The Stimulation of the Phospholipase A₂-Acylation System of Synaptic Membranes of Brain by Cyclic Nucleotides

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1. Hydrolysis of phosphatidylcholine by phospholipase A₂ of synaptic membranes in Tris-HCl buffer was stimulated by cyclic AMP, cyclic GMP, cyclic CMP, cyclic UMP and adenosine (0.1 mM). 2. In the presence of 1 mM-NaF and cofactors, the same cyclic nucleotides and adenosine (10 μ M) stimulated the incorporation of added oleate into the choline glycerophospholipids of synaptic membranes. 3. Cyclic AMP and noradrenaline stimulated the incorporation of added oleate into position 2 of choline glycerophospholipid. Stimulation of net acylation was increased by preincubation in conditions which stimulated hydrolysis of phosphatidylcholine. 4. Cyclic AMP only slightly stimulated the transfer of oleate from oleoyl-CoA into choline glycerophospholipid. 5. The optimum concentration of CaCl₂ for the stimulation of hydrolysis by phospholipase A₂ by cyclic AMP was 1 μ M. Stimulation of the incorporation of added oleate was maximal in the CaCl₂ concentration range 1 μ M-1 mM. MgCl₂ also enhanced stimulations, maximum effects being obtained with concentrations of 10 μ M and 0.5 mM for hydrolysis by phospholipase A₂ and incorporation of added oleate respectively. 6. ATP enhanced the stimulation of incorporation of oleate but had no effect on the cyclic nucleotide stimulation of hydrolysis of added phosphatidylcholine by phospholipase A₂. 7. Adenosine, guanosine, ADP and 5'-AMP (all at 1 mM) inhibited the stimulation of incorporation of oleate by cyclic nucleotides and inhibited the transfer of oleate from oleoyl-CoA to phospholipid. They did not inhibit the stimulation of hydrolysis of added phosphatidylcholine (by phospholipase A₂) by cyclic nucleotides, but inhibited the stimulation by noradrenaline, acetylcholine, 5-hydroxytryptamine, dopamine (3,4-dihydroxyphenethylamine) and histamine. 8. Preincubation of synaptic membranes in the water or buffer increased the net activity of phospholipase A₂. Preincubation with a mixture of ATP and MgCl₂ increased the initial rate of acylation of membrane lipid.

In a previous paper (Gullis & Rowe, 1975a) we reported that a series of transmitters and putative transmitters of the central nervous system, namely noradrenaline, acetylcholine, 5-hydroxytryptamine, dopamine (3,4-dihydroxyphenethylamine), histamine, γ -aminobutyric acid, glutamic acid, aspartic acid and taurine, stimulated the hydrolysis of phosphatidylcholine by phospholipase A₂ of synaptic membranes. The first six of these compounds also stimulated the acylation of phospholipids of isolated synaptic membranes in the presence of phosphate or fluoride (Gullis & Rowe, 1975b). These neurohormones and putative transmitter substances stimulate the formation of cyclic nucleotides in brain. Cerebral injection of noradrenaline increased the concentration of cyclic AMP in brain by 50% (Burkard, 1972). Noradrenaline, 5-hydroxytryptamine, histamine, and adenosine increased the concentration of cyclic AMP in brain slices (Klainer *et al.*, 1962; Kakiuchi & Rall, 1968a,b;

Krishna *et al.*, 1970; Rall & Sattin, 1970; Shimizu *et al.*, 1970; Haung *et al.*, 1971; Chasin *et al.*, 1973). A dopamine-sensitive adenylate cyclase is present in the mammalian superior cervical ganglion (Kebabian & Greengard, 1971) and in the cerebral cortex of rats (von Hungen & Roberts, 1973). Acetylcholine, on the other hand, stimulated the formation of cyclic GMP in slices of rat brain and rabbit cerebral cortex (Lee *et al.*, 1972; Kuo *et al.*, 1972). γ -Aminobutyric acid and glutamic acid (which is also a putative transmitter substance in the central nervous system) stimulated the formation of cyclic GMP in mouse cerebellum (Ferrendelli *et al.*, 1974).

Siggins *et al.* (1971) showed that the depression of electrical discharge of single Purkinje cells of rat by noradrenaline was mimicked by cyclic AMP. Pre-ganglionic stimulation of the cervical ganglion of rabbit increased the post-ganglionic concentration of cyclic AMP (McAfee *et al.*, 1971). More-

over the phosphodiesterase inhibitor theophylline potentiated both the inhibitory synaptic potential and the dopamine-induced hyperpolarization of the post-ganglionic membrane (Greengard & McAfee, 1972).

In synaptic membranes the stimulations of the phospholipase A2-acylase system were pulse-like and their effects were rapidly reversed (Gullis & Rowe, 1975*a,b*). This might be expected from phenomena resulting from sudden increases in the concentration of cyclic nucleotide able to be hydrolysed by endogenous phosphodiesterase. Moreover, the reversal was prevented by theophylline (Gullis & Rowe, 1975*a,b*). NaF (10mM) stimulates adenylate cyclase in the cerebral cortex of ox brain (Bradham *et al.*, 1970). It also stimulated the hydrolysis of phospholipid by phospholipase A2 and the incorporation of oleate into choline glycerophospholipids, although in this case the stimulation of phospholipid hydrolysis was independent of ATP (Gullis & Rowe, 1975*b*).

From the above observations it seemed likely that the stimulations of phospholipid hydrolysis, and the acylation of membrane phospholipids, by neurohormones, were mediated by cyclic nucleotides. The present paper reports the effects of cyclic AMP, cyclic GMP, cyclic CMP and cyclic UMP on the phospholipase A2-acylation system of synaptic membranes of guinea-pig brain. Stimulations by adenosine are also reported.

