A Membrane-Bound Activity Catalysing Phosphatidylinositol Breakdown to 1,2-Diacylglycerol, D-*myo*Inositol 1:2-Cyclic Phosphate and D-*myo*Inositol 1-Phosphate

PROPERTIES AND SUBCELLULAR DISTRIBUTION IN RAT CEREBRAL CORTEX

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1. Breakdown of phosphatidylinositol was studied in homogenates and subcellular fractions of rat cerebral cortex by using both membrane-bound and externally added [32 P]phosphatidylinositol as substrate. 2. In the presence of deoxycholate breakdown followed first-order kinetics at low substrate concentrations (≤ 1 mM) and zero-order kinetics at higher concentrations (6–9 mM). 3. Maximum breakdown by cerebral-cortex homogenates was approximately $0.5 \mu\text{mol/h}$ per mg of protein and occurred at pH 7.0 in the presence of 8 mM-phosphatidylinositol, 2 mM- CaCl_2 and 2 mg of deoxycholate/ml. Activity was abolished by 1 mM-ethanedioxybis(ethylamine)tetra-acetate. 4. The products of phosphatidylinositol breakdown were 1,2-diacylglycerol and a mixture of D-*myo*-inositol 1:2-cyclic phosphate (55%) and D-*myo*-inositol 1-phosphate (45%). The two phosphate esters appeared to be produced together and in constant proportions. 5. Some 51% of the activity was particle-bound, with the highest activities in small nerve endings, microsomal material and two synaptic membrane fractions (fractions Mic_{20} , Mic_{100} , M_1 1.0 and M_1 0.9 respectively), all of which were also rich in acetylcholinesterase and which have been shown to be rich in other surface-membrane enzymes. Much of the particle-bound activity therefore appears to be present in cerebral-cortex plasma membranes. 6. The results are discussed in relation to previously described soluble activities that catalyse the same reaction, and to a possible role of the membrane-bound enzyme in enhanced phosphatidylinositol turnover in externally stimulated cells.

There have been several reports of enzymes from animal tissues that degrade phosphatidylinositol. These have usually been soluble cytoplasmic activities that initially appeared to act as phosphatidylinositol inositolphosphohydrolases (reaction 1); they require Ca^{2+} and have acid pH optima (Kemp *et al.*, 1961; Atherton & Hawthorne, 1968; Friedel *et al.*, 1967, 1969; Thompson, 1967). Recent work by Dawson and his colleagues (Jungalwala *et al.*, 1971; Dawson *et al.*, 1971) has shown that these enzymes are cyclizing phosphotransferases that also catalyse reaction (2), the release from phosphatidylinositol of D-*myo*inositol 1:2-cyclic phosphate.

Durell and his collaborators have also obtained results that suggest that a similar, but as yet uncharacterized, membrane-bound activity might exist in brain and be stimulated in the presence of acetylcholine (Durell *et al.*, 1969).

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We have now detected a membrane-bound enzyme activity in rat cerebral cortex that releases *myo*-inositol 1:2-cyclic phosphate from phosphatidylinositol. Some characteristics of this enzyme and its subcellular distribution are reported in this paper. A brief description of this work has been published (Michell & Lapetina, 1972).

Experimental

Materials

Most materials were obtained as described previously (Lapetina & Hawthorne, 1971; Lapetina & Michell, 1972). D-*myo*Inositol 1-phosphate was a gift from Dr. R. M. C. Dawson and *myo*inositol 2-phosphate was obtained from the Sigma (London) Chemical Co., London S.W.6, U.K. or was prepared from phytic acid (McCormick & Carter, 1952). DL-*myo*-Inositol 1:2-cyclic phosphate was prepared from *myo*inositol 2-phosphate (Pizer & Ballou, 1959) and D-*myo*inositol 1:2-cyclic phosphate was prepared

from phosphatidylinositol essentially as described by Dawson & Clarke (1972): both were purified electrophoretically (Dawson & Clarke, 1972).

