The Stimulation by Transmitter Substances and Putative Transmitter Substances of the Net Activity of Phospholipase A2 of Synaptic Membranes of Cortex of Guinea-Pig Brain

By ROBERT J. GULLIS and CHARLES E. ROWE

*Department of Biochemistry, University of Birmingham, P.O. Box 363,
Birmingham B15 2TT, U.K.*

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1. The distribution of the hydrolyses of phosphatidylcholine by phospholipase A2 and phospholipase A1, and the hydrolysis of lysophosphatidylcholine by lysophospholipase, in subcellular and subsynaptosomal fractions of cerebral cortices of guinea-pig brain, was determined. 2. Noradrenaline stimulated hydrolysis by phospholipase A2 in whole synaptosomes, synaptic membranes and fractions containing synaptic vesicles. 3. Stimulation of hydrolysis by phospholipase A2 in synaptic membranes by noradrenaline was enhanced by CaCl_2 , and by a mixture of ATP and MgCl_2 . The optimum concentration of CaCl_2 , in the presence of ATP and MgCl_2 , for stimulation by $10\text{ }\mu\text{M}$ -noradrenaline was in the range $1\text{--}10\text{ }\mu\text{M}$. The optimum concentration for ATP-2 MgCl_2 in the presence of $1\text{ }\mu\text{M}$ - CaCl_2 was in the range $0.1\text{--}1\text{ mM}$. 4. Hydrolysis by phospholipase A2 of synaptic membranes was also stimulated by acetylcholine, carbamoylcholine, 5-hydroxytryptamine, dopamine (3,4-dihydroxyphenethylamine), histamine, γ -aminobutyric acid, glutamic acid and aspartic acid. With appropriate concentrations of cofactors, sigmoidal dose-response curves were obtained, half-maximum stimulations being obtained with concentrations of stimulant in the range $0.1\text{--}1\text{ }\mu\text{M}$. 5. Taurine also stimulated hydrolysis of phosphatidylcholine by phospholipase A2. There were only slight stimulations with methylamine, ethylenediamine or spermidine. No stimulation was obtained with glucagon.

Injection of the anticholinesterase eserine into the ventricles of rat brain increased the concentration of endogenous unesterified fatty acid 30-fold (Lunt & Rowe, 1971). The inference from this observation was that the increase in fatty acid was due to increased concentration of endogenous acetylcholine. Injection of the cholinergic agonist carbamoylcholine increased the concentration of cerebral unesterified fatty acid 50-fold (Lunt & Rowe, 1971). Electroshock and certain convulsive drugs also increased the concentration of unesterified fatty acid in rat brain (Bazán, 1970, 1971a), suggesting a connexion between fatty acid formation and electrical activity involving synaptic transmission.

Noradrenaline and 5-hydroxytryptamine, which are synaptic transmitters in brain (cf. Curtis, 1969), increased the concentration of unesterified fatty acids of synaptosomes and synaptic membranes *in vitro* (Price & Rowe, 1972). Moreover the concentration in incubated osmotically ruptured synaptosomes was enhanced by the monoamine oxidase inhibitor iproniazid phosphate (Price & Rowe, 1972), suggesting that endogenous amines have the same effect. These experiments linked the formation of unesterified fatty acid with the release of transmitter, thus confirming the inferences drawn from cerebral

injections of eserine and carbamoylcholine. The source of the unesterified fatty acid, however, was unknown.

Bazán (1970) reported that during electroshock the concentration of triglyceride in the brain fell. Guinea-pig brain contains triglyceride, diglyceride and monoglyceride lipases (Vyvoda & Rowe, 1973). Moreover, noradrenaline and 5-hydroxytryptamine stimulated the hydrolysis of triglyceride and diglyceride by osmotically ruptured synaptosomes (Vyvoda & Rowe, 1973), suggesting that the stimulation of glyceride lipases could contribute to the previously observed increases in fatty acid. The concentration of triglyceride in brain, however, is low (approx. $0.2\text{ }\mu\text{mol/g}$ of tissue; Rowe, 1969) and the complete hydrolysis of endogenous triglyceride could account for only part of the observed increases in fatty acid.

Brain contains high concentrations of phospholipids, which are a potential source of fatty acid. Phospholipase A1 (Gatt *et al.*, 1966; Gatt, 1968), phospholipase A2 (Cooper & Webster, 1970, 1972) and lysophospholipase (Leibovitz & Gatt, 1968) are present in brain, and phospholipase A1 and phospholipase A2 have been demonstrated in synaptosomes (Bazán, 1971b; Woelk & Porcellati, 1973). It was thus decided to investigate the effects of

transmitter substances, and putative transmitter substances on these phospholipases. The present paper reports the stimulation of the hydrolysis of phosphatidylcholine by phospholipase A2 in synaptic membranes, by noradrenaline, acetylcholine, carbamoylcholine, 5-hydroxytryptamine, dopamine, histamine, γ -aminobutyric acid, glutamic acid, aspartic acid and taurine. Preliminary communications of some of this work have been given (Gullis & Rowe, 1973a,b).

Experimental

Materials

Male guinea pigs of Dunkin-Hartley strain, 2-3 months of age, were used. Egg lecithin (phosphatidylcholine), methylamine hydrochloride and inorganic chemicals were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. L-Noradrenaline hydrochloride, L-5-hydroxytryptamine creatine sulphate, L-adrenaline bitartrate, dopamine (3,4-dihydroxyphenethylamine) hydrochloride, histamine dihydrochloride, γ -aminobutyric acid, carbamoylcholine, taurine, spermidine trihydrochloride, ethylenediamine dihydrochloride, tranlycypromine, coenzyme A (free acid, grade 1 from yeast) and dithiothreitol were supplied by Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Tris-ATP, used throughout, was prepared from the disodium salt of adenosine 5'-triphosphate (obtained from Kyowa Kakko Kogyo Co. Ltd., Tokyo, Japan) by ion exchange on Dowex 50, as described by Schwartz *et al.* (1962). Silicic acid (100 mesh), manufactured by Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A., was supplied by Camlab, Cambridge, U.K. Kiesegel G, made by E. Merck, Darmstadt, Germany, was supplied by Anderman and Co. Ltd., London S.E.1, U.K. Triton X-100 was supplied by Lennig Chemicals, Croydon, U.K. [9,10- ^3H]oleic acid and [1- ^{14}C]palmitic acid were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Phosphor used for the determination of radioactivity consisted of 2 vol. of a mixture of 0.6% (w/v) 2,5-diphenyloxazole and 0.15% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene, dissolved in xylene, mixed with 1 vol. of Triton X-100. Solvents were supplied by Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K., and were distilled before use.