Experimental

Materials and methods

Guinea pigs and materials were as described previously (Gullis & Rowe, 1975*a*). Cyclic nucleotides were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Synaptic membranes were prepared and hydrolyses by phospholipase A2 and net rates of acylation of membrane phosphatidylcholine were determined as described previously (Gullis & Rowe, 1975*a,b*).

Cofactors used as a routine for the determination of hydrolysis by phospholipase A2 were ATP (0.20mM), MgCl₂ (0.40mM), CaCl₂ (1.0μM) and theophylline (0.50mM). Standard cofactors for the measurement of the incorporation of added oleate into choline glycerophospholipid of synaptic membranes were ATP (0.50mM), MgCl₂ (3.0mM), CaCl₂ (1.0μM), CoA (1.0μM), dithiothreitol (10μM) and theophylline (0.50mM). Incubations were carried out in 10mM-Tris-HCl buffer, pH7.4, unless otherwise indicated.

Results

Cyclic AMP (1nM) stimulated the hydrolysis of phosphatidylcholine by phospholipase A2 of syn-

aptic membranes incubated in 10mM-Tris-HCl buffer 13-fold (Fig. 1). When the buffer was changed to 10mM-sodium phosphate, the stimulation decreased to fivefold. In 100mM-sodium phosphate stimulation of phospholipid hydrolysis was not detected, and cyclic AMP stimulated the uptake of oleate into the membrane. In the absence of theophylline, stimulations both of phospholipid hydrolysis and of the uptake of oleate were of short duration, the effects of the stimulations being reversed after 1 min (Fig. 2). Stimulations of hydrolysis of phospholipid by membranes incubated in Tris-HCl buffer were dependent on the concentration of added CaCl₂, the optimum concentration being 1.0μM (Fig. 3*a*). At a concentration of CaCl₂ of 1mM, however, hydrolysis of phospholipid was not

Fig. 1. Stimulation of hydrolysis of phosphatidylcholine by phospholipase A2 and the uptake of oleate into synaptic membranes, by cyclic AMP

Cofactors were: ATP, 0.20mM; MgCl₂, 0.40mM; CaCl₂, 1.0μM; theophylline, 0.50mM. The concentration of cyclic AMP when present was 1.0nM. ○, Membranes in 10mM-Tris-HCl; ●, membranes with cyclic AMP in 10mM-Tris-HCl; ◆, membranes in 10mM-sodium phosphate; ◇, membranes with cyclic AMP in 10mM-sodium phosphate; △, membranes in 50mM-sodium phosphate buffer; ▲, membranes with cyclic AMP in 50mM-sodium phosphate buffer; □, membranes in 100mM-sodium phosphate buffer; ■, membranes with cyclic AMP in 100mM-sodium phosphate buffer.

Fig. 2. Effects of theophylline on stimulation by cyclic AMP

Cofactors, excluding theophylline, were as described for Fig. 1. □, Membranes in 10mM-Tris-HCl; ○, membranes with cyclic AMP (1.0nM) in 10mM-Tris-HCl; ●, membranes with cyclic AMP (1.0nM) and theophylline (0.50mM) in 10mM-Tris-HCl; ■, membranes in 100mM-sodium phosphate; △, membranes with cyclic AMP (1.0nM) in 100mM-sodium phosphate; ▲, membranes with cyclic AMP (1.0nM) and theophylline (0.50mM) in 100mM-sodium phosphate.

detected and the incorporation of added oleate was stimulated by the cyclic AMP. $MgCl_2$ slightly enhanced stimulations of added phosphatidylcholine but was less effective than $CaCl_2$ (Table 1, Expt. 1). EGTA [ethanedioxybis(ethylamine)tetracetic acid] prevented appreciable stimulation (Expt. 2). In the presence of $CaCl_2$, ATP and $MgCl_2$ combined were without effect (Table 1, Expt. 2).

Sigmoidal dose-response curves were obtained for the stimulation of phospholipid hydrolysis by cyclic AMP, cyclic GMP, cyclic CMP and cyclic UMP (Fig. 4). The concentrations of cyclic nucleotides giving half-maximal stimulations were $10^{-11.2}M$, $10^{-10.4}M$, $10^{-9.8}M$ and $10^{-9.8}M$ respectively. Cyclic AMP was thus more effective than cyclic GMP, which was in turn more effective than cyclic CMP and cyclic UMP. The maximum stimulation obtained with cyclic AMP was approximately twice that obtained with cyclic UMP.

The effect of different concentrations of NaF on cyclic AMP-stimulated phospholipid hydrolysis is shown in Fig. 5. NaF, at concentrations in the

range $10\mu M$ –1mM, suppressed the stimulation of phospholipid hydrolysis by cyclic AMP. In the experiment shown, the rate of hydrolysis in the presence of 1mM-NaF was slightly above the unstimulated rate (in the absence of NaF and cyclic AMP). In another experiment, it was below the unstimulated rate.

Cyclic AMP (1nM) stimulated the incorporation of added oleate into choline glycerophospholipids of synaptic membranes (Table 2a). With NaF, $CaCl_2$ and theophylline, stimulation was nearly threefold (Table 2a). When CoA, dithiothreitol and $ATP-Mg^{2+}$ were included in the incubation, the stimulation by cyclic AMP was over 16-fold, which was greater than that obtained with $10\mu M$ -nor-adrenaline (13-fold), with the same cofactors. The degree of stimulation was dependent on the concentration of added $CaCl_2$, maximum stimulations being obtained in the concentration range 1–100 μM (Fig. 3b). $MgCl_2$ also enhanced stimulation of acylation, the maximum effects being obtained with a concentration of 5mM (Table 2b). ATP and $MgCl_2$ combined enhanced the sensitivity to cyclic AMP approximately 300-fold (Fig. 6).