Phosphatidylinositol. This was isolated from unlabelled or ^{32}P -labelled tissues. ^{32}P -labelled lipids were obtained from rats injected with 1.0mCi of $^{32}\text{P}_i$ either intraperitoneally (for liver) or intraventricularly (for brain). Unlabelled lipids were obtained from rat or pig liver. The method of extraction was based on that of Bligh & Dyer (1959), as modified by Michell *et al.* (1970), and the isolation of phosphatidylinositol on that of Kai *et al.* (1966).

Tissues were homogenized in a Waring Blendor with 3ml of chloroform-methanol (1:2, v/v) per g of tissue. A further 3.0ml of chloroform-methanol (1:2, v/v) and 0.8ml of 2M-KCl per g of tissue was added and the mixture thoroughly stirred. After 1h the mixture was filtered on a Buchner funnel under suction and chloroform and water (2.0ml of each/g of original tissue) were mixed thoroughly with the filtrate. The lower phase was dried *in vacuo* and the isolated lipids were dissolved in chloroform (1ml/10g of tissue). Ethanol (5 vol.) was added slowly, with continuous mixing, to 1 vol. of this chloroform solution and the mixture was kept overnight at -20°C . The precipitate was dissolved in a minimum volume of chloroform and the precipitation was repeated. Solvent was removed from the precipitate *in vacuo* and the lipids were dissolved in chloroform. The chloroform solution was applied to a column of aluminium oxide (1mg of lipid P/g of alumina; 'for chromatographic adsorption analysis, Brockman, Activity II', obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.). Short, wide columns with the height not exceeding $1.5 \times$ the diameter were essential and elution of the column was completed in a single day. Elution was initially with chloroform-methanol-water (23:23:4, by vol.), which removed neutral lipids, phosphatidylcholine, sphingomyelin and phos-

phatidylethanolamine in that order. Phosphatidylinositol was then eluted with chloroform-methanol (1:1, v/v) to which the maximum amount of water compatible with the maintenance of a single phase was added: the solvent was initially made with 14% (v/v) of water and further water was carefully added until a bluish tinge was clearly apparent. The compositions of the eluted fractions were determined by t.l.c. after removal of the solvent (Skipski *et al.*, 1964).

Fractions of the latter eluate that were rich in phosphatidylinositol were combined and water or 2M-KCl was added to give a chloroform-methanol-water composition of 1.0:1.0:0.9 (by vol.) (Bligh & Dyer, 1959). The lower phase was dried *in vacuo* and the residue was dissolved in chloroform and applied to a column of silicic acid [1mg of lipid P per g of Mallinkrodt SilicaR CC-7 (100-200 mesh), obtained from Camlab (Glass) Ltd., Cambridge, U.K.; column length = $8 \times$ column diameter]. The column was prepared by slurring the silicic acid in methanol, decanting fine particles, packing the column in methanol and then passing chloroform through the column until it was clear throughout. Elution was with chloroform, followed by chloroform-methanol mixtures containing 20, 25 and 30% methanol. Pure phosphatidylinositol usually emerged in the latter two solvents and was identified by t.l.c. and chromatography on formaldehyde-treated paper (Lapetina & Michell, 1972). In a typical preparation 34mg of phosphatidylinositol P were obtained from 900g of pig liver. The radiochemical purity of ^{32}P -labelled phosphatidylinositol was checked by examination of a thin-layer chromatogram in a Radiochromatogram Spark Chamber (Birchover Instruments, Hitchin, Herts., U.K.). Unlabelled and ^{32}P -labelled phosphatidylinositol were mixed in appropriate proportions before use as substrate for studies of phosphatidylinositol breakdown.

Methods

Determination of protein, phosphate and cholinesterase. These were assayed as described by Lapetina & Michell (1972).

Measurement of phosphatidylinositol breakdown. This was assayed by using as substrate either the [^{32}P]phosphatidylinositol of the membrane fractions containing the enzyme activity being measured, or externally added [^{32}P]phosphatidylinositol.

