cut and collected in cold Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) that contained 11 mM-glucose. After collection, they were transferred within 5 min from killing the first animal to a 100 ml conical flask containing 15 ml of the same buffer, with the addition of 50–80 μCi of $^{32}\text{P}_i$ /ml (carrier free, type PBS1, from The Radiochemical Centre, Amersham, Bucks., U.K.). Incubations were at 37°C in a shaking incubator (type SSB4; Grant

Instruments, Barrington, Cambridge, U.K.). When acetylcholine chloride and eserine sulphate were included the final concentrations were always 10mM and 0.1 mM respectively.

In each experiment two pairs of flasks with the same amount of radioactivity in each were incubated either: (a) for 2 h in the presence (S) and absence (C) of acetylcholine and eserine, or (b) for 2 h in the absence of acetylcholine and eserine, and then for a further 10 min in the presence (S) and absence (C) of acetylcholine and eserine.

After incubation the slices were washed twice with 50 ml of ice-cold Krebs–Ringer bicarbonate buffer and once with 50 ml of 0.32M-sucrose. Slices from incubation flasks (S) were then combined, as were those from flasks (C). The two sets of slices were then subjected to subcellular fractionation in parallel.

Subcellular fractionation

For each fractionation, slices (approx. 2g) were suspended in 15 ml of 0.32M-sucrose (adjusted to pH 7.4 with tris) and homogenized in a stainless-steel tube, with a Teflon pestle (radial clearance 0.10–0.15 mm) that was rotated at 600 rev./min. Six complete strokes were used during 1 min of homogenization and this was repeated after 1 min. The homogenate was made to 30 ml with 0.32M-sucrose, a sample was retained for analysis, and the remainder was subjected to subcellular fractionation as described by Rodriguez de Lores Arnaiz *et al.* (1967) and Kataoka & De Robertis (1967). The composition of the isolated fractions, as established by those authors, is summarized in Table 1.

Extraction of lipids and isolation of phosphatidylinositol

The subcellular fractions were suspended in a known volume of 0.32M-sucrose. Samples of these suspensions and of the homogenate were mixed thoroughly with 3.75 ml of chloroform–methanol (1:2, v/v)/ml of suspension. After at least 20 min at room temperature chloroform was added, followed by 2M-KCl (1.25 ml of each/ml of fraction). After being shaken vigorously the mixture was centrifuged. The lower phase was removed and the upper aqueous phase was washed once with chloroform equal in volume to the original lower phase (Michell *et al.*, 1970). The lower phases were combined, dried under a stream of N₂ and the lipids dissolved in a known volume (0.2–0.5 ml) of chloroform–methanol (1:1, v/v). Phosphatidylinositol was separated from other phospholipids by chromatography of samples of the lipid extracts on formaldehyde-impregnated papers as described by Kai & Hawthorne (1966), except that Whatman no. 1 papers were used. The spots corresponding to phosphatidylinositol on the stained

chromatograms were cut out, digested and assayed for phosphate (Galliard *et al.*, 1965). Paper blanks of the same size were analysed at the same time.

Measurement of radioactivity

The samples from the phosphate determination were made up to 10 ml with water and counted for radioactivity in a 10 ml liquid Geiger–Müller tube.

Materials

Reagents were obtained as described previously (Lapetina & Hawthorne, 1971).

Results and Discussion

Subcellular fractions from incubated slices

Table 1 gives information on the composition of the different fractions obtained from rat cerebral cortex incubated at 37°C for 2 h. The protein distribution is similar to reported values for fractions from rat brain cortex (Lapetina *et al.*, 1968) except for a decrease in the amount of material in the two microsomal fractions. All the membrane fractions contained phosphatidylinositol, with the highest concentrations in synaptic vesicles, microsomal fractions and nerve-ending membranes (Table 1). In adult guinea-pig brain (Eichberg *et al.*, 1964) and in young rat brain (Kai & Hawthorne, 1966) an enrichment of phosphatidylinositol has been reported in microsomal fractions and in intact nerve-endings. The phosphatidylinositol concentrations quoted by these authors and by Michell *et al.* (1970) for fresh brain are close to those in Table 1. This suggests that there was no substantial change in the phosphatidylinositol content of the slices during 2 h of incubation.