Phosphatidylcholine was purified from egg lecithin by chromatography on alumina (Long & Staples, 1961) followed by chromatography on silicic acid (Robertson & Lands, 1962).

Isolation of choline plasmalogen

Lipid was extracted from ox heart with 19 vol. of chloroform-methanol (2:1, v/v; Folch *et al.*, 1957).

Choline-containing phospholipids were isolated by the method of Rouser *et al.* (1961) by using samples containing up to 100 μmol of lipid P on columns (40 cm $\times$ 2.5 cm diam.) containing 15 g of DEAE-cellulose (acetate form). The column was washed with 5 column volumes of chloroform to remove the neutral lipids. Choline-containing phospholipids were then collected by washing with 8 vol. of chloroform-methanol (9:1, v/v).

A mixture of phosphatidylcholine and choline plasmalogen was isolated from the choline-containing phospholipids (which contained lysophosphatidylcholine and sphingomyelin) by chromatography on silicic acid (Robertson & Lands, 1962) as follows. Lipid, containing 50 μg of P, dissolved in 0.50 ml of chloroform-methanol (9:1, v/v) was passed down a column (25 cm $\times$ 1.5 cm diam.) of 5 g of silicic acid. After washing with 70 ml of chloroform-methanol (9:1, v/v), to remove traces of neutral lipids, the mixture of phosphatidylcholine and choline plasmalogen was collected by elution of the column with 250 ml of chloroform-methanol (3:2, v/v).

Plasmalogen was isolated from the mixture of plasmalogen and phosphatidylcholine by the method of Coleman (1962). The procedure is based on the observation that diacylglycerophosphorylcholine (phosphatidylcholine) is hydrolysed by phospholipase D more rapidly than is 1-alkenyl-2-acylglycerophosphorylcholine (choline plasmalogen). We are grateful to Dr. R. Coleman for advice on the preparation of phospholipase D, and the hydrolysis conditions used. Phospholipase D was prepared by extraction of 60 g of cabbage (*Brassica oleracea*) with 120 ml of 0.2 M-sodium acetate buffer, pH 5.6, for 1 min in a Waring blender (see Davidson & Long, 1958). Fibrous material was removed by filtration through gauze and the filtrate was centrifuged at 36000g for 30 min at 2°C. The supernatant was heated at 55°C for 5 min and the precipitate formed removed by centrifugation for 100000g for 1 h at 2°C. Glycerophosphorylcholine-containing lipid (containing 180 μmol of P and 72 μmol of alkenyl ether), dissolved in 6 ml of diethyl ether, was mixed with 8.0 ml of phospholipase D preparation. The mixture, after dilution to 20 ml with water and addition of CaCl_2 (2 mmol), was left at room temperature ($\sim 20^\circ\text{C}$) for 3 h with occasional shaking. Diethyl ether (10 ml) was added and shaken. The diethyl ether was then removed and the aqueous layer extracted three times with diethyl ether, the two layers being separated by centrifugation in an MSE bench centrifuge. The combined extracts in diethyl ether were concentrated to dryness. Water was removed by repeated distillation to dryness in methanol. The lipid was dissolved in chloroform. Plasmalogen was separated from phosphatidic acid (produced from the hydrolysis of diacylglycerophosphorylcholine) by chromatography on

silicic acid. Phospholipid (50 μ mol of P) dissolved in chloroform (1.0ml) was passed through a column (1.5cm diam.) of silicic acid (5g). The column was washed successively with chloroform (25ml) and chloroform-methanol (9:1, v/v) (50ml). Plasmalogen was then collected by elution with 100ml of chloroform-methanol (3:2, v/v). The solution was concentrated to dryness and the residue dissolved in chloroform (10 μ mol of P/ml) and stored under N₂ at 4°C. Only one component was detectable by t.l.c. The i.r. spectrum of the isolated product could be distinguished from that of phosphatidylcholine by a strong band at 6.0 μ m, which is attributable to C=C stretching (see Göttfried & Rapport, 1962). The ratio phosphate:vinyl ether:ester was 1.0:0.98:0.92.

Preparation of 1-acyl-2-[9,10-³H]oleoyl-sn-glycero-3-phosphorylcholine and [1-¹⁴C]palmitoyl-2-acyl-sn-glycero-3-phosphorylcholine

1-Acyl-2-[9,10-³H]oleoyl-sn-glycero-3-phosphorylcholine was prepared by the acylation of 1-acyl-sn-glycero-3-phosphorylcholine with [9,10-³H]oleic acid (specific radioactivity 1 Ci/mmol) by the procedure of Lands (1960). Similarly, [1-¹⁴C]palmitoyl-2-acyl-sn-glycero-3-phosphorylcholine was prepared by the acylation of 2-acyl-sn-glycero-3-phosphorylcholine with [1-¹⁴C]palmitic acid (specific radioactivity 50 Ci/mol). 1-Acyl-sn-3-phosphorylcholine was prepared from the action of snake (*Crotalus adamanteus*) venom on egg phosphatidylcholine as described by Robertson & Lands (1962). 2-Acyl-sn-3-phosphorylcholine was prepared by the removal of the 1-alkenyl ether from choline plasmalogen by iodination immediately before use, as described by Lands (1965).

Radioactive phosphatidylcholine synthesized was isolated by t.l.c. on a layer (0.5mm thick) of Kieselgel G by using the solvent mixture chloroform-methanol-acetic acid-water (50:30:8:5, by vol). 1-Acyl-2-[9,10-³H]oleoyl-sn-glycero-3-phosphorylcholine contained 0.06mol % unesterified fatty acid, which contained 0.4% of the total radioactivity.