(a) A ^{32}P -labelled homogenate or total particulate fraction (see below) was prepared from cerebral cortices of rats injected intraventricularly with $50\mu\text{Ci}$ of carrier-free $^{32}\text{P}_i$ 18h before death. This preparation provided both the enzymic activity and the ^{32}P -labelled substrate. Standard incubation conditions were: 50mM-Tris-maleate buffer, pH7.0 (Long, 1961), 2mM- CaCl_2 , 2mg of sodium deoxycholate/ml. Reactions were started by the addition of the tissue preparation (0.3–2.5mg of protein, in 0.2ml of 0.32M-sucrose or 5mM-Tris-maleate, pH7.0). They were incubated in a final volume of 0.5ml at 37°C for 1h. Reactions were stopped and lipids extracted as described by Lapetina & Michell (1972). Unincubated samples were extracted similarly. Phosphatidylinositol was isolated and its radioactivity determined as described by Lapetina & Michell (1972). In some experiments ^{32}P was determined by Čerenkov counting (Jungalwala *et al.*, 1971) by using a Philips liquid-scintillation counter. The degree of breakdown of phosphatidylinositol was calculated from the observed decrease in radioactivity.

(b) Unlabelled tissue fractions and externally added [^{32}P]phosphatidylinositol were used in these assays. The standard conditions were as in (a) except for the addition of [^{32}P]phosphatidylinositol to a final concentration of 8mM. A chloroform solution of the lipid was placed in the assay tubes and the solvent was removed with a stream of N_2 . Deoxycholate was added, the contents of the tubes mixed, and the other components of the assay mixture added. Incubation, termination of the reaction, extraction of the lipids and determination of the radioactivity of phosphatidylinositol were as in (a). In later experiments, phosphatidylinositol breakdown was measured by Čerenkov counting of the upper aqueous phases from the lipid extraction, after dilution with 10ml of water. This method was only adopted after it had been established that the only major products of phosphatidylinositol breakdown were diacylglycerol and water-soluble phosphate esters.

Identification of the products of phosphatidylinositol breakdown. The lipid products were identified by t.l.c. (Skipski *et al.*, 1968).

The water-soluble phosphate esters were separated by electrophoresis on paper at pH3.5 in pyridine-acetic acid-water (1:10:90, by vol.) at 40V/cm for

2.5h and by descending chromatography in ethanol-13.5M- NH_3 (3:2, v/v) as described by Dawson & Clarke (1972). Phosphate-containing spots were detected by the method of Dawson (1960). The stained spots were cut out, digested with 72% HClO_4 , diluted to 10ml with water and the radioactivity was measured by Čerenkov counting. If the water-soluble products of the reaction were to be studied water was substituted for 2M-KCl when obtaining aqueous phases during termination of enzyme incubations and extraction of lipids. These aqueous phases were freeze-dried and the residues dissolved in water before study.

Preparation of total particulate fraction. Rat cerebral cortices were homogenized (stainless-steel tube with a Teflon pestle rotated at 600rev./min, radial clearance 0.1–0.15mm) in 5mM-Tris-maleate buffer, pH7.0.

The homogenates were diluted with buffer to 10ml/g of tissue and centrifuged at $6 \times 10^6 \text{g} \cdot \text{min}$. The pellets, suspended in 0.32M-sucrose, were used as a total particulate fraction.

Preparation of homogenates and subcellular fractions. For each fractionation the grey matter (3.8–3.9g) was obtained from five rats (approx. 300g), suspended in 16ml of 0.32M-sucrose (adjusted to pH7.4 with Tris) and homogenized and fractionated exactly as described before (Lapetina & Michell, 1972). The composition of the isolated fractions is summarized in Table 2 and by Lapetina & Michell (1972).

Results

Properties of an activity catalysing phosphatidylinositol breakdown in ^{32}P -labelled rat cerebral cortex

[^{32}P]Phosphatidylinositol in fresh homogenates was stable; no appreciable breakdown was detected during 60min incubations at pH7.0 (Michell & Lapetina, 1972). When homogenates had previously been frozen or had been sonicated for 2min breakdown of phosphatidylinositol did occur, with about 10% of the lipid disappearing in 60min. Breakdown in either fresh or frozen preparations was greatly increased in the presence of deoxycholate (Fig. 1a), but not Triton X-100 (0.1%, w/v). It was also increased in the presence of Ca^{2+} ions, with the highest activity at 1–4mM, and was abolished by 1mM-ethanedioxybis(ethylamine)tetra-acetate (EGTA) (Fig. 1b). Maximum activity was at pH7.0 (Fig. 1c). Phosphatidylinositol breakdown did not occur in homogenates that had been heated at 100°C for 2.5min, even in the presence of deoxycholate.