Cyclic AMP had a relatively small effect on the transfer of oleate from oleoyl-CoA to glycerophosphorylcholine. In the experiment shown in Table 2(a) it increased the rate of transfer by 24%. Transfer of oleate from oleoyl-CoA was inhibited by adenosine, ADP and AMP (Table 2c). The transfer of oleate from oleoyl-CoA is compared with stimulated transfer from oleate-albumin in Fig. 7.

Sigmoidal dose-response curves for the stimulation of the incorporation of oleate into choline glycerophospholipid were obtained with cyclic AMP, cyclic GMP, cyclic CMP and cyclic UMP (Fig. 6). The concentrations giving half-maximum stimulation were $10^{-11.4}M$, $10^{-11.0}M$, $10^{-9.6}M$ and $10^{-10.6}M$ respectively. Thus the sensitivities, and the order of sensitivities, to the four cyclic nucleotides tested were similar to those exhibited for the stimulation of the hydrolysis of added phospholipid. In conditions where membranes were incubated in 150mM-sodium phosphate containing 1.0mM-ATP, 2.0mM- $MgCl_2$, 0.10mM-CoA and 1.0mM-dithiothreitol, the concentration of cyclic AMP giving half-maximum stimulation was $10^{-12.3}M$ (Fig. 6e).

The stimulation of the hydrolysis of phosphatidylcholine by cyclic nucleotides and by 50mM-NaF was unaffected by adenosine, guanosine, ADP and AMP (Table 3). This contrasted with the stimulation of phospholipid hydrolysis by neurotransmitters, which was inhibited by these substances. On the other hand, the above-mentioned substances inhibited the stimulation of incorporation of oleate by both cyclic nucleotides and neurotransmitters (Table 4).

Fig. 3. Effects of the concentration of CaCl_2 on (a) the stimulation of hydrolysis by phospholipase A2 and (b) the stimulation of the incorporation of oleate into choline glycerophospholipid

Cofactors used were as described in the text. $\square$, Membranes; $\bullet$, membranes with cyclic AMP (1.0 nM); $\circ$, membranes with EGTA (1.0 mM); $\triangle$, membranes with cyclic AMP (1.0 nM) and EGTA (1.0 mM).

Table 1. Effects of CaCl_2 , MgCl_2 and ATP on the cyclic AMP-stimulated hydrolysis of phosphatidylcholine by phospholipase A2

Membranes were incubated in the presence of 0.50 mM theophylline. Concentrations of reagents were: cyclic AMP, 1.0 nM; CaCl_2 , 1.0 μM ; ATP, 0.20 mM; EGTA, 1.0 mM.

Expt.	Addition to membrane	Hydrolysis of phosphatidylcholine (nmol of fatty acid released/min per mg of protein)
1	Cyclic AMP	7.4
	Cyclic AMP + MgCl_2 1 μM	9.0
	10 μM	12.2
	0.10 mM	12.0
	Cyclic AMP + CaCl_2	19.7
2	None	1.5
	Cyclic AMP + CaCl_2	20.0
	Cyclic AMP + CaCl_2 + ATP + MgCl_2 (0.40 mM)	20.0
	Cyclic AMP + CaCl_2 + EGTA	2.7

The stimulation of oleate incorporation by a mixture of phosphate and fluoride was also inhibited. Adenosine was more effective than guanosine in

inhibiting stimulations by noradrenaline and cyclic AMP (Tables 3 and 4). Guanosine was more effective than adenosine in inhibiting the stimulations by acetylcholine and cyclic GMP, although, in the case of stimulation of phospholipid hydrolysis, the difference was marginal. These observations parallel the relative efficacies of GTP and ATP in enhancing stimulations by acetylcholine and noradrenaline respectively (Gullis & Rowe 1975a,b). They also correlate with the formation of cyclic GMP and cyclic AMP in brain by cholinergic and adrenergic agonists respectively (Kuo *et al.*, 1970).

Adenosine itself, at low concentrations, stimulated both the hydrolysis of phosphatidylcholine and the incorporation of oleate in the presence of fluoride. The dose-response curves are shown in Fig. 8. At higher concentrations, adenosine inhibited its own stimulation. In contrast with the stimulation of phospholipid hydrolysis by noradrenaline (Gullis & Rowe, 1975a) stimulation by concentrations of adenosine greater than 0.1 mM were not enhanced by 10 μM - Ca^{2+} . High concentrations of ATP (5 mM) and MgCl_2 (10 mM) did increase stimulation, however (Fig. 8b), and thus competed with the blocking effect of adenosine on its own stimulation. The

Fig. 4. Dose-response curves for the cyclic nucleotide-stimulated hydrolyses of phosphatidylcholine by phospholipase A2 of synaptic membranes

●, Cyclic AMP; ■, cyclic GMP; ○, cyclic CMP; □, cyclic UMP.

Fig. 5. Effect of NaF on the cyclic AMP-stimulated hydrolysis of phosphatidylcholine by phospholipase A2

Cofactors were as described in the text. The unstimulated activity of phospholipase was equivalent to 2.8 nmol of fatty acid released/min per mg of protein.

optimum concentrations of adenosine for stimulations of hydrolysis of added phosphatidylcholine and incorporation of oleate into membrane phosphatidylcholine, under the experimental conditions normally used, were 0.1 mM and 10 μ M respectively. Stimulations by adenosine were blocked by theophylline and by caffeine (Table 5).