The distribution of two 'marker' enzymes, acetylcholinesterase and cytochrome oxidase, is also included in Table 1 and indicates that cell fractionation similar to that obtained with fresh tissue had been achieved. The distribution of acetylcholinesterase, with enrichment in nerve-ending membranes and in the microsomal fractions, is not markedly different from that observed in subcellular fractions prepared from fresh rat brain cortex (Lapetina *et al.*, 1967). Most of the cytochrome oxidase was recovered in the fraction previously characterized as mitochondria (Rodriguez de Lores Arnaiz *et al.*, 1967) with much less activity in the fractions expected to contain synaptic membranes. Moreover, the activities of both enzymes in the homogenates were similar to those reported for homogenates from fresh tissue (Lapetina & Hawthorne, 1971) suggesting that the slices were well preserved. This confirms the conclusions of Lunt & Lapetina (1970) who made an electron-microscopic examination of rat cerebral-cortex slices after 2 h of incubation and observed that

Table 1. *Distribution of protein, phosphatidylinositol, acetylcholinesterase and cytochrome c oxidase in subcellular fractions isolated from incubated rat brain-cortex slices*

The percentages are averages $\pm$ S.D., based on total recovered activity. Recoveries are given both relative to the immediate parent fraction (homogenate, crude mitochondrial fraction or M_1) of the fraction under study and relative to the original homogenate ('overall recovery'). Relative specific activity (RSA) [or relative specific concentration (RSC)] is defined for each fraction as percentage of the total recovered activity (or phosphatidylinositol) divided by percentage of the total recovered protein. The RSA and RSC values were calculated relative to the immediate parent fraction of the fraction under study. The numbers of experiments are given in parentheses. n.a., Not assayed. Descriptions of the contents of each fraction are derived from Lapetina *et al.* (1967) and refer to the structures present in equivalent fractions derived from fresh rat cerebral cortex.

	Protein (3) %	Phosphatidyl- inositol (10)		Acetyl cholinesterase (3)		Cytochrome <i>c</i> oxidase (3)	
		%	RSC	%	RSA	%	RSA
Primary fractions							
Nuclear	14.0 $\pm$ 0.4	13.6 $\pm$ 3.2	0.97	12.6 $\pm$ 1.0	0.90	12.6 $\pm$ 3.5	0.90
Crude mitochondrial	60.2 $\pm$ 7.0	62.0 $\pm$ 6.7	1.03	52.1 $\pm$ 7.8	0.87	81.1 $\pm$ 4.1	1.35
Mic ₂₀ (mainly small nerve endings)	10.2 $\pm$ 1.3	15.2 $\pm$ 6.6	1.49	20.9 $\pm$ 1.8	2.04	5.3 $\pm$ 0.4	0.52
Mic ₁₀₀ (microsomal fraction)	4.7 $\pm$ 1.6	9.2 $\pm$ 2.8	1.96	12.3 $\pm$ 4.3	2.62	1.0 $\pm$ 0.4	0.21
Supernatant	10.9 $\pm$ 4.6	0.0	0.0	2.1 $\pm$ 1.6	0.19	0.0	0.0
Recovery (% of homogenate)	98.4 $\pm$ 8.2	93.8 $\pm$ 19.3		85.5 $\pm$ 12.6		94.5 $\pm$ 8.1	
Subfractions after osmotic shock of crude mitochondrial fraction							
M_1 (myelin, synaptic membranes and mitochondria)	81.5 $\pm$ 6.6	89.1 $\pm$ 2.6	1.09	93.8 $\pm$ 2.4	1.15	99.7 $\pm$ 0.7	1.22
M_2 (mainly synaptic vesicles)	4.4 $\pm$ 1.5	10.9 $\pm$ 2.6	2.46	6.2 $\pm$ 2.4	1.41	0.3 $\pm$ 0.0	0.07
M_3 (soluble)	14.1 $\pm$ 6.7	n.a.	n.a.	0.0	0.0	0.0	0.0
Recovery (% of crude mitochondrial fraction)	82.4 $\pm$ 6.8	90.7 $\pm$ 22.0		89.1 $\pm$ 13.1		86.0 $\pm$ 24.9	
Subfractions of M_1 from a discontinuous sucrose gradient							
M_1 0.8 (myelin)	17.0 $\pm$ 4.7	19.3 $\pm$ 2.4	1.13	32.3 $\pm$ 16.0	1.90	12.7 $\pm$ 4.6	0.75
M_1 0.9 (synaptic membranes)	6.7 $\pm$ 0.7	9.2 $\pm$ 1.4	1.37	13.8 $\pm$ 3.2	2.06	3.5 $\pm$ 0.5	0.52
M_1 1.0 (synaptic membranes)	8.9 $\pm$ 2.7	13.6 $\pm$ 2.3	1.53	17.6 $\pm$ 6.8	1.98	2.5 $\pm$ 0.5	0.28
M_1 1.2 (synaptic membranes)	17.8 $\pm$ 1.2	24.0 $\pm$ 6.3	1.35	20.3 $\pm$ 7.2	1.14	8.3 $\pm$ 0.2	0.47
M_1 (p) (mitochondria)	49.5 $\pm$ 2.6	33.9 $\pm$ 6.8	0.69	16.0 $\pm$ 3.2	0.32	73.0 $\pm$ 7.7	1.48
Recovery (% of M_1)	81.8 $\pm$ 7.2	75.5 $\pm$ 22.0		82.5 $\pm$ 4.2		89.0 $\pm$ 9.2	
Overall recoveries	79.7 $\pm$ 5.2	74.6 $\pm$ 12.2		73.4 $\pm$ 7.8		74.8 $\pm$ 10.1	
Total values of homogenate (slices from 10 cortices)	236 $\pm$ 27 (mg)	99 $\pm$ 17 (μ g of phosphatidylinositol P)		16.7 $\pm$ 3.8 (μ mol/min)		3.5 $\pm$ 1.1 (μ mol/min)	