Fig. 1(d) shows the time-course of the reaction, which was exponential for at least 100min in incubations with either an homogenate or a total particulate fraction. This suggested that at the low substrate

Fig. 1. Breakdown of endogenous $[^{32}\text{P}]$ phosphatidylinositol in homogenates and the total particulate fraction

(a) Effect of deoxycholate concentration (3.3 mg of protein). (b) Effect of Ca^{2+} on the enzyme activity, enzyme preparation as (a). The arrow indicates the addition of 1 mM-ethanedioxybis(ethylamine)tetra-acetate. (c) Effect of pH: ●, 4.5 mg of protein; ▲, 3.0 mg of protein. The two curves were obtained with different cerebral-cortex homogenates. (d) Time-course of the enzyme reaction: ▲, total homogenate (4.5 mg of protein); ●, total particulate fraction (2.2 mg of protein). Homogenates had been previously frozen. The total particulate fraction used in (d) was freshly prepared. As the kinetics of phosphatidylinositol breakdown in these experiments were first-order the data have been plotted on semi-logarithmic scale.

concentrations prevailing in these assays, and with membrane-bound phosphatidylinositol as substrate, the breakdown followed first-order kinetics. Under such conditions the extent of phosphatidylinositol breakdown/min was approximately 1.5% of the total. Under conditions in which phosphatidylinositol breakdown was rapid there was no breakdown of other phospholipids (Michell & Lapetina, 1972).

Phosphatidylinositol breakdown: comparison of the utilization of membrane-bound and externally added substrate

The fact that the kinetics of the breakdown of endogenous phosphatidylinositol were first-order made these incubation conditions difficult to apply to the study of the subcellular distribution of the activity catalysing this breakdown. However, it

Fig. 2. Breakdown of endogenous and externally added phosphatidylinositol by total particulate fractions

(a) □, Various amounts of added [^{32}P]phosphatidylinositol were incubated with unlabelled total particulate fraction (2.2mg of protein; 16.4nmol of unlabelled phosphatidylinositol). ○, Various amounts of added unlabelled phosphatidylinositol were incubated with ^{32}P -labelled total particulate fraction (2.2mg of protein, 7.4nmol of [^{32}P]phosphatidylinositol). ▲, 0.18 μmol of added [^{32}P]phosphatidylinositol with ^{32}P -labelled total particulate fractions (2.2mg of protein, 7.4 nmol of [^{32}P]phosphatidylinositol). (b) As in (a). (c) □, Data from (a), compared with release of water-soluble ^{32}P -labelled products in the same incubations (●). Incubations were for 60 min.

seemed likely that in the presence of such high concentrations of deoxycholate endogenous phosphatidylinositol and membrane proteins would be dissociated from one another, and therefore endogenous and externally added phosphatidylinositol would be broken down similarly. Experiments were therefore performed to test whether (a) externally added unlabelled phosphatidylinositol would modify the breakdown of endogenous [^{32}P]phosphatidylinositol, (b) externally added [^{32}P]phosphatidylinositol would be attacked in a way similar to endogenous substrate, and (c) whether ^{32}P -labelled endogenous and exogenous substrates, if present together, would both be broken down to the same extent.

The results are shown in Fig. 2. If it was assumed in calculating the results that endogenous and exogenous substrate were incorporated into an homogeneous pool before breakdown, then it was found that the kinetics of breakdown were first-order up to about 1 mM, irrespective of substrate source (Fig. 2a). Secondly, as would be expected with first-order kinetics, the proportion of the labelled phosphatidylinositol broken down in a standard time was the same in all assays in which the substrate concentration was 1 mM or less (Fig. 2b). The data were therefore consistent with complete mixing of the endogenous and

externally added substrate before attack and indicated that in the presence of deoxycholate (2mg/ml) the same enzyme activity was responsible for phosphatidylinositol breakdown irrespective of the source of the substrate. Thus a valid assay for the activity could be developed by using externally added substrate.