When 1-acyl-2-[9,10-³H]-oleoyl-sn-glycero-3-phosphorylcholine was treated with snake (*Crotalus adamanteus*) venom for 3h and the resulting hydrolysate, to which 0.50 μ mol of oleic acid, 0.20 μ mol of phosphatidylcholine and 0.20 μ mol of lysophosphatidylcholine had been added as carriers, was analysed by t.l.c.: 97–98% of the recovered radioactivity was in fatty acid; 1–2% of the recovered radioactivity remained in unhydrolysed lipid. When [1-¹⁴C]palmitoyl-2-acyl-sn-glycero-3-phosphorylcholine was treated in the same manner, 97% of the recovered radioactivity was in the monoacylglycero-phosphorylcholine and 2% remained in unhydrolysed lipid.

All radioactive phospholipids were stored in solutions in chloroform under N₂ at –20°C.

Preparation of synaptic membranes and other subcellular fractions

Guinea pigs were killed by a blow on the back of the head. Cortices were removed and subcellular fractions were prepared by the method of Eichberg *et al.* (1964). Synaptosomes were osmotically ruptured as described by Whittaker *et al.* (1964). Preparations were checked for complete rupture by ascertaining that the activities of lactate dehydrogenase determined in water and in 0.1% (w/v) Triton X-100 were identical. Subsynaptosomal fractions were prepared by the procedure of Whittaker *et al.* (1964). Fraction G, containing synaptosome 'ghosts', was used in experiments with synaptic membranes. Tissue fractions were suspended in water (5mg of protein/ml) and used immediately.

Determination of phospholipase A₂, phospholipase A₁ and lysophospholipase

Preparation of emulsions of phospholipid. Phospholipase A₂ was determined from the release of [9,10-³H]oleic acid from 1-acyl-2-[9,10-³H]oleoyl-sn-glycero-3-phosphorylcholine, and phospholipase A₁ from the release of [1-¹⁴C]palmitic acid from [1-¹⁴C]palmitoyl-2-acyl-sn-glycero-3-phosphorylcholine. The procedure was based upon that of Rahman & Verhagen (1970). An emulsion of the substrate was prepared as follows. A mixture of radioactive lipid and egg phosphatidylcholine, with a specific radioactivity of approx. 15 Ci/mol was concentrated to dryness on a rotary evaporator. The residue was dissolved in approx. 3ml of diethyl ether. Water was added to yield a final concentration of lipid of 1 μ mol/ml. Diethyl ether was removed by evaporation at room temperature in a rotary evaporator. The emulsion obtained was sonicated (18000–20000Hz) in a vessel surrounded by ice for three periods of 1 min, with 30s between each period.

Lysophospholipase was determined from the release of [1-¹⁴C]palmitate from [1-¹⁴C]palmitoyl-sn-glycero-3-phosphorylcholine (lysolecithin). Emulsions used for the determination of lysophospholipase were prepared as described above for phosphatidylcholine except that the use of diethyl ether was omitted and that emulsification was achieved by vigorous agitation with water with a Whirlimixer (made by Fisons Scientific Apparatus) to give a final concentration of 0.5 μ mol/ml. All emulsions of phospholipid were used within 1h of preparation.

Conditions of incubation. Tissue (50–100 μ g of protein), suspended in water (20 μ l), was incubated with 50mM-Tris-HCl buffer, pH 7.4 (20 μ l), at 37°C for approx. 3min. Cofactors in water (10 μ l) were added individually or in mixtures, followed 10s later by radioactive phospholipid (20nmol, 20 μ l).

Lysophosphatidylcholine (10 nmol) was used for the determination of lysophospholipase. Neurohormones and other stimulants under investigation, dissolved in water (10 μ l), were then added immediately. All solutions added were maintained at 37°C before use. The final concentration of buffer was 10 mM and the total volume of the incubation mixture was 100 μ l. The use of cofactors, including ATP, MgCl₂, CaCl₂ and theophylline, is indicated in the legends to Figures and Tables. The incubation was stopped by the addition of 2.0 ml of chloroform-methanol (2:1, v/v; Folch *et al.*, 1957). Phosphatidylcholine (1.0 μ mol), lysophosphatidylcholine (1-acyl-*sn*-glycero-3-phosphorylcholine; 1.0 μ mol) and fatty acid (oleic acid or palmitic acid; 5 μ mol) were added as carriers in volumes of 10 μ l from standard solutions in chloroform.

Time-courses of the release of fatty acid, over a period of at least 3 min from the addition of substrate, were determined from measurements made at intervals of 30 s. Most experiments were repeated at least once. Rates of hydrolyses given in the Tables were calculated from the radioactive fatty acid released after 1 min, assuming that radioactive and carrier phospholipid were hydrolysed at the same rate.

Determination of radioactive fatty acid. Protein was removed by centrifugation. The pellet was washed with chloroform-methanol (2:1, v/v) equal in volume to the supernatant previously removed. The combined supernatants were washed with one-fifth of their volume of 0.1 M-KCl. The aqueous layer was washed again with 1.0 ml of chloroform. The combined extracts in organic solvent were concentrated to dryness at 40°C under N₂, and the residue was dissolved in 0.20 ml of chloroform. The solution of lipid was chromatographed in a band 2 cm wide on a layer of Kieselgel G, 0.5 mm thick, in the solvent chloroform-methanol-acetic acid-water (25:15:4:2, by vol.; Skipski *et al.*, 1964). Components were detected by immersion of the dried plate for a short time into iodine vapour. The positions of the components were marked, and after evaporation of the iodine (normally 30 min) the part of the chromatogram containing oleic acid was transferred to a vial and mixed thoroughly with Triton X-100-phosphor (1:2, v/v). When the Kieselgel had settled radioactivity was determined by liquid-scintillation counting in a Philips counter. Radioactivity was corrected to 100% efficiency by the use of an external standard.

Analyses. Esters were determined by the procedure of Snyder & Stevens (1959). $\alpha\beta$ -Unsaturated esters of plasmalogen were determined by interaction with I₂ by the method of Norton (1960) as described by Gottfried & Rapport (1962). Phosphate was determined by the method of King (1932). Protein was determined, by either the method of Lowry *et al.* (1951) or its modification by Whittaker & Barker (1972).