the general structure of the tissue was well preserved, although some increase in the extracellular space was visible.

The recoveries of the enzyme activities, protein and phosphatidylinositol through each stage of the fractionation procedure were satisfactory (Table 1). However, slight losses throughout the complex fractionation of relatively small amounts of tissue inevitably led to overall recoveries that were a little low, but very consistent with each other (Table 1, Figs. 1 and 2).

Effect of acetylcholine on phosphatidylinositol labelling

It has been known for many years that when brain cortex slices are incubated with $^{32}\text{P}_i$, acetylcholine and eserine for 2h there is a considerable increase in the labelling of the tissue phospholipids (Hokin & Hokin, 1955). This effect is largely confined to phosphatidic acid and phosphatidylinositol (Hokin, 1969). As it is not clear from the available information at what sites in the tissue this effect occurs (see below and Table 3) we have studied the intracellular localization of the phosphatidylinositol

that is responsive to acetylcholine. These experiments were done with cerebral-cortex slices that had been incubated under two sets of conditions (see the Methods section). After incubation for 2 h with $^{32}\text{P}_i$, acetylcholine and eserine, the conditions usually used by previous workers, the stimulation of phosphatidylinositol labelling was about 100% (Table 2), a value similar to that reported by Hokin & Hokin (1955) and considerably more than that observed by Lunt *et al.* (1971).

When slices were incubated for 120 and 130 min with ^{32}P orthophosphate the incorporation of ^{32}P into phosphatidylinositol was proportional to the total period of the incubation, i.e. the increase in labelling in these 10 min was about 8%. However, in slices incubated with $^{32}\text{P}_i$ for 130 min, but treated with acetylcholine and eserine for only the last 10 min, the specific radioactivity of phosphatidylinositol was still raised by about 40% (Table 2). This reflects an approximately fivefold increase in the rate of incorporation of ^{32}P into phosphatidylinositol during the 10 min period after addition of acetylcholine.

Previous studies on the intracellular site of the stimulation of phosphatidylinositol metabolism

Increased phosphatidylinositol metabolism evoked by acetylcholine or electrical stimulation in nervous tissue is only observed in tissues that contain synapses (Hokin *et al.*, 1960) and the site of the effect is postsynaptic (Larrabee & Leicht, 1965). This could be either in the phosphatidylinositol of the postsynaptic membrane of the synaptic junction or rather less localized in the membranes of the cell body of the postsynaptic neurone. The latter alternative is supported by radioautographic evidence (Hokin, 1965) which showed that the phosphatidylinositol effect was located in neurones but provided no evidence for any specific intracellular localization of the effect within the cell body. Lunt *et al.* (1971) suggest, however, that the acetylcholine-responsive phosphatidylinositol of cerebral cortex is associated

with an acetylcholine-receptor proteolipid and that this proteolipid is concentrated in the synaptic function (De Robertis, 1971).