Further, it was shown that breakdown, as calculated from the decrease in the amount of labelled phosphatidylinositol during incubation, could be determined with equal validity and much more simply from measurements of the release of water-soluble ^{32}P radioactivity that occurred during phosphatidylinositol breakdown (Fig. 2c). This conclusion was later validated by the analyses of the products of phosphatidylinositol breakdown (see below).

Conditions for assay of phosphatidylinositol breakdown with zero-order kinetics

Maximum rates of phosphatidylinositol breakdown were observed at concentrations of externally added substrate of 6–9 mM (Fig. 3); 8 mM was used thereafter. Activity was proportional to the concentration of the added tissue preparation and to the period of incubation.

Fig. 3. Effect of phosphatidylinositol concentration on an enzyme activity catalysing breakdown of external [^{32}P]phosphatidylinositol

A freshly prepared total particulate fraction (2.2mg of protein) was used. Assays were done by method (b) described in the Experimental section.

Identification of the products of phosphatidylinositol breakdown

The lipid-soluble products from phosphatidylinositol breakdown catalysed by cerebral cortical total particulate fractions were analysed by t.l.c. Almost all of the non-polar lipids released during the reaction were 1,2-diacylglycerols, with a small amount of 1,3-diacylglycerol (probably derived from 1,2-diacylglycerol by isomerization) and traces of unesterified fatty acids and monoacylglycerol. When different concentrations (2, 4, 8 and 12mM) of added substrate were included in the incubations, maximum production of 1,2-diacylglycerol occurred at 8mM, with a decrease at 12mM. This is in agreement with the phosphatidylinositol concentration curve shown in Fig. 3. No 1,2- or 1,3-diacylglycerol was seen in these chromatograms when external phosphatidylinositol was not added.

The water-soluble products from incubations with [^{32}P]phosphatidylinositol were separated by electrophoresis. P_i and two further phosphate-containing areas were detected by spraying marker strips from the electrophoretograms. The latter two components had mobilities relative to P_i which indicated that they were probably *myo*inositol 1:2-cyclic phosphate and *myo*inositol 1-phosphate; they were absent in incubations to which phosphatidylinositol had not been added. All three components, however, migrated

more slowly than the standards with which they were being tentatively identified, due to the presence of residual buffer from the enzyme incubations. They were therefore eluted from the appropriate unsprayed areas of the papers with water, freeze-dried and dissolved in water. Samples were then subjected to both electrophoresis and paper chromatography together with standards of *D*-*myo*inositol 1:2-cyclic phosphate, *D*-*myo*inositol 1-phosphate, and P_i , with which they appeared to be identical. The radioactivity released from the [^{32}P]phosphatidylinositol was recovered in the areas corresponding to *myo*inositol 1:2-cyclic phosphate and *myo*inositol 1-phosphate (Table 1, Expt. 1). Negligible radioactivity was present in either P_i or the material remaining at the origin. When a sample of the tentatively identified *myo*inositol 1:2-cyclic phosphate was subjected to acid hydrolysis (1M-HCl, 80°C, 3min) it was quantitatively converted into *myo*inositol 1-phosphate, which was identified by both electrophoresis and chromatography.

In Expt. 1 of Table 1 the period of incubation was 30min and *myo*inositol 1:2-cyclic phosphate and *myo*inositol 1-phosphate were produced in about equal quantities. This might have been a result of either their simultaneous release (Dawson *et al.*, 1971) or of production of the cyclic phosphate ester, followed by its breakdown by phosphodiesterase activity (Dawson & Clarke, 1972). The products generated by a total particulate fraction during a 10min incubation and during various periods by a fraction enriched in the activity catalysing phosphatidylinositol breakdown (M_1 1.0, see Table 2) were therefore examined (Table 1, Expts. 2 and 3). In all cases the cyclic ester formed 51–60% of the products and 40–49% was *myo*inositol 1-phosphate, suggesting that both were produced simultaneously.