Results

Subcellular distributions of hydrolyses by phospholipases

The distributions of hydrolysis of phospholipid by phospholipase A₂, phospholipase A₁ and lysophospholipase in subcellular fractions and subsynaptosomal fractions of cortices of guinea-pig brain are shown in Table 1. Phospholipase A₂ activity was distributed throughout and was of highest specific activity with respect to the hydrolysis of added phosphatidylcholine in mitochondrial and synaptosomal fractions. There was little activity in the supernatant fraction of homogenized cortex, or in the cell sap of osmotically shocked synaptosomes, indicating that the enzyme was bound to membrane. In the subsynaptosomal fractions activity was again concentrated in the mitochondria, and there was considerable activity in the synaptic vesicles.

In contrast with phospholipase A₂, the activity of phospholipase A₁ was concentrated to a high degree in the microsomal fractions prepared from whole cortex (fraction P₃) and from synaptosomes (fraction E). There was also considerable activity in the synaptic-vesicle fraction. Again the enzyme was mostly membrane-bound. The distribution of the observed activity of lysophospholipase was similar to that of phospholipase A₁, except that there was appreciable activity in the primary supernatant fraction (S₃) of cortex (relative specific activity = 0.18), and considerable concentration of activity in the soluble fraction of synaptosomes (relative specific activity = 5.31). Thus lysophospholipase was either active in solution in synaptosomes or had been activated or removed from membranes during the preparation of subsynaptosomal fractions.

The effects of noradrenaline on hydrolyses by phospholipase A₂, phospholipase A₁ and lysophospholipase in the subcellular and subsynaptosomal fractions are shown in Table 2. Hydrolysis by phospholipase A₂ of synaptosomes was stimulated fivefold by 10 μ M-noradrenaline under the conditions used. Other primary fractions were relatively little affected. Of the subsynaptosomal fractions hydrolyses by the phospholipase A₂ of fractions containing membranes and vesicles were stimulated. Hydrolysis by phospholipase A₂ in the mitochondrial and soluble fractions of synaptosomes was little affected by noradrenaline. Phospholipase A₁ and lysophospholipase of all tissue fractions were relatively unaffected.

In all fractions the values of the observed activities of phospholipase A₂ and phospholipase A₁ in both the absence and presence of noradrenaline, determined from the production of unesterified fatty acid, were close to those determined from the measurement of production of lysophosphatidylcholine (Table 2). Thus the production of fatty acid measured was due

Table 1. *Subcellular and subsynaptosomal distributions of hydrolyses by phospholipase A2, phospholipase A1 and lysophospholipase in the cortex of guinea-pig brain*
Tissue was incubated in 10mM-Tris-HCl buffer, pH 7.4, containing ATP (0.10mM), MgCl₂ (0.20mM), CaCl₂ (1.0 μ M) and theophylline (0.50mM). R.S.A. is the relative specific activity, which is defined as specific activity of the fraction/specific activity of the homogenate.

Primary fractions	Content	Phospholipase A2			Phospholipase A1			Lysophospholipase		
		Protein recovered (%)	Recovery (%)	Specific activity (nmol of fatty acid released/min per mg of protein)	Recovery (%)	Specific activity (nmol of fatty acid released/min per mg of protein)	Recovery (%)	Recovery (%)	Specific activity (nmol of fatty acid released/min per mg of protein)	R.S.A.
Homogenate	—	100.0	100.0	7.3	1.00	8.3	1.00	100.0	0.80	1.00
P1	Nuclei, large myelin, cell debris	25.1	8.0	2.3	0.32	2.0	0.24	9.3	0.30	0.38
P2A	Small myelin	8.1	4.5	4.1	0.56	1.7	0.20	5.7	0.57	0.71
P2B	Synaptosomes	12.0	16.9	8.3	1.14	7.7	0.64	3.5	0.23	0.29
P2C	Mitochondria	12.1	45.7	27.3	3.76	1.1	0.13	2.2	0.14	0.18
P3	Microsomal fraction	14.3	2.3	1.1	0.16	39.5	4.75	55.6	3.12	3.90
S3	Cell sap	26.2	1.3	0.4	0.05	0.1	0.01	4.7	0.14	0.18
Total recovery (%)		97.8	78.7			84.9		81.4		
Subsynaptosomal fractions										
O	Cell sap	8.0	0.6	0.7	0.07	0.8	0.15	41.0	1.18	5.13
D	Synaptic vesicles	4.0	3.9	10.0	0.98	12.5	2.31	4.2	0.24	1.04
E	Microsomal fraction, synaptic vesicles	3.7	1.9	5.3	0.50	43.6	8.07	12.4	0.77	3.35
F	Membranes	8.1	2.6	3.2	0.32	9.5	1.76	3.5	0.10	0.43
G	Synaptosome 'ghosts'	10.2	4.6	4.6	0.45	4.1	0.76	3.1	0.07	0.30
H	Damaged synaptosomes	27.6	14.4	5.3	0.52	3.7	0.69	11.9	0.10	0.43
I	Mitochondria	35.0	52.7	15.5	1.51	1.4	0.26	12.1	0.08	0.34
Total recovery (%)		96.6	80.7			90.5		88.2		

Table 2. *Effects of noradrenaline on hydrolyses by phospholipase A₂, phospholipase A₁ and lysophospholipase of subcellular and subsynaptosomal fractions of cortex of guinea-pig brain*

Hydrolysis by phospholipase A₂ was determined from the release of either [9,10-³H]oleate from 1-acyl-2-[9,10-³H]oleoyl-*sn*-glycero-3-phosphorylcholine, or radioactive lysophosphatidylcholine from [1-¹⁴C]palmitoyl-2-acyl-*sn*-glycero-3-phosphorylcholine. Hydrolysis by phospholipase A₁ was determined from the release of [1-¹⁴C]palmitate from [1-¹⁴C]palmitoyl-2-acyl-*sn*-glycero-3-phosphorylcholine, or radioactive lysophosphatidylcholine from 1-acyl-2-[9,10-³H]oleoyl-*sn*-glycero-3-phosphorylcholine. Membranes were incubated in 10 mM-Tris-HCl, pH 7.4, with 0.20 mM-ATP, 0.40 mM-MgCl₂, 1.0 μM-CaCl₂ and 0.50 mM-theophylline. Final concentration of noradrenaline was 50 μM.

