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Fatty Acid Stimulation of Membrane Phosphatidylinositol Hydrolysis by Brain Phosphatidylinositol Phosphodiesterase

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The hydrolysis of membrane-bound phosphatidylinositol in rat liver microsomal fraction by the soluble phosphatidylinositol phosphodiesterase from rat brain was markedly stimulated by oleic acid or arachidonic acid. The stimulation did not require added calcium, although it was abolished by EDTA. Lysophosphatidylcholine also totally suppressed the stimulation. A possible role for the fatty acid content of a membrane in controlling phosphatidylinositol turnover is suggested.

It is now generally accepted that the phenomenon of stimulated phosphatidylinositol turnover (for review see Michell, 1975) is initiated by the increased hydrolysis of phosphatidylinositol into phosphoinositol and diacylglycerol. The enzyme possibly responsible for this is the Ca^{2+}-dependent phosphatidylinositol phosphodiesterase (EC 3.1.4.10) found in the cytosol of many mammalian tissues, being especially rich in the brain. Of the several hypotheses advanced to explain stimulated phosphatidylinositol turnover, the one that has received most attention recently is that of Durell *et al.* (1969), De Robertis (1971) and Michell (1975) who suggest that it is an integral part of receptor function in the plasma membrane, specifically of Ca^{2+}-ion 'gating' (Michell, 1975).

This hypothesis would require, however, the existence of a membrane-bound phosphatidylinositol phosphodiesterase and such an activity has been reported in brain (Friedel *et al.*, 1969; Lapetina & Michell, 1973). We have recently shown that the evidence for this membrane-bound enzyme may be an artifact (Irvine & Dawson, 1978*a*,*b*); furthermore, we also showed that the soluble brain phosphodiesterase produced little hydrolysis of phosphatidylinositol in a membrane (rat liver microsomal fraction) unless the substrate was activated by the detergent deoxycholate. We suggested (Irvine & Dawson, 1978*a*,*b*) that deoxycholate may be mimicking some endogenous anionic amphiphile that regulates the activity of the soluble phosphatidylinositol phosphodiesterase *in vivo*, hence possibly controlling phosphatidylinositol turnover. We provide evidence in the present paper that this amphiphilic regulator may be free fatty acid.

Materials and Methods

^{32}P- and [^{3}H]inositol-labelled microsomal fraction

^{32}P-labelled microsomal fraction was prepared by the method of Higgins & Dawson (1977) from the livers of rats injected 22h previously with 0.5–2mCi of [^{32}P]P_i (carrier-free from The Radiochemical Centre, Amersham, Bucks., U.K.). The fraction was washed and resedimented twice in 0.3M-sucrose (all solutions were made with double-distilled water) and then sonicated for 5min in a Mullard 60W sonicator with a 1cm probe turned to maximum cavitation, and repelleted at 100000$g_{av.}$ for 60min. The sonication removes entrapped liver cytosol enzymes, including the liver phosphatidylinositol phosphodiesterase, and probably helps to ensure access of exogenous soluble enzyme to the phosphatidylinositol, even if this phospholipid were predominantly in the inner leaflet of the bilayer of the membrane.

[^{3}H]Inositol-labelled microsomal fraction was prepared in identical fashion to the ^{32}P-labelled microsomal fraction, from the livers of rats injected 16h previously with 100μCi of [2-^{3}H]inositol (sp. radioactivity 5Ci/nmol; The Radiochemical Centre). Analysis of the free-fatty acid content of microsomal fraction prepared in this manner showed that the concentration in fresh preparations was of the order of 1nmol of fatty acid/30nmol of phospholipid.

Identification of water-soluble products of phosphatidylinositol hydrolysis

Water-soluble ^{3}H-labelled products were separated by the method of Hübscher & Hawthorne (1957) and identified by using standards prepared as described by Dawson & Clarke (1972).

Brain supernatant

A 30% homogenate of rat brain in 0.32M-sucrose was centrifuged at 100000$g_{av.}$ for 90min to give a soluble protein supernatant.

Assay of phospholipid hydrolysis

Unless otherwise stated, the assay was conducted in a final volume of 0.5ml containing 155nmol of

membrane phospholipid, 30mM-Tris/maleate buffer, pH7.0, and 0.2ml of brain extract. The phospholipids were extracted (Lapetina & Michell, 1973) and separated by t.l.c. (Irvine & Dawson, 1978*b*) and, after locating the phospholipids by radioautography, the phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol spots were scraped off for scintillation counting. For every incubation condition (i.e. no additions, fatty acid added etc.) three tubes were used; the first was kept on ice and the brain extract added at the end of the incubation; the second tube was incubated at 37°C for 60min with 0.32M-sucrose in place of brain extract and the latter added at the end of the incubation; the third tube was incubated with brain supernatant for 60min at 37°C. Thus any minor differences in the radioactivities of phospholipid fractions caused by differential carry-over during extraction or by the membrane-bound phospholipases in the endoplasmic reticulum etc. were always measured; only hydrolysis of phospholipids by the brain supernatant is considered in this paper.