Subcellular localization of the activity catalysing phosphatidylinositol breakdown

It was of particular interest to determine the cellular site of the particulate activity; this was compared with the distribution of acetylcholinesterase, which is concentrated particularly in surface membranes. Such a comparison is shown in Fig. 4, for which specific activities were calculated relative to the total activity recovered in all of the particulate fractions. Phosphatidylinositol-degrading and acetylcholinesterase activities were both concentrated particularly in fractions M_1 0.9 and M_1 1.0, which are rich in surface membranes derived from nerve-endings, and in fractions Mic_{20} and Mic_{100} , which include surface-membrane fragments from other locations. This argues for the localization of an appreciable proportion of the activity degrading phosphatidylinositol in cell surface-membranes in the cerebral cortex.

Table 1. *Water-soluble products from phosphatidylinositol hydrolysis*

Assay conditions were as indicated in the Experimental section except that phosphatidylinositol concentration and protein were respectively as follows: 10.8mM and 10mg (Expt. 1), 3.6mM and 3.3mg (Expt. 2), 0.34mM and 0.6mg (Expt. 3). Both *myo*inositol phosphate esters were separated electrophoretically and their radioactivity measured (see the Experimental section).

Expt.	Fraction studied	Incubation time (min)	<i>myo</i> Inositol 1:2-cyclic phosphate (%)	<i>myo</i> Inositol 1-phosphate (%)
1	Total particulate	30	51	49
2	Total particulate	10	58	42
3	Synaptic membranes (M_1 1.0)	5	60	40
		15	51	49
		60	56	44

Table 2. *Distribution of protein, activity catalysing phosphatidylinositol breakdown and acetylcholinesterase activity in subcellular fractions of rat brain cortex*

The percentages are based on total recovered protein or activity. All values are given as means $\pm$ S.D. Numbers in parentheses indicate the numbers of experiments.

	Protein (%) (3)	Activity catalysing phosphatidylinositol breakdown (%) (3)	Acetylcholinesterase (%) (3)
Nuclear	19.6 $\pm$ 2.5	14.5 $\pm$ 0.9	14.7 $\pm$ 3.3
Myelin* (M_1 0.8)	12.3 $\pm$ 2.2	5.9 $\pm$ 0.0	19.4 $\pm$ 1.5
Synaptic membranes* (M_1 0.9)	3.9 $\pm$ 0.0	3.4 $\pm$ 0.4	6.8 $\pm$ 1.3
Synaptic membranes* (M_1 1.0)	2.6 $\pm$ 0.3	2.4 $\pm$ 0.1	4.2 $\pm$ 0.8
Synaptic membranes* (M_1 1.2)	4.7 $\pm$ 0.3	2.6 $\pm$ 0.3	4.3 $\pm$ 0.8
Mitochondria* (M_1 p)	12.3 $\pm$ 0.4	2.0 $\pm$ 0.9	2.8 $\pm$ 1.7
Synaptic vesicles* (M_2)	2.9 $\pm$ 0.2	1.9 $\pm$ 0.2	6.3 $\pm$ 2.4
Small nerve endings (Mic_{20})	10.7 $\pm$ 1.4	11.3 $\pm$ 0.6	19.7 $\pm$ 2.8
Microsomal (Mic_{100})	7.7 $\pm$ 1.2	7.8 $\pm$ 0.3	15.4 $\pm$ 1.4
Soluble* (M_3)	6.9 $\pm$ 0.6	18.5 $\pm$ 1.3	1.8 $\pm$ 0.0
Soluble (Sup)	15.8 $\pm$ 1.5	30.6 $\pm$ 1.0	4.3 $\pm$ 2.5
Recoveries	94 $\pm$ 6	115 $\pm$ 22	95 $\pm$ 23
Absolute values for homogenate (mg/g of fresh tissue)	146 $\pm$ 6 (3)	—	—
(nmol/min per mg of protein)	—	8.0 $\pm$ 0.5 (5)	119 $\pm$ 10 (3)

* Fractions derived from osmotically shocked crude mitochondrial fraction.

Discussion

The reaction catalysed by the membrane-bound enzyme reported here is clearly similar to that catalysed by the previously described soluble enzymes, being a Ca^{2+} -dependent cleavage of phosphatidylinositol between the phosphate group and the glycerol backbone, with release of 1,2-diacylglycerol and a mixture of *D*-*myo*inositol 1:2-cyclic phosphate and *D*-*myo*inositol 1-phosphate. It is also clearly different from a particulate activity responsible for degradation of phosphatidylinositol in liver and

intestinal mucosa, which removes the fatty acids from phosphatidylinositol, releasing glycerylphosphoryl-inositol (Kemp *et al.*, 1961; Atherton & Hawthorne, 1968).