Primary fractions	Phospholipase A ₂ (nmol of fatty acid released/min per mg of protein)				Phospholipase A ₁ (nmol of fatty acid released/min per mg of protein)				Lysophospholipase (nmol of fatty acid released/min per mg of protein)			
	Control		Noradrenaline present		Control		Noradrenaline present		Control		Noradrenaline present	
	From [³ H]-fatty acid	From [¹⁴ C]-lysophosphatidylcholine	From [³ H]-fatty acid	From [¹⁴ C]-lysophosphatidylcholine	From [¹⁴ C]-fatty acid	From [³ H]-lysophosphatidylcholine	From [¹⁴ C]-fatty acid	From [³ H]-lysophosphatidylcholine	From [¹⁴ C]-fatty acid	From [³ H]-lysophosphatidylcholine	From [¹⁴ C]-fatty acid	From [³ H]-lysophosphatidylcholine
Homogenate	6.59	6.00	10.3	9.91	8.30	7.41	7.71	7.41	0.91	1.01	0.47	0.42
Nuclei, large myelin, cell debris	2.51	2.37	3.01	2.84	2.46	2.12	2.07	2.12	0.41	0.45	0.28	0.31
Small myelin	3.78	3.61	4.52	4.12	1.54	1.40	1.21	1.40	0.91	1.01	0.18	0.20
Synaptosomes	6.27	5.42	31.6	29.1	6.02	5.74	5.12	4.94	0.41	0.45	0.12	0.13
Mitochondria	29.3	28.7	32.4	30.1	1.51	1.01	1.24	1.09	0.91	1.01	0.18	0.20
Microsomal fraction	1.50	0.86	1.97	1.74	35.2	34.2	31.1	31.7	0.91	1.01	0.18	0.20
Cell sap	0.27	0.21	0.32	0.29	0.20	0.20	0.20	0.20	0.91	1.01	0.12	0.13
Subsynaptosomal fractions												
Cell sap	0.65	0.62	0.72	0.59	0.72	0.70	0.70	0.65	1.32	1.37	0.27	0.31
Synaptic vesicles	8.27	7.87	28.8	27.8	10.5	9.4	8.72	9.01	0.27	0.31	0.62	0.62
Microsomal fraction, synaptic vesicles	4.64	4.02	6.1	4.0	37.1	35.4	36.1	34.2	0.62	0.62	0.13	0.16
Membranes	2.64	2.30	7.1	6.1	9.1	9.0	8.84	8.72	0.13	0.16	0.09	0.10
Synaptosome 'ghosts'	4.10	3.87	19.9	18.2	4.8	4.3	4.12	4.13	0.09	0.10	0.12	0.14
Damaged synaptosomes	4.87	4.56	14.7	13.3	3.6	3.1	3.17	3.00	0.12	0.14	0.06	0.08
Mitochondria	15.0	14.6	16.1	14.1	1.3	1.1	1.29	1.00	0.06	0.08		

solely to the activity of a phospholipase A, and was not due to the consecutive actions of phospholipase A and lysophospholipase. The observation also indicates that the assumption, made in calculating activities, of lack of specificity between radioactive oleate and other non-radioactive fatty acids in identical steric positions in the molecule, was correct.

Attention was concentrated on the hydrolysis of phospholipid by synaptic membranes. The time-courses for hydrolyses by phospholipase A1, phospholipase A2 and lysophospholipase are shown in Fig. 1. Observed activities of the three enzymes were proportional to concentration of membrane in the range 0–200 µg of protein per incubation. Inclusion of 0.1% (v/v) bovine serum albumin in the incubation mixture decreased hydrolysis by phospholipase A2 of synaptic membranes by 56%.

Table 3 shows the effects of MgCl₂, CaCl₂, ATP, EDTA and EGTA [ethanedioxybis(ethylamine)tetraacetate] on the stimulation by noradrenaline of hydrolysis by phospholipase A2 of synaptic membranes. Noradrenaline without cofactors stimulated hydrolysis nearly 2.5-fold (Table 3). Stimulation was enhanced slightly by the addition of CaCl₂ and MgCl₂, CaCl₂ being the most effective. Stimulation was decreased by EDTA and more so by EGTA, which preferentially complexes Ca²⁺. In the presence of noradrenaline (5 µM) and theophylline (0.50 mM), a combination of ATP (0.20 mM), CaCl₂ (1.0 µM) and MgCl₂ (40 µM) increased hydrolysis of phosphatidylcholine from 2.0 to 21.4 nmol of fatty acid released/min per mg

of protein. Increasing the concentration of MgCl₂ to 1.0 mM, 1.5 mM and 5.0 mM decreased hydrolysis to 21.2, 18.0 and 16.0 nmol of fatty acid released/min per mg of protein respectively. In the presence of a combination of noradrenaline (5.0 µM), ATP (0.20 mM) and MgCl₂ (0.40 mM), EDTA (1.0 mM) decreased hydrolysis from 21.4 to 5.4 nmol of fatty acid released/min per mg of protein. EGTA (1.0 mM) decreased hydrolysis to 3.0 nmol of fatty acid released/min per mg of protein. CaCl₂ without noradrenaline, at concentrations greater than 1.0 µM, decreased hydrolysis, and this decrease was enhanced by combined ATP and MgCl₂ (Table 4).

In the presence of 0.20 mM-ATP and 0.40 mM-MgCl₂ there was an optimum for the concentration of CaCl₂ for stimulation by 10 µM-noradrenaline, which was in the range 1–10 µM (Fig. 2). In the presence of 1 µM-CaCl₂, ATP–2MgCl₂ enhanced stimulation, the maximum effect being obtained with ATP in the concentration range 0.1–1 mM (Fig. 3). The interrelationships between noradrenaline, ATP and bivalent cations, and the stimulation they produce, are illustrated by the noradrenaline dose–response curve shown in Fig. 4. Under conditions used as a routine there was optimum stimulation at a noradrenaline concentration of 10 µM, higher concentrations being less effective. Increased concentrations of CaCl₂ (10 µM) increased stimulation by high concentrations of noradrenaline. With 10 µM-CaCl₂ and with high concentrations of ATP (5 mM) and MgCl₂ (10 mM) a sigmoidal dose–response curve was obtained.