The effects of the preincubation of the membranes on the phospholipase A2-acylation system are shown in Figs. 9 and 10 and Table 6. Pretreatment of membranes with concentrations of CaCl_2 , ATP and MgCl_2 optimal for stimulation of phospholipid hydrolysis, before the addition of lipid substrate, completely and unexpectedly removed the ability of the membranes to hydrolyse phosphatidylcholine (Fig. 9). On the contrary, when substrate was added, oleate was taken up into the membrane rather than being released from added phospholipid. Stimulation of the incorporation of oleate by preincubation with cofactors could be demonstrated with respect to the transfer of oleate from oleate-albumin to phospholipid (Fig. 10a). On addition of cyclic AMP or noradrenaline to preincubated membranes, hydrolysis of phosphatidylcholine was stimulated as usual, and the effects of preincubation were nullified (Figs. 9 and 10a). This was not the case, however, when adenosine or ADP had been included in the preincubation. In these circumstances, both the unstimulated and stimulated incorporations of oleate were prevented when oleate-albumin was subsequently added (Fig. 10a).

When membranes were preincubated alone in water, or in Tris-HCl buffer, for periods up to 15 h before the addition of substrate, the ability of the membranes to hydrolyse phospholipid was considerably enhanced, as shown in Table 6. The net rates of hydrolysis approached the levels that could be obtained by stimulation with cyclic AMP or

Table 2. *Effects of cofactors on the stimulation by cyclic AMP of the transfer of oleate from oleate-albumin and oleoyl-CoA to choline glycerophospholipids of synaptic membranes*

(a) Effects of ATP, MgCl_2 , CoA and dithiothreitol. Membranes were incubated in the presence of 1.0mM-NaF, 1.0 μM - CaCl_2 and 0.50mM-theophylline. Concentrations of other reagents were: ATP, 0.50mM; MgCl_2 , 3.0mM; CoA, 1.0 μM ; dithiothreitol, 10 μM ; noradrenaline, 10 μM .

Additions to the incubation mixture	Incorporation of oleate (nmol/min per mg of protein)
Oleate-albumin	0.21
Oleate-albumin+cyclic AMP	0.56
Oleate-albumin+CoA+dithiothreitol+ATP+ MgCl_2	0.53
Oleate-albumin+CoA+dithiothreitol+ATP+ MgCl_2 +cyclic AMP	8.35
Oleate-albumin+CoA+dithiothreitol+ATP+ MgCl_2 +noradrenaline	6.50
Oleate-albumin+CoA+dithiothreitol+cyclic AMP	1.75
Oleoyl-CoA+ATP+ MgCl_2	6.43
Oleoyl-CoA+ATP+ MgCl_2 +cyclic AMP	8.00

(b) Effects of the concentration of MgCl_2 on the transfer of oleate from oleate-albumin. Membranes were incubated in the presence of 1.0mM-NaF, 1.0 μM - CaCl_2 , 0.50mM-ATP, 1.0 μM CoA, 10 μM -dithiothreitol, 0.50mM-theophylline and 1.0nM-cyclic AMP.

Concentration of MgCl_2 (mM)	Incorporation (nmol/min per mg of protein)
0.7	5.5
1.2	8.0
5.2	10.0
100	9.7

(c) Effects of adenosine and adenosine phosphates on the transfer of oleate from oleoyl-CoA. Membranes were incubated in the presence of 1.0 μM - CaCl_2 , 0.50mM-ATP and 3.0mM- MgCl_2 . Concentrations of other reagents, when present, were: adenosine, ADP and 5'-AMP, 3.0mM; theophylline, 0.50mM.

Additions to incubation mixture	Incorporation (nmol/min per mg of protein)
Oleoyl-CoA	7.0
Oleoyl-CoA+adenosine	2.95
Oleoyl-CoA+ADP	1.00
Oleoyl-CoA+5'-AMP	1.20
Oleoyl-CoA+adenosine+theophylline	2.10

noradrenaline. When such membranes were incubated with ATP and MgCl_2 for a further 5min, incorporation of oleate predominated when substrate was subsequently added. Thus combined ATP and MgCl_2 nullified the effects of preincubation in water or buffer.

Most (approx. 90%) of membrane preparations isolated by the standard procedures had unstimulated activities, with respect to hydrolysis of phosphatidylcholine by phospholipase A₂, and the incorporation of oleate into membrane choline glycerophospholipids, shown in the above Figures and Tables. However, membranes which hydrolysed added phosphatidylcholine at rates equivalent to 0.0–7.8nmol of fatty acid released/min per mg of protein (eight experiments) were isolated. In these cases, stimulation with noradrenaline (10 μM) gave values in the range equivalent to 11.2–14.2nmol of fatty acid released/min per mg of protein, and stimulation with cyclic AMP (1nM) gave values equivalent to 13.8–17.2nmol of fatty acid released/min per mg of protein. Likewise, membranes with acylating activities equivalent to 0.1–5.4nmol of added oleate incorporated/min per mg of protein (five experiments) were isolated. Stimulation of these membranes with cyclic AMP gave values in the range 3.9–6.2nmol of oleate incorporated/min per mg of protein. Thus membranes were stimulated to similar extents, regardless of their unstimulated activities.

Preincubation of the membrane with a mixture of ATP and MgCl_2 also increased the initial transfer of oleate from added oleoyl-CoA (Fig. 10b). After 25s, a 2½-fold increase, attributable to preincubation for 1min, was detected. Only the initial rate was stimulated. The incorporation of oleate attained after 3min was the same as that obtained with membranes which had not been preincubated.

Pre-stimulation of membrane without NaF, followed by stimulation in the presence of NaF, resulted in an increase in the initial rate of incorporation of oleate. With both cyclic AMP and noradrenaline the acylation of membrane choline glycerophospholipid was most affected (Table 7). Ethanolamine glycerophospholipid and phosphatidylinositol were also involved. The same phenomenon was observed with stimulations by carbamoylcholine, 5-hydroxytryptamine, dopamine, histamine and γ -aminobutyric acid. The stimulation of acylation of choline glycerophospholipid was mainly into position 2 of the phospholipid molecule (Table 8).