However, the membrane-bound activity differs in several respects from the soluble enzymes reported previously and it seems possible that the two activities are distinct entities rather than simply membrane-bound and soluble forms of the same activity.

The pH optimum (7.0) of the membrane-bound activity is close to the pH within cells, in contrast with

Fig. 4. Distribution patterns of activity catalysing phosphatidylinositol breakdown (a) and of acetylcholinesterase (b) in particulate fractions

Relative specific activities are expressed relative to the total activity recovered in the particulate fraction (i.e. all but fractions Sup and M_3). On the abscissa each fraction is represented by its protein content, expressed as a percentage of the total recovered particulate protein. The composition of the different fractions is summarized in Table 2, where recoveries are also given. Results are the average of three experiments.

the soluble enzymes which are most active at pH values between 5 and 6. For maximum activity to be expressed against either membrane-bound lipid or externally added lipid the particulate enzyme requires the presence of an anionic detergent, deoxycholate. In the presence of the detergent, substrate from both sources is treated similarly. It is not clear whether the main effect of the detergent is to disrupt the membranes, allowing access of substrate to the enzyme, or to improve the dispersion or the surface charge properties of the substrate. In contrast the soluble enzyme, at least in intestinal mucosa, is strongly inhibited by detergents (Atherton & Hawthorne, 1968). The phosphatidylinositol concentrations needed for maximum activity of the membrane-bound activity (6–9 mM) is much higher than the requirement of the soluble enzymes (1–3 mM; Kemp *et al.*, 1961; Thompson, 1967; Atherton & Hawthorne, 1968). However, it is not clear what a substrate optimum for a membrane enzyme under such conditions might mean in physiological terms, as we lack evidence to indicate the state in which the substrate is presented to the enzyme inside the cell.

Subcellular localization of activity catalysing phosphatidylinositol breakdown

Under the optimum zero-order assay conditions reported here, 51% of the activity was membrane-bound. The 49% that was soluble was not studied further, and we have not determined whether this is the previously described cerebral-cortex soluble activity (Thompson, 1967; Friedel *et al.*, 1967, 1969) or a new activity that is active at pH 7.0 and which is not inhibited by detergents. The membrane-bound activity was concentrated in fractions (Mic₂₀, Mic₁₀₀, M_1 0.9, M_1 1.0) known from previous work on the subcellular distribution of 5'-nucleotidase, acetylcholinesterase, adenylyl cyclase and $Na^+ + K^+$ -stimulated adenosine triphosphatase (Lapetina & Hawthorne, 1971; De Robertis, 1967), and from their content of acetylcholinesterase in our experiments, to be enriched in plasma-membrane fragments (Fig. 4). The data, as with any studies of subcellular fractions from brain, are not sufficiently clear-cut to assign activity solely to any one intracellular site, but they do indicate that much of the membrane-bound activity carrying phosphatidylinositol breakdown in cerebral cortex is probably in cell surface-membranes. The high activity of the nuclear fractions in our experiments and their unusually high protein content (Table 2; cf. Lapetina & Hawthorne, 1971) were almost certainly due to a rather large number of unbroken cells, leading to a high amount of entrapped soluble material.

The only previous studies of the subcellular distribution in brain of enzymes attacking phosphatidylinositol are those of Friedel *et al.* (1969) and Canessa

de Scarnati & Rodriguez de Lores Arnaiz (1972). The former authors measured activity at pH 7.0, but in the absence of detergent or added Ca^{2+} and with a low substrate concentration (2 mM). As might be expected, they found activity predominantly in the soluble fraction (84%), with the remainder concentrated in microsomal and nerve-ending fractions. In the more recent study by Canessa de Scarnati & Rodriguez de Lores Arnaiz (1972) assays were at pH 6.5 with 1 mM-phosphatidylinositol, 1 mM- Ca^{2+} and no detergent; under such conditions the activity probably displayed first-order kinetics, but activities were calculated in units ($\mu\text{mol/h}$) that assume zero-order conditions. Only 20% of the activity was soluble, which is unexpected as these again are conditions which would be expected to favour the soluble activity. A possible explanation may lie in the use of dialysed and freeze-dried subcellular fractions, as dialysis inactivates the soluble enzyme (Kemp *et al.*, 1961; Thompson, 1967; Atherton & Hawthorne, 1968). The membrane-bound activity was concentrated in nerve-ending membranes in their experiments, in agreement with our information (Fig. 4). They did not, however, observe high activity in their microsomal fractions.