Fig. 1. Time-courses of the hydrolyses of added phospholipid by phospholipase A2, phospholipase A1 and lysophospholipase

Membranes were incubated with 0.20 mM-ATP, 0.40 mM-MgCl₂ and 1.0 µM-CaCl₂. Δ, Release of [9,10-³H]oleate from 1-acyl-2-[9,10-³H]oleoyl-*sn*-glycero-3-phosphorylcholine; ○, release of [1-¹⁴C]palmitate from [1-¹⁴C]-palmitoyl-2-acyl-*sn*-glycero-3-phosphorylcholine; □, release of [1-¹⁴C]palmitate from [1-¹⁴C]palmitoyl-*sn*-glycero-3-phosphorylcholine.

Table 3. Effects of CaCl₂, MgCl₂, EDTA, EGTA and noradrenaline on the hydrolysis of phosphatidylcholine by phospholipase A2 of synaptic membranes

Comparison of effects of CaCl₂ and MgCl₂ on the stimulation of hydrolysis by noradrenaline. Membranes were incubated in the presence of 0.50 mM-theophylline.

Additions	Hydrolysis of phosphatidylcholine (nmol of fatty acid released/min per mg of protein)
None	3.0
Noradrenaline (5 µM)	7.2
Noradrenaline (5 µM)+MgCl ₂ (10 µM)	7.5
Noradrenaline (5 µM)+EDTA (1.0 mM)	8.4
Noradrenaline (5 µM)+CaCl ₂ (1.0 µM)	6.5
Noradrenaline (5 µM)+EGTA (1.0 mM)	9.0
Noradrenaline (5 µM)+MgCl ₂ (1.0 mM)	5.0

Table 4. *Effects of CaCl_2 , in the absence of noradrenaline, on the hydrolyses of phosphatidylcholine by phospholipase A2*

Other cofactors	Concn. of CaCl_2	Time (min) ...	Hydrolysis of phosphatidylcholine (nmol of fatty acid released/mg of protein)						
			0.25	0.50	1.00	1.50	2.00	2.50	3.00
None	1.0 μM		1.20	1.90	2.75	3.20	3.15	3.25	3.05
	10.0 μM		1.20	1.90	2.55	2.90	2.90	2.80	2.55
	0.10 mM		1.00	1.50	2.30	2.65	2.50	2.30	1.90
	1.0 mM		0.85	1.30	2.05	2.10	2.20	1.70	1.40
ATP (0.20 mM)† MgCl ₂ (0.41 mM)	1.0 μM		1.35	1.80	2.60	2.50	2.50	2.00	1.85
	10.0 μM		0.85	1.35	1.90	2.10	2.70	1.35	1.00
	0.10 mM		0.60	1.00	1.50	1.50	1.25	0.90	0.95
	1.0 mM		0.35	1.15	1.10	0.95	0.75	0.35	0.05

Fig. 2. *Effect of CaCl_2 on the noradrenaline-stimulated hydrolysis by phospholipase A2*

Synaptic membranes were incubated with radioactive phospholipid in the presence of 0.20 mM-ATP, 0.40 mM-MgCl₂, 0.50 mM-theophylline and 10 μM -noradrenaline.

The stimulation of hydrolysis by phospholipase A2 was reversed after a few minutes unless theophylline was present. This is illustrated for stimulation by noradrenaline in Fig. 5. The monoamine oxidase inhibitor, tranylcypromine, was without effect, suggesting that the reversal was not due to removal of amine by oxidation. Hydrolysis of phosphatidyl-

Fig. 3. *Effect of combined ATP and MgCl₂ on the noradrenaline-stimulated hydrolysis by phospholipase A2*

ATP and MgCl₂ were present in the molar ratio 1:2. Concentrations of ATP are shown on the abscissa. CaCl₂ (1.0 μM) and theophylline (0.50 mM) were present. $\circ$, Noradrenaline (10 μM) present; $\square$, noradrenaline absent.

choline by phospholipase A2 of synaptic membranes was stimulated by acetylcholine and by carbamoylcholine (Fig. 6). Without theophylline, the effects of both stimulations were reversed, the reversal of the stimulation by acetylcholine being the most rapid. With theophylline the release of fatty acid induced by carbamoylcholine, but not acetylcholine, was sustained. The reversal of stimulation by acetylcholine, which was less rapid than that obtained in the absence of theophylline, is likely to have been due to hydrolysis of acetylcholine by acetylcholinesterase in the synaptic membranes. GTP was more effective than ATP in enhancing the stimulation of hydrolysis by

phospholipase A2 by acetylcholine (Table 5) and ATP was more effective than GTP in enhancing the stimulation by noradrenaline.

Hydrolysis by phospholipase A2 was stimulated by 5-hydroxytryptamine, dopamine, histamine and γ -aminobutyric acid. Again, theophylline was necessary for the effects to be sustained. The dose-response

curves for these stimulants, together with those of noradrenaline, acetylcholine and carbamoylcholine, are shown in Fig. 7. Stimulation was also obtained with glutamic acid, aspartic acid and taurine (Table 6), but not with glucagon. Stimulation by methylamine, ethylenediamine and spermidine was relatively small.

Fig. 4. Effects of CaCl_2 and combined ATP and MgCl_2 on the dose-response curve for the noradrenaline-stimulated hydrolysis by phospholipase A2

□, Cofactors consisted of ATP, 0.20 mM; MgCl_2 , 0.40 mM; CaCl_2 , 1.0 μM ; theophylline, 0.50 mM. ◇, cofactors were as described above except that the concentration of CaCl_2 was 10 μM . △, ATP, 5.0 mM; MgCl_2 , 10 mM; CaCl_2 , 10 μM ; theophylline, 0.50 mM. ●, Noradrenaline was present with equimolar quantities of ATP and CaCl_2 , concn. of theophylline was 0.50 mM.

Fig. 5. Effects of theophylline and tranylcypromine on the noradrenaline-stimulated hydrolysis by phospholipase A2

All membranes were incubated in the presence of ATP (0.20 mM), MgCl_2 (0.40 mM) and CaCl_2 (1.0 μM). □, Membranes without other additions; ●, membranes with noradrenaline (10 μM) and theophylline (0.50 mM); ▲, membranes with noradrenaline (10 μM) and tranylcypromine (0.50 mM).