Discussion

Influence of acyl acceptors on the stimulation of acylation

In phosphate buffer, lysophosphatidylcholine increased the stimulation of the incorporation of added oleate into choline glycerophospholipids by cyclic AMP (Fig. 6e). This can be interpreted as being due to the increased availability of acyl receptor. An additional conclusion is that, under the experimental

Fig. 6. Stimulation of the incorporation of oleate into choline glycerophospholipids by cyclic nucleotides

○, Membranes incubated with 1.0 nM-cyclic nucleotide; □, membranes incubated with 1.0 nM-cyclic AMP, with ATP and $MgCl_2$ omitted; ●, 5 nmol of 1-acyl-*sn*-glycero-3-phosphorylcholine present.

Fig. 7. Comparison of the transfer of oleate from oleoyl-CoA to choline glycerophospholipids of synaptic membranes, with the transfer from oleate-albumin

Membranes were incubated with cofactors and: ■, oleate-albumin; ○, oleoyl-CoA; □, oleate-albumin and 1.0 nM-cyclic AMP; △, oleate-albumin and 10 μ M-noradrenaline; ●, oleoyl-CoA and cyclic AMP (1.0 nM) with NaF omitted.

conditions used, exogenous lysophosphatidylcholine had access to the acyl-CoA acyltransferase in the membrane. When net phospholipase A2 activity was stimulated with cyclic AMP, thus increasing the concentration of endogenous lysophospholipid, and the system was then switched to acylation, the subsequent stimulated rate of acylation was greater than that normally obtained. Thus, under the conditions of the experiment, the availability of endogenous lysophospholipid was a rate-limiting factor. The observation that acylation of choline glycerophospholipid was stimulated to the greatest extent suggests that this class of endogenous phospholipid was hydrolysed most by the stimulated phospholipase A2.

Under conditions of non-stimulated acylation, 67% of the oleate was incorporated into position 2 of the choline glycerophospholipids of the membrane (Table 8). When acylation was stimulated by cyclic AMP and by noradrenaline, 87% and 88% respectively of the stimulated incorporation into choline glycerophospholipid was into position 2 of the phospholipid. When net phospholipase activity was stimulated before stimulation of acylation, 100 and 97% respectively of the increase in the acylation due to pre-stimulation of the phospholipase

Table 3. *Effects of adenosine, guanosine, ADP and 5'-AMP on the hydrolyses and stimulated hydrolyses of phosphatidylcholine by phospholipase A2 in synaptic membranes*

Membranes were incubated with 0.20mM-ATP, 0.40mM-MgCl₂, 1.0μM-CaCl₂ and 0.50mM-theophylline. In experiments with acetylcholine and carbamoylcholine, GTP was present in place of ATP. The concentrations of other reagents were amines and choline derivatives (50μM), cyclic nucleotides (1.0nM) and NaF (50mM). Adenosine, guanosine, ADP and 5'-AMP (final concn. 1.0mM) were added, where indicated, immediately before the addition of phospholipid and stimulant.

Stimulant	Hydrolysis of phosphatidylcholine (nmol of fatty acid released/min per mg of protein)									
	None		Adenosine		Guanosine		ADP		5'-AMP	
	Unstimulated	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated
Cyclic AMP	1.6	23.8	2.3	21.9	1.9	21.0	2.6	22.2	4.1	21.9
Cyclic GMP	1.8	16.5	2.7	16.0	2.3	15.9	3.4	16.1	3.7	15.9
Cyclic CMP	1.4	13.0	2.3	12.1	1.7	13.5	2.7	13.0	3.0	12.5
Cyclic UMP	1.2	13.0	1.9	13.5	1.9	12.2	3.1	12.7	3.5	11.7
NaF	1.6	10.2	2.2	11.0	2.1	10.7	2.9	11.7	3.2	12.1
Noradrenaline	1.2	16.5	1.8	3.6	1.7	5.2	2.4	3.3	4.9	5.2
Acetylcholine	1.8	15.2	2.5	4.6	2.4	3.7	2.9	4.2	5.0	8.7
Carbamoylcholine	2.1	17.1	2.9	4.8	2.2	3.5	2.8	4.5	5.6	8.2
5-Hydroxytryptamine	1.6	14.6	2.6	4.7	1.9	4.2	2.8	3.6	5.1	5.6
Dopamine	2.4	17.2	3.1	3.9	3.2	4.7	3.0	3.6	5.5	6.1
Histamine	1.7	16.5	2.4	4.1	2.1	4.3	3.4	4.1	5.0	5.2

Table 4. *Effects of adenosine, guanosine, ADP and 5'-AMP on the incorporation of oleate into choline glycerophospholipids of synaptic membranes*

Membranes were incubated with 0.50mM-ATP, 3.0mM-MgCl₂, 1.0μM-CaCl₂, 0.50mM-theophylline and 1.0mM-NaF. In experiments with acetylcholine and carbamoylcholine, GTP was present in place of ATP. The concentration of amines and choline derivatives was 50μM and that of cyclic nucleotides was 1.0nM. Adenosine, guanosine, ADP and 5'-AMP (final concn. 1.0mM) were added immediately before the addition of oleate-albumin and stimulant. In the experiment where the mixture of NaF (1.0mM) and sodium phosphate was the stimulant, membranes were incubated in 80mM-sodium phosphate, pH 7.4.