Fatty acids

Oleic and arachidonic acids were added as sonicated dispersions or as the potassium salts. On addition of the latter to the buffer at pH7.0 the solution turned cloudy, indicating that a suspension of the free acid had formed. Stearic acid was added as the sodium salt by dissolving the acid in 0.05M-NaOH at 80°C, readjusting the pH to 7.5 with 0.3M-ethyl formate, and adding the hot solution down the side of the incubation tube (in these experiments, other tubes had hot water added in a similar way as a control).

Hydrolysis of pure [^{32}P]phosphatidylinositol

The preparation of substrate and the assay procedure was as described by Irvine *et al.* (1978). The Ca^{2+} concentration for the assay was routinely 1mM, the phosphatidylinositol content was 4.34μg of phospholipid P/tube, i.e. 0.14mM, and the incubation was for 15min at 37°C. Assays were performed at low (20mM) and high (120mM) K^+ concentrations (see Allan & Michell, 1974).

Results and Discussion

The results in Table 1 show clearly that addition of oleic or arachidonic acids stimulates specifically the hydrolysis of membrane phosphatidylinositol by the brain supernatant. Considerable quantitative variations were apparent between the different membrane or brain-extract preparations, but the overall picture from nine separate experiments is consistent. The products of hydrolysis of [^{3}H]inositol-labelled phospholipids (see the Materials and Methods section) at 1.4μmol of added oleic acid were identified as phosphoinositol (58%), cyclic phosphoinositol (15%) (Dawson *et al.*, 1971) and inositol (26%), the last of these presumably being formed by phosphatase activity on the first. No ^{3}H-labelled glycerophosphoinositol was detected, even in the presence of a 1mM-glycerophosphoinositol trap, which was hydrolysed less than 50%, confirming it is the phosphatidylinositol phosphodiesterase that is stimulated.

The data in Table 1 are derived only from experiments with ^{32}P-labelled microsomal fraction kept frozen for less than 3 weeks; over a longer period of time the effect of low fatty acid concentrations became difficult to detect. Concurrently the hydrolysis of

Table 1. *Stimulation of hydrolysis of membrane phospholipids by soluble brain extract*

For incubation details see the Materials and Methods section. The results given are the percentage hydrolysis of phospholipid fractions by rat brain extract in excess of that in duplicate or triplicate controls with no added fatty acid. The results are pooled from nine separate experiments, and individual results given or (in one case) the mean±standard error with the number of experiments in brackets. A value of 0 represents no stimulation detectable over the experimental error.

Quantity of oleic acid per tube (μmol)	Hydrolysis of phosphatidylethanolamine (%)	Hydrolysis of phosphatidylinositol (%)	Hydrolysis of phosphatidylcholine (%)
0.04	0	8	0
0.08	0, 0, 11	9, 12, 14	0, 0, 0
0.5	0, 0	15, 18	0, 0
0.75	0, 0, 0	45, 50, 58	0, 0, 0
1.4	0 (9)	50±5 (9)	0 (9)
Quantity of arachidonic acid per tube (μmol)			
0.45	0	18	0
0.97	0, 0	22, 38	0, 0
1.4	0, 0	40, 53	0, 0

phosphatidylinositol by the brain enzyme with no fatty acid added, instead of being undetectable by this method, increased to 10–20%, suggesting that on storage some deacylation of phospholipid may have occurred.

It is evident from Table 1 that, although a measurable effect does occur at low fatty acid concentrations, the amounts of fatty acid generating a large effect are generally high compared with the microsomal phospholipid present (155 nmol). However, we do not know what proportion of added fatty acid enters the lipid bilayer of the membrane to mimic a fatty acid generated *in situ*. We attempted to measure this degree of entry of fatty acids into the membrane by using unlabelled microsomal fraction and [^{14}C]oleic acid, but we found that the brain extract itself bound some of the radioactive isotope and precipitated it on centrifugation, so we could draw no clear conclusions as to the actual concentration of fatty acid in the membrane during the incubation.

After incubation with 1.4 μmol of oleic acid, the microsomal fraction could still be pelleted at 100000*g*, and under the electron microscope still appeared as microsomal membrane vesicles, suggesting that the structure of the membrane is not dissolved even with the highest oleic acid concentrations.

Furthermore, if pure phosphatidylinositol were used as a substrate (Fig. 1), the oleic acid could be mixed with it as a chloroform solution before removing the solvent and resuspending the two in water as mixed micelles. Under these conditions the amount of oleic acid required to stimulate the phosphatidylinositol phosphodiesterase was about 30-fold less, relative to phospholipid, than when using a membrane substrate (Table 1). This suggests that only a small proportion of the oleic acid actually enters the lipid bilayer when microsomal fraction is used as substrate.