Fig. 6. Stimulation by acetylcholine and carbamoylcholine of hydrolysis of phosphatidylcholine by phospholipase A2: the effects of theophylline

All membranes were incubated in the presence of GTP (0.20 mM), MgCl_2 (0.40 mM) and CaCl_2 (1.0 μM). (a) ○, Membranes without other additions; □, acetylcholine (10 μM) present; ■, acetylcholine (10 μM) and theophylline (0.50 mM); (b) △, carbamoylcholine (10 μM); ▲, carbamoylcholine (10 μM) and theophylline (0.50 mM).

Table 5. Comparison of the effects of GTP and ATP on the acetylcholine- and noradrenaline-stimulated hydrolyses by phospholipase A2

Membranes were incubated in the presence of 0.50mM-theophylline. Concentrations of reagents were: acetylcholine, 10 μ M; noradrenaline, 10 μ M; MgCl₂, 0.40mM; GTP, 0.20mM; ATP, 0.20mM.

Additions	Hydrolysis of phosphatidylcholine (nmol of fatty acid released/min per mg of protein)
None	1.60
Acetylcholine	9.00
Acetylcholine+MgCl ₂ +GTP	18.30
Acetylcholine+MgCl ₂ +ATP	13.60
Noradrenaline	8.60
Noradrenaline+MgCl ₂ +ATP	17.00
Noradrenaline+MgCl ₂ +GTP	12.20

Discussion

It is usually assumed that hydrolysis of phospholipid added to particulate fractions is a measure of phospholipase activity (e.g. Bazán, 1971*b*; Woelk & Porcellati, 1973; Nachbaur *et al.*, 1972). When synaptic membranes were incubated with oleate-albumin, oleate was incorporated into the choline glycerophosphate of the membrane (Gullis & Rowe, 1975*a*). The acylating activity was low compared with the release of fatty acid from phosphatidylcholine and was negligible compared with the stimulated fatty acid release. However, the possibility that part or all of the observed transmitter-induced increase of phospholipid hydrolysis was due to suppression of the reacylation of lysophosphatidylcholine cannot be discounted.

The subcellular distributions of hydrolysis of phospholipid by phospholipase A2 and phospholipase A1 in cerebral cortex of the guinea pig were similar to these reported previously for cerebral cortex and whole brain, of rat (Bazán, 1971*b*; Woelk & Porcellati, 1973). These authors, however, measured hydrolysis after 1 h and either used detergents (Woelk & Porcellati, 1973), or different fractionation procedures from that reported in the present paper. In brain of both guinea pig and rat, however, there is a relatively high observable activity of phospholipase A2 in mitochondria and a high observable activity of phospholipase A1 in the microsomal fraction.

The hydrolyses by phospholipase A2 and phospholipase A1 in synaptosome ghosts were similar and higher than the hydrolysis by lysophospholipase. A characteristic of the preparation was that phospholipase A2 and phospholipase A1 hydrolysed added phosphatidylcholine for a short time only. The reac-

tion was usually complete after 1 min and only 0.5% of the available substrate was hydrolysed. Product inhibition is a possible explanation for the short reaction time. The lack of observable stimulation of phospholipase A2 by added serum albumin, however, suggests that either lysophosphatidylcholine was the inhibitor or that the liberated fatty acid, including that produced by endogenous lipid, was not readily accessible to albumin. Initial rates of reaction under the experimental conditions used were approximately equivalent to 2 nmol of fatty acid released/min per mg of protein (cf. Fig. 6). The total fatty acid released was usually 3 nmol/g of protein. This is less than the increase in endogenous fatty acid produced by noradrenaline and 5-hydroxytryptamine (Price & Rowe, 1972).

An unexpected finding was that ATP, or in the case of cholinergic stimulants, GTP, was necessary for maximum stimulation of hydrolysis by phospholipase A2. A possible explanation is that stimulant-ATP-cation complexes are necessary for the binding of stimulant to membrane receptors. Rajan *et al.* (1972) have shown that catecholamine-ATP-Mg²⁺ complexes are formed in solution. High concentrations of amines decreased stimulation unless the amines were reinforced with additional ATP and bivalent cation. Thus the stimulants appeared to remove their cofactors, thereby diminishing their own effects. *In vivo* this could be part of a self-regulatory system gearing stimulation to metabolic state and protecting the neurons from stimulation beyond reversibility.

Another possible explanation for the effect of ATP is that stimulation of phospholipase is mediated by cyclic AMP and that ATP-2Mg²⁺ is needed as a substrate for adenylate kinase. These possibilities are discussed elsewhere (Gullis & Rowe, 1975*b*).

With the exception of carbamoylcholine all the substances reported which stimulated hydrolysis by phospholipase A2 are present in the central nervous system. Acetylcholine, noradrenaline, 5-hydroxytryptamine and γ -aminobutyric acid are transmitters and there is evidence that dopamine and histamine may also be. A transmitter role has been suggested for taurine (Davison & Kaczmarek, 1971). As transmitters these substances would be released into the synaptic cleft and be in contact with synaptic membranes. In the absence of theophylline, stimulations were temporary, which is a characteristic expected for changes in membrane properties leading to reversible changes in permeability to cations during synaptic transmission. Release and re-uptake of fatty acids *in vitro* after addition of neurohormone lasted a few minutes. Endogenous pools of substrates and cofactors might be in closer proximity to relevant enzymes *in vivo* than those added *in vitro*, which might lead to more rapid reactions, necessary for synaptic

Fig. 7. Dose-response curves for the stimulation by transmitter substances, and putative transmitter substances, of the hydrolysis of phosphatidylcholine by phospholipase A2

All membranes were incubated in the presence of theophylline (0.50mM). (a) Noradrenaline, (c) 5-hydroxytryptamine, (d) histamine, (e) dopamine, (f) γ -aminobutyric acid: $\circ$, stimulant present with equimolar quantities of ATP and CaCl_2 ; Δ , stimulant present with 0.20mM-ATP, 0.40mM- MgCl_2 and 1.0 μM - CaCl_2 ; $\square$, ATP and MgCl_2 omitted, 1.0 μM - CaCl_2 present. (b) Acetylcholine and carbamoylcholine: $\circ$, membranes incubated with acetylcholine in the presence of 0.20mM-GTP, 0.40mM- MgCl_2 and 1.0 μM - CaCl_2 ; $\square$, acetylcholine and 1.0 μM - CaCl_2 present, GTP and MgCl_2 omitted; $\bullet$, membranes incubated with carbamoylcholine in the presence of 0.20mM-GTP, 0.40mM- MgCl_2 and 1.0 μM - CaCl_2 .