Stimulant	Incorporation of oleate (nmol/min per mg of protein)									
	None		Adenosine		Guanosine		ADP		5'-AMP	
	Unstimulated	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated
Cyclic AMP	0.40	10.6	0.24	0.51	0.32	1.12	0.21	0.31	0.20	0.27
Cyclic GMP	0.30	9.4	0.17	0.90	0.21	0.40	0.15	0.20	0.09	0.12
Cyclic CMP	0.40	7.1	0.14	0.36	0.22	1.76	0.14	0.25	0.07	0.13
Cyclic UMP	0.50	8.3	0.23	0.29	0.27	1.70	0.25	0.23	0.12	0.09
NaF+sodium phosphate	0.47	4.3	0.19	0.24	0.32	1.80	0.17	0.26	0.10	0.05
Noradrenaline	0.25	5.7	0.14	0.21	0.20	0.70	0.12	0.14	0.03	0.04
Acetylcholine	0.37	6.4	0.29	0.45	0.29	0.20	0.08	0.12	0.02	0.04
Carbamoylcholine	0.20	5.8	0.11	0.43	0.16	0.18	0.09	0.18	0.04	0.06
5-Hydroxytryptamine	0.31	5.4	0.17	0.33	0.26	0.70	0.13	0.20	0.07	0.10
Dopamine	0.47	5.4	0.21	0.30	0.39	0.80	0.10	0.10	0.08	0.10
Histamine	0.62	6.6	0.42	0.57	0.47	0.85	0.15	0.21	0.05	0.07

Fig. 8. Stimulation of the phospholipase A₂-acylation system of synaptic membranes by adenosine

(a) Stimulation of phospholipase. ○, Cofactors were as described in the text; □, concn. of CaCl₂ increased to 10 μ M; ●, concn. of ATP increased to 5.0 mM and concn. of MgCl₂ increased to 10 mM; ■, cofactors were as described in the text, 0.5 mM-theophylline present. (b) Incorporation of oleate. ○, Cofactors as described in the text; ●, 5.0 mM-ATP and 10 mM-MgCl₂ present.

Table 5. Inhibition of adenosine-induced stimulations by theophylline and caffeine

Standard conditions were used for the measurement of the hydrolysis of phospholipase A₂ and the incorporation of oleate into choline glycerophospholipids. The concentration of adenosine was 50 μ M.

Additions		Hydrolysis of phosphatidylcholine (nmol fatty acid released/ min per mg of protein)	Incorporation of oleate into choline glycerophospholipids (nmol/min per mg of protein)
None		2.20	0.25
Adenosine		17.8	8.90
Adenosine+theophylline	1 μ M	17.6	8.80
	10 μ M	13.2	6.20
	0.1 mM	7.5	1.85
	0.5 mM	3.3	0.21
None		2.00	0.30
Adenosine		20.6	9.80
Adenosine+caffeine	1.0 μ M	19.8	9.70
	10 μ M	9.5	6.40
	0.1 mM	4.7	2.80
	0.5 mM	3.0	1.15
	1.0 mM	3.0	0.22

was into position 2. Thus, assuming that the increase in acylation was a direct response to an increase in lysophosphatidylcholine, phospholipase A₂ was predominantly, if not exclusively, stimulated. This agrees with the conclusion, from observations of the hydrolyses of exogenous phosphatidylcholine (Gullis & Rowe, 1975a), that phospholipase A₂, but not phospholipase A₁ or lysophospholipase, is stimulated by noradrenaline.

Acylation by oleoyl-CoA was affected only to a

small extent by noradrenaline (Gullis & Rowe, 1975b), cyclic AMP (Table 2a) or NaF (Gullis & Rowe, 1975b). Selective stimulation of net phospholipase activity by noradrenaline or cyclic AMP, in the absence of NaF, drastically decreased acylation by oleoyl-CoA (Fig. 7). In the absence of NaF, hydrolysis by phospholipase A₂ would have been selectively stimulated. This observation suggests that the molecules being acylated were hydrolysed by the activated phospholipase A₂. Thus there is

Fig. 9. Effects of preincubation on the phospholipase A₂-acylation system of synaptic membranes

Membranes were incubated with cofactors as described in the text for the determination of phospholipase A₂. (a) ○, Membranes incubated by standard procedure; □, membranes incubated with cofactors for 5 min before addition of substrate; △, membranes incubated with cofactors 10 min before addition of substrate; ▲, cyclic AMP (1.0 nM) added after preincubation with cofactors for 5 min. (b) ●, Stimulation by noradrenaline (10 μM) under standard conditions; ■, substrate and noradrenaline (10 μM) added after preincubation with cofactors for 5 min; ▲, substrate and noradrenaline added after preincubation with cofactors for 10 min.

evidence for a stimutable system in the membrane which deacylates and reacylates specific phospholipid molecules.

Role of cyclic nucleotides in stimulations

The stimulations of hydrolysis by phospholipase A₂, and the acylation of choline glycerophospholipids, by noradrenaline, acetylcholine, 5-hydroxytryptamine, dopamine, histamine and γ -aminobutyric acid, reported previously (Gullis & Rowe, 1975a,b), were mimicked by cyclic nucleotides. The sensitivity

of the phospholipase A₂-acylation system to cyclic nucleotides was high, stimulations being obtained with cyclic AMP in the concentration range 0.1 pM–1 nM. This range is lower than that reported for the stimulation of protein kinase isolated from ox (20 nM–2.5 μM; Miyamoto *et al.*, 1969) or protein kinase in the synaptic membrane of ox (20 nM–10 μM; Weller & Rodnight, 1970).