Previous attempts to define the intracellular localization of this effect by evoking it in isolated subcellular fractions have been inconclusive (see Table 3). The stimulation by acetylcholine of phosphatidylinositol labelling has only once been reported in a broken-cell preparation, which was essentially a nuclei-free homogenate of cerebral cortex (Redman & Hokin, 1964). An effect of acetylcholine on phosphatidic acid metabolism, which may be related to the phosphatidylinositol effect, has been found in isolated particulate subcellular fractions (Hokin & Hokin, 1959) and localized to intact nerve-ending particles (Durell & Sodd, 1966). The effect is only observed in intact nerve-ending particles and its apparent localization may reflect the ability of these particles to generate and retain relatively high concentrations of highly labelled ATP. Further, the enzymic site of the stimulation has not yet been clearly localized. It seems that diglyceride kinase, once suggested as the site of action of acetylcholine (Redman & Hokin, 1964), is not directly affected (Lapetina & Hawthorne, 1971).

It has been suggested that the controlled step is the hydrolysis of phosphatidylinositol by a phosphodiesterase (Durell *et al.*, 1969), but the evidence for this is still not convincing either in nervous tissue (Lapetina *et al.*, 1971) or in pancreas (White & Hawthorne, 1970).

Subcellular localization of the phosphatidylinositol responsive to acetylcholine

As a step towards defining the site and mechanism of the action of acetylcholine in this process we decided to study the intracellular localization of the acetylcholine-responsive phosphatidylinositol in cerebral-cortex slices. The results of these experiments are shown in Figs. 1 and 2. Phosphatidylinositol was highly labelled in all of the fractions, with the highest

Table 2. *Labelling of phosphatidylinositol in rat cerebral-cortex slices treated with acetylcholine and eserine*
Addition of acetylcholine (10 mM) and eserine (0.1 mM) during the indicated periods is denoted by +. Results are expressed as percentages of the appropriate control values and are the means $\pm$ S.D. (numbers of experiments).

	Radioactivity of phosphatidylinositol		P (Student's <i>t</i> test)
	Control	Experimental	
120 min of stimulation	100 $\pm$ 14 (6) (0-120 min: -)	206 $\pm$ 31 (6) (0-120 min: +)	<0.001
10 min of stimulation	100 $\pm$ 19 (8) (0-120 min: - 120-130 min: -)	138 $\pm$ 14 (8) (0-120 min: - 120-130 min: +)	<0.001

Table 3. Cellular site of phosphatidylinositol (and phosphatidic acid) labelling in stimulated nervous tissue

Authors	Tissue	Methods of study	Location of effect
Larrabee & Leicht (1965); Larrabee (1970)	Cervical ganglia	Electrical stimulation Study of effects on phosphatidylinositol labelling of inhibitors blocking transmission	Post-synaptic
Hokin (1965, 1969)	Cervical ganglia (intact)	Acetylcholine treatment followed by radioautography with [³ H]inositol	Throughout the cytoplasm of the neuronal cell-bodies
Durell & Sodd (1966); Durell & Garland (1969); Durell <i>et al.</i> (1969)	Guinea-pig brain (isolated subcellular fractions)	Acetylcholine-stimulated phosphatidic acid synthesis from ³² P _i , using only internally-generated ATP	Confined to nerve-ending particles
Lunt <i>et al.</i> (1971); De Robertis (1971)	Rat cerebral cortex (slices)	Hydrolysis of phosphoinositides, stimulated by acetylcholine Isolation of 'acetylcholine-receptor' proteolipid, a component of the synaptic junction	Only observed in crude mitochondrial fraction: not further localized Stimulated phosphatidylinositol was isolated together with the proteolipid
Lapetina & Michell (this paper)	Rat cerebral cortex (slices)	Subcellular fractionation after stimulation by acetylcholine	No evidence of specific localization of the stimulated phosphatidylinositol

specific radioactivities in the nuclear, microsomal and synaptic-vesicle fractions and the lowest in the myelin fraction from both the control and stimulated slices. After 2h, the most frequently used period of exposure to acetylcholine (Hokin & Hokin, 1955; Lunt *et al.*, 1971), there was a marked stimulation of phosphatidylinositol labelling. This was not localized in any particular fraction but was relatively evenly