Table 6. Stimulation by glutamic acid, aspartic acid and taurine of the hydrolysis of phosphatidylcholine by phospholipase A2

Membranes were incubated with ATP (0.20mM), MgCl_2 (0.40mM), CaCl_2 (1.0 μM) and theophylline (0.50mM).

Substance added	Concn. of substance added (μM) ...	Hydrolysis of phosphatidylcholine (nmol of fatty acid released/min per mg of protein)				
		0	0.1	1.0	10	100
Glutamic acid		2.4	3.7	8.4	12.9	13.0
Aspartic acid		2.5	3.2	7.2	11.1	11.5
Taurine		2.2	2.2	4.5	8.5	10.2
Glucagon		2.7	2.9	2.8	2.7	2.4
Methylamine		1.9	2.1	2.3	2.7	2.6
Ethylenediamine		2.3	2.3	2.7	2.6	3.2
Spermidine		2.8	3.0	3.5	3.4	3.6

transmission. The effects of ATP, $MgCl_2$ and $CaCl_2$ suggest that transmission could be modulated by substances other than transmitters, in or close to the membrane. Such modulation would relate electrical activity to the metabolic and physiological state of the neurons and give greater flexibility to the neuronal network.

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References

- Bazán, N. G. (1970) *Biochim. Biophys. Acta* **218**, 1–10
 Bazán, N. G. (1971a) *J. Neurochem.* **18**, 1379–1385
 Bazán, N. G. (1971b) *Acta Physiol. Lat. Amer.* **21**, 101–106
 Coleman, R. (1962) Ph.D. Thesis, University of Birmingham
 Cooper, M. F. & Webster, G. R. (1970) *J. Neurochem.* **17**, 1543–1554
 Cooper, N. F. & Webster, G. R. (1972) *J. Neurochem.* **19**, 333–340
 Curtis, D. R. (1969) in *Basic Mechanisms of the Epilepsies* (Jasper, H. H., Ward, A. A. & Pope, A., eds.), pp. 105–129, J. and A. Churchill, London
 Davidson, F. M. & Long, C. (1958) *Biochem. J.* **69**, 458–466
 Davison, A. N. & Kaczmarek, L. K. (1971) *Nature (London)* **234**, 107–108
 Eichberg, J., Whittaker, V. P. & Dawson, R. M. C. (1964) *Biochem. J.* **92**, 91–100
 Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
 Gatt, S. (1968) *Biochim. Biophys. Acta* **159**, 304–316
 Gatt, S., Barenholz, Y. & Roitman, A. (1966) *Biochem. Biophys. Res. Commun.* **24**, 169–172
 Gottfried, E. L. & Rapport, M. M. (1962) *J. Biol. Chem.* **237**, 329–333
 Gullis, R. J. & Rowe, C. E. (1973a) *Biochem. Soc. Trans.* **1**, 849
 Gullis, R. J. & Rowe, C. E. (1973b) *Abstr. Int. Congr. Biochem.* 9th p. 415
 Gullis, R. J. & Rowe, C. E. (1975a) *Biochem. J.* in the press
 Gullis, R. J. & Rowe, C. E. (1975b) *Biochem. J.* in the press
 King, E. J. (1932) *Biochem. J.* **26**, 292–297
 Lands, W. E. M. (1960) *J. Biol. Chem.* **235**, 2233–2237
 Lands, W. E. M. (1965) *J. Amer. Oil Chem. Soc.* **42**, 465–467
 Leibovitz, Z. & Gatt, S. (1968) *Biochim. Biophys. Acta* **164**, 439–441
 Long, C. & Staples, D. A. (1961) *Biochem. J.* **80**, 557–562
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
 Lunt, G. G. & Rowe, C. E. (1971) *Brain Res.* **35**, 215–220
 Nachbaur, J., Cobleau, A. & Vignais, P. M. (1972) *Biochim. Biophys. Acta* **274**, 426–446
 Norton, W. T. (1960) *Biochim. Biophys. Acta* **38**, 340–342
 Price, C. J. & Rowe, C. E. (1972) *Biochem. J.* **126**, 575–585
 Rahman, Y. E. & Verhagen, J. (1970) *Biophys. Biochem. Res. Commun.* **38**, 670–677
 Rajan, K. S., Davis, J. M., Colburn, R. W. & Jarke, F. H. (1972) *J. Neurochem.* **19**, 1099–1166
 Robertson, A. F. & Lands, W. E. M. (1962) *Biochemistry* **1**, 804–810
 Rouser, G., Bauman, A. J., Kritchevsky, G., Heller, D. & O'Brien, J. S. (1961) *J. Amer. Oil Chem. Soc.* **38**, 544–555
 Rowe, C. E. (1969) *J. Neurochem.* **16**, 205–214
 Schwartz, A., Bachelard, H. S. & McIlwain, H. (1962) *Biochem. J.* **84**, 626–637
 Skipski, V. P., Peterson, R. F. & Barclay, M. (1964) *Biochem. J.* **90**, 374–378
 Snyder, F. & Stevens, N. (1959) *Biochim. Biophys. Acta* **34**, 244–245
 Vyvoda, O. S. & Rowe, C. E. (1973) *Biochem. J.* **132**, 233–248
 Whittaker, V. P. & Barker, L. A. (1972) in *Methods of Neurochemistry* (Fried, R., ed.), vol. 2, pp. 1–52, M. Dekker, New York
 Whittaker, V. P., Michaelson, I. A. & Kirkland, R. J. A. (1964) *Biochem. J.* **90**, 293–303
 Woelk, H. & Porcellati, G. (1973) *Hopper-Seyler's Z. Physiol. Chem.* **354**, 90–100