Stimulations by cyclic nucleotides, and by the above-mentioned transmitters and putative transmitters, were similar in their behaviours in response to phosphate, fluoride, theophylline and preincubation

Fig. 10. Effects of preincubation on the transfer of oleate from oleate-albumin (a) and oleoyl-CoA (b)

(a) (I) ○, Membranes were incubated under standard conditions. (II) Membranes were incubated with cofactors for 5 min before the addition of: □, oleate-albumin; ▲, oleate-albumin and 1.0nM-cyclic AMP; ■, oleate-albumin and 10μM-noradrenaline. (III) Before the addition of oleate-albumin and 1.0nM-cyclic AMP, or oleate-albumin and 10μM-noradrenaline, membranes were incubated for 5 min with cofactors and: ●, 1.0mM-adenosine; △, 1.0mM-ADP. (b) ○, Membranes were incubated with oleoyl-CoA, 0.50mM-ATP, 3.0mM-MgCl₂ and 1.0μM-CaCl₂; □, ATP and MgCl₂ omitted; ●, membranes were preincubated with 0.50mM-ATP, 3.0mM-MgCl₂ and 1.0μM-CaCl₂ 1 min before the addition of oleoyl-CoA.

Table 6. Effects of preincubation of synaptic membranes on the hydrolysis of phosphatidylcholine by phospholipase A2

The concentrations of reagents used were as follows: Tris-HCl, 10mM (pH7.4); sodium phosphate, 100mM (pH7.4); cyclic AMP, 1.0nM; noradrenaline, 50μM; CaCl₂, 1.0μM; ATP, 0.20mM; MgCl₂, 0.40mM; theophylline, 0.50mM.

Preincubation medium	Assay conditions	Time of preincubation (h)	Hydrolysis of phosphatidylcholine (nmol of fatty acid released/min per mg of protein)					
			0	2	4	8	10	15
Water	Tris-HCl	...	3.2	4.5	7.1	11.7	13.4	12.7
Water	Tris-HCl+cyclic AMP+CaCl ₂ +theophylline	...	15.7	15.8	17.9	17.4	16.2	17.7
Tris-HCl	Tris-HCl*	...	3.7	8.4	13.0	15.2	15.0	15.0
Tris-HCl	Tris-HCl*+cyclic AMP+CaCl ₂ +theophylline	...	17.9	18.4	17.0	17.7	18.2	18.9
Tris-HCl	Tris-HCl+noradrenaline+CaCl ₂ +ATP+MgCl ₂ +theophylline	...	20.9	19.9	19.2	18.4	17.2	15.4
Tris-HCl+ATP+MgCl ₂	Tris-HCl†	...	2.7	0‡	3.9	10.7	13.9	14.2
Sodium phosphate	Sodium phosphate*	...	3.8	4.2	4.5	5.5	6.0	6.5
Sodium phosphate	Sodium phosphate+cyclic AMP+CaCl ₂ +theophylline	...	8.7	9.0	8.8	9.1	8.9	8.4

* When the assay mixture was incubated with 0.20mM-ATP and 0.40mM-MgCl₂ for 5 min before addition of substrate, no hydrolysis of phosphatidylcholine was detected and oleate was taken up into the membrane.

† Assay mixture contained a small quantity of ATP-Mg²⁺ from preincubation mixture.

‡ Oleate taken up by the membrane.

Table 7. *Effects of pre-stimulation of phospholipase on subsequent stimulation of acylation*

(a) Stimulation by cyclic AMP and noradrenaline of incorporation of oleate into different classes of phospholipids. Membranes were incubated for 1 min with 1.0 nM-cyclic AMP or 10 μ M-noradrenaline in the presence of 0.20 mM-ATP, 0.40 mM-MgCl₂, 1.0 μ M-CaCl₂ and 0.50 mM-theophylline. NaF (final concn. 1.0 mM) was then added and the concentrations of ATP and MgCl₂ were increased to 0.50 mM and 3.0 mM respectively. After an additional 1 min, oleate-albumin was added.

Phospholipid	Incorporation of oleate (nmol/min per mg of protein)			
	Stimulation of acylation by		Stimulation of phospholipase followed by stimulation of acylation by	
	Cyclic AMP	Noradrenaline	Cyclic AMP	Noradrenaline
Choline glycerophospholipid	5.3	4.1	14.2	13.5
Ethanolamine glycerophospholipid	2.9	2.0	3.5	2.3
Phosphatidylinositol	2.0	1.7	2.7	3.4
Phosphatidylserine	1.3	1.5	1.5	1.7
Phosphatidic acid	1.1	1.2	1.2	1.2

(b) Effects of different stimulants. The procedure was as described for (a). 10 μ M of stimulant was used. In the experiment with carbamoylcholine, GTP was used in place of ATP.

Stimulant	Incorporation of oleate into choline glycerophospholipids (nmol/min per mg of protein)	
	Stimulation of acylation	Stimulation of phospholipase followed by stimulation of acylation
Noradrenaline	6.1	14.0
Carbamoylcholine	6.2	13.5
5-Hydroxytryptamine	5.0	9.0
Dopamine	5.6	10.5
Histamine	6.5	11.9
γ -Aminobutyric acid	5.2	12.4

Table 8. *Positional distribution of the incorporation of oleate into choline glycerophospholipid of synaptic membrane*

Experimental conditions were those described for Table 7(a).