When extrapolating the results presented (Table 1) to conditions *in vivo* the following points should also be considered. First, the amount of membrane hydrolysed *in vivo* is very small (see for example Pickard & Hawthorne, 1978), whereas in the present paper large amounts of membrane have been employed so that the percentage hydrolysis in our experiments is relatively small. Secondly, the phosphatidylinositol phosphodiesterase is at least 7 times more concentrated *in vivo* than the concentration used in the present paper and it may be that the concentration of the phospholipase (rather than that of the substrate) limits the rate of hydrolysis of membranes (Verger & de Haas, 1976). Also high local concentrations of fatty acids may be generated *in vivo*, for example near a receptor protein. Thirdly, other membranes may well show a greater sensitivity in this effect than rat liver endoplasmic reticulum, which is here acting only as a convenient source of ^{32}P-labelled membrane. Thus the stimulations observed in Table 1 could, *in vivo*, indeed be of physiological significance.

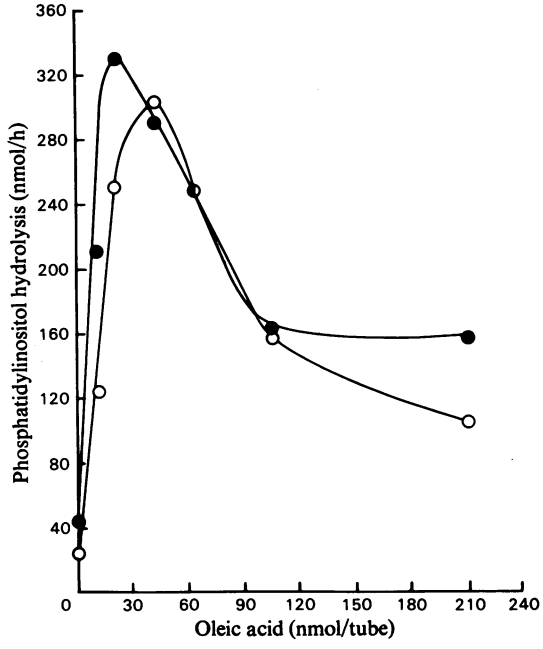

Fig. 1. *Effect of oleic acid on the hydrolysis of pure* [^{32}*P*]-*phosphatidylinositol by soluble rat brain extract*
For method of assay see the Materials and Methods section. Symbols: ●, K^+ concentration 20mM; ○, K^+ concentration 120mM.

A saturated fatty acid (stearate) gave no detectable stimulation of phosphatidylinositol hydrolysis, but it is likely that because of its high melting point it does not enter the membrane at all; if 0.7 μmol of stearate were added after 0.7 μmol of potassium oleate, the stearate had no effect or a slight inhibitory effect on the oleate stimulation. If the two were mixed together (at 80°C; see the Materials and Methods section) and then added, this inhibitory effect was almost total, suggesting again that entry of fatty acids into the membrane is the limiting factor in the activation phenomenon.

The biological detergents palmitoyl-CoA and lysophosphatidylinositol at concentrations up to 1.5 μmol/tube had no detectable effect on phosphatidylinositol hydrolysis. The effects of lysophosphatidylcholine and lysophosphatidylethanolamine were noteworthy as they not only gave no stimulation themselves, but markedly inhibited the oleic acid-stimulated hydrolysis of phosphatidylinositol. As little as 1 lysophosphatidylcholine molecule/6 oleic acid molecules can completely inhibit the hydrolysis (results not shown), though again we do not know how much of the lysophospholipid actually enters

the membrane. It is possible that this inhibition by lysophospholipids may have physiological significance in that only when phospholipids are completely deacylated would stimulated phosphatidylinositol breakdown occur.

The concentration of free Ca^{2+} in the brain extract was low, as indicated by a large increase in phosphatidylinositol hydrolysis (with the pure phospholipid substrate) on addition of Ca^{2+}. Yet we found that phosphatidylinositol hydrolysis in microsomal fraction with 1.4μmol of oleic acid added (Table 1) was consistently increased only by 5–10% on the addition of 2mM-Ca^{2+}; this confirms our previous suggestion (Irvine & Dawson, 1978*a*,*b*) that, once stimulated in this manner, phosphatidylinositol hydrolysis will not be limited by Ca^{2+}, there already being sufficient bound to the membrane to satisfy the enzyme. It is relevant to add that the addition of EDTA completely inhibited the oleic acid-stimulated hydrolysis.

In conclusion, we have shown in the present paper that a soluble brain extract has little activity against phosphatidylinositol in a membrane and that the specific activation by deoxycholate of phosphatidylinositol hydrolysis (Irvine & Dawson, 1978*a*,*b*) can be mimicked by free fatty acids. Thus phosphatidylinositol turnover in a membrane *in vivo* may well be regulated by the activity of the soluble phosphatidylinositol phosphodiesterase against the membrane; this in turn could be controlled by the membrane's content of free fatty acids.

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