Possible role of the membrane-bound enzyme in stimulated phosphatidylinositol turnover

The possible role of enzymes cleaving phosphatidylinositol in the reactions that lead to enhanced phosphatidylinositol labelling in a variety of stimulated tissues, including acetylcholine-treated cerebral cortex, has been discussed several times (Hokin, 1969; Durell *et al.*, 1969; Lapetina & Hawthorne, 1971; Jungalwala *et al.*, 1971; De Robertis, 1971; Dawson *et al.*, 1971; Lapetina & Michell, 1972; Michell & Lapetina, 1972).

It has, however, always been difficult to develop a model in which phosphatidylinositol breakdown catalysed by the known soluble enzyme might participate. Durell and his collaborators have presented some evidence for the participation of a membrane-bound phosphatidylinositol phosphodiesterase, but have never fully characterized such an enzyme (Durell *et al.*, 1969; Durell & Garland, 1969). It is now clear that a highly active enzyme of this type exists in cell surface-membranes in cerebral cortex. This enzyme may be the stimulated step in the cycle of reactions leading to enhanced phosphatidylinositol labelling.

Canessa de Scarnati & Rodriguez de Lores Arnaiz (1972) claimed that a membrane-bound enzyme in cerebral cortex which they assayed as phosphatidylinositol phosphohydrolase, but which was probably the activity reported here, is directly stimulated by acetylcholine. We have as yet been unable to confirm

this observation (E. G. Lapetina & R. H. Michell, unpublished work).

Production of D-myoinositol 1:2-cyclic phosphate

The water-soluble products at optimum pH of the previously studied soluble enzymes catalysing phosphatidylinositol breakdown were about 75% (Dawson *et al.*, 1971; tissue not specified) or 62–65% (Jungalwala *et al.*, 1971; thyroid) D-myoinositol 1:2-cyclic phosphate, the remainder being D-myoinositol 1-phosphate. Dawson *et al.* (1971) therefore proposed that this activity be called phosphatidylinositol 2-inositol phosphotransferase (cyclizing), rather than phosphatidylinositol inositolphosphohydrolase, although at pH values above about 6.5 the proportion of the cyclic ester fell to less than 50% of the products. The particulate activity described here is clearly similar, but produces slightly less (about 55%) D-myoinositol 1:2-cyclic phosphate at its optimum pH of 7.0. This is, however, a relatively physiological value and is probably close to the pH at which it operates in the cell. As in the case of the soluble activity, both D-myoinositol 1:2-cyclic phosphate and D-myoinositol 1-phosphate are produced simultaneously and it seems very likely that the release of both is catalysed by the same enzyme. Due to the fairly even balance of products in our studies we have preferred to refer to 'activity catalysing phosphatidylinositol breakdown', even though the synthesis of D-myoinositol 1:2-cyclic phosphate seems likely to be the cellular function of this enzyme (Michell & Lapetina, 1972).

This is the first time that production of D-myoinositol 1:2-cyclic phosphate has been demonstrated as a result of a plasma membrane-bound enzyme. This observation and the recent report of a specific phosphodiesterase converting the cyclic ester (Dawson & Clarke, 1972) into inositol 1-phosphate increase the obvious parallels between the reactions involved in production and breakdown of D-myoinositol 1:2-cyclic phosphate and adenosine 3':5'-cyclic phosphate, particularly as situations in which D-myoinositol 1:2-cyclic phosphate production may well increase are situations involving external stimulation of cells (Dawson *et al.*, 1971; Dawson & Clarke, 1972; Michell & Lapetina, 1972).

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