Fig. 1. Effects of acetylcholine treatment (2h) on the Relative specific radioactivities of phosphatidylinositol in subcellular fractions isolated from rat cerebral-cortex slices

The slices were incubated (a) for 2h without acetylcholine (control: filled bars), and (b) in the presence of acetylcholine and eserine for 2h (stimulated: open bars). The relative specific radioactivity is defined as the specific radioactivity of phosphatidylinositol in the specified fraction divided by the specific radioactivity of phosphatidylinositol in the homogenate from the control slices. The degree of stimulation of phosphatidylinositol labelling observed in each fraction is indicated by the ratio of the relative specific radioactivities [RSR(S)/RSR(C)]. This is the relative specific radioactivity of the particular fraction derived from the stimulated slices (S) divided by the relative specific radioactivity of the equivalent fraction isolated from the control slices (C). Overall recoveries (see legend to Table 1) were, for radioactivity 72.1% (control) and 72.3% (stimulated), and for phosphate 63.0% (control) and 68.8% (stimulated). The results are the means of two similar experiments.

Fig. 2. Effects of acetylcholine treatment (10 min) on the relative specific radioactivities (see Fig. 1) of phosphatidylinositol in subcellular fractions isolated from rat cerebral-cortex slices

The slices were incubated (a) in the absence of acetylcholine for 130 min (control: filled bars), and (b) Without acetylcholine and eserine for 120 min, and then in their presence for 10 min (stimulated: open bars). Overall recoveries were, for radioactivity 75.0% (control) and 71.2% (stimulated), and for phosphate 72.6% (control) and 77.0% (stimulated). The results are the means of three similar experiments.

distributed so that no fraction showed a stimulation markedly higher than that in the homogenate (Fig. 1).

Two possible interpretations of these results were apparent. Either (1) the stimulation of phosphatidylinositol labelling did not occur at any single site in the cell, or (2) the effect occurred at a single site, but the newly synthesized phosphatidylinositol was rapidly distributed throughout the membranes of the cell by an exchange reaction similar to that described in liver by Wirtz & Zilversmit (1968).

A possible way to minimize any exchange and thus to distinguish between these alternatives was to label slices with ^{32}P for a considerable period and then to stimulate phosphatidylinositol labelling for only a short period (10 min). The results obtained by this approach (Fig. 2) were similar to those obtained in the preceding experiments. The stimulation was lower than that induced in 2 h, but there was still no clear localization of the extra labelling in the phosphatidylinositol of any single fraction.

Thus it seems that, even if the synthesis of phosphatidylinositol that is involved in the effect of acetylcholine occurs at a localized site within the postsynaptic neurone, the responsive phosphatidylinositol is very rapidly redistributed over all of the membrane structures of the cell. This lack of localization is consistent with the experiments of Hokin (1965) who, by using radioautography of acetylcholine-treated sympathetic ganglia, could detect no localization of the stimulated incorporation within the neurones. A rapid equilibrium of newly synthesized phosphatidylinositol between the different cellular structures in cerebral cortex is supported by the relatively rapid equalization of the specific radioactivities of the phosphatidylinositol of the microsomal fraction and of myelin after a single injection of ^{32}P , *in vivo* (Jungalwala & Dawson, 1971) and also by the high capacity of a soluble fraction from brain to catalyse exchange of phosphatidylinositol between labelled liver cell fractions (Wirtz, 1971).

It is difficult to reconcile our results with those of Lunt *et al.* (1971), who found an association between acetylcholine-responsive phosphatidylinositol and a proteolipid that was concentrated in the synaptic junction. This proteolipid had the properties expected of an acetylcholine receptor. The association was found in material from slices incubated with acetylcholine for 2 h, both in a mixed proteolipid fraction prepared by ether precipitation of a lipid extract and also in a fraction of the proteolipids that binds cholinergic ligands and can be isolated by chromatography on Sephadex LH-20. We have now shown that in slices incubated with acetylcholine under similar conditions the phosphatidylinositol effect is observed in all membranes. These findings suggest that the previously observed association of acetylcholine-responsive phosphatidylinositol with an acetylcholine-receptor proteolipid might have been coincidental, and reflected the presence of more than one proteolipid in this fraction.

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