Acylation conditions	Incorporation of oleate (nmol/min per mg of protein)	
	Position 2	Position 1
Unstimulated acylation	0.10	0.05
Cyclic AMP-stimulated acylation	6.9	1.1
Noradrenaline-stimulated acylation	6.3	0.9
Cyclic AMP-stimulated phospholipase A followed by cyclic AMP-stimulated acylation	17.2	1.1
Noradrenaline-stimulated phospholipase A followed by noradrenaline-stimulated acylation	13.0	1.1

of the membrane. Both cyclic AMP and noradrenaline had relatively little effect on the transfer of oleate from oleoyl-CoA. Further, the rate of transfer from oleoyl-CoA was similar to the rate of transfer from oleate-albumin stimulated by cyclic AMP (Table 2a, Fig. 7). These last two observations suggest that cyclic AMP and noradrenaline stimulated the formation of oleoyl-CoA rather than the oleoyl-CoA-lysophospholipid acyltransferase. Moreover, transfer of oleate from oleoyl-CoA was not reversed in the

absence of theophylline (Fig. 10b), suggesting that cyclic AMP was not involved in this step. The effects of cyclic AMP and noradrenaline on the positional distribution of incorporated oleate (Table 8) and on the relative rates of acyl transfer from oleate-albumin (Table 2) were also similar. All these results described above, when taken together, are consistent with the view that the stimulations produced by the transmitters were mediated by the activation of nucleotide cyclases, which produce increased con-

Fig. 11. Effects of adenosine, guanosine and adenosine phosphates on the phospholipase A₂-acylation system of synaptic membranes

----->, Stimulation; —|----->, inhibition.

centrations of cyclic nucleotides. This is supported by a consideration of the effects of adenosine and theophylline. Adenosine in low concentrations stimulated both hydrolysis by phospholipase A₂ and acylation (Fig. 8), and these stimulations were blocked by theophylline (Table 5). Adenosine stimulates adenylate cyclase in brain slices, and the effect is specifically blocked by theophylline (Sattin & Rall, 1970). Thus the stimulation and blocking of the phospholipase A₂-acylation system correlates with the stimulation and blocking of adenylate cyclase.

Three differences were observed between the behaviour of transmitters and cyclic nucleotides. The first difference was that, in 10mM- or 50mM-sodium phosphate buffers, noradrenaline initially stimulated the uptake of oleate, and there were delays of up to 3min before hydrolysis by phospholipase A₂ was stimulated to the maximum extent (Gullis & Rowe, 1975b). With cyclic AMP the stimulations of hydrolysis by phospholipase A₂ were immediate and there was no detectable lag (Fig. 1). A possible explanation for this difference is that, with noradrenaline, time was needed for the formation of sufficient cyclic AMP to overcome the phosphate-induced predisposition of the membranes to acylate. The stimulation of acylation then preceded stimulation of phospholipase as the concentration of endogenous cyclic nucleotide increased.

The second difference is that, in the case of hydrolysis by phospholipase A₂ and in the presence of MgCl₂, ATP enhanced the stimulation by transmitters, but not the stimulation by cyclic AMP. A possible explanation for this difference is that, in the case of transmitters, ATP-Mg²⁺ was required for the synthesis of cyclic AMP. A need for ATP-Mg²⁺ in transmitter-receptor interaction is another possible explanation.

The third difference is that the stimulation of hydrolysis by phospholipase A₂ by transmitters, but not by cyclic nucleotides, was blocked by adenosine, guanosine, ADP and ATP. A possible explanation is that at high concentrations adenosine and its derivatives block the formation of cyclic nucleotide. Addition of cyclic nucleotide thus by-passes this block, giving stimulation. Adenosine has been reported to inhibit the activation of adenylate cyclase in 'ghosts' of rat fat-cells (Fain *et al.*, 1972). In the case of acylations, adenosine, ADP and AMP inhibit the acyltransferase (Table 2c), thus blocking the effects of both added transmitter and cyclic nucleotide, and masking the inhibition of nucleotide cyclase. These relationships are illustrated in Fig. 11. It must be noted that adenosine inhibits isolated cyclic nucleotide-stimulated protein kinase (Kuo *et al.*, 1970; Miyamoto *et al.*, 1969;

Weller & Rodnight, 1970), which is therefore a third possible site for inhibition.

Possible control mechanisms

The stimulated acylation is essentially into position 2 of the choline glycerophospholipids. Thus the stimulations of acylation and phospholipase A2 are directly opposed. These reactions must be controlled, presumably, by different mechanisms. One possible mechanism of control is suggested from the effects of Ca^{2+} . The stimulation of acylation by 1 nM-cyclic AMP was considerably enhanced by CaCl_2 in the concentration range 1 μM –1 mM (Fig. 3). Hydrolysis by phospholipase A2, however, in the presence of the same concentration of cyclic AMP, was stimulated by 1 μM - CaCl_2 but inhibited by 1 mM- CaCl_2 . Thus a high concentration of Ca^{2+} would control the direction of the stimulations by cyclic AMP by selectively switching off phospholipase A2. The concentrations of Ca^{2+} and Mg^{2+} can also control the activities of adenylate cyclase (Bradham *et al.*, 1970), phosphodiesterase (Kakiuchi *et al.*, 1972) and cyclic AMP-stimulated protein kinase (Miyamoto *et al.*, 1969).

Some information about another possible mechanism is obtained from the effects of treatment of the membranes on the unstimulated rate of acylation and the unstimulated level of net activity of phospholipase A2. The presence of a mixture of ATP and MgCl_2 significantly lowered the activity of unstimulated phospholipase A2 (Gullis & Rowe, 1975a). Pre-incubation of membranes with ATP- MgCl_2 for short periods before the addition of substrate eliminated detectable hydrolysis by phospholipase A2 and stimulated subsequent acylation. This might have been due to the production of lysophosphatidylcholine and fatty acid. On the other hand, pre-incubation of membranes in the absence of ATP or MgCl_2 considerably enhanced the hydrolysis by phospholipase A2. It has been shown by Weller & Rodnight (1971) that incubation of synaptic membranes in the presence of Mg^{2+} , with and without ATP, leads to a net phosphorylation and dephosphorylation respectively of membrane protein. The observations on lipid metabolism are thus consistent with the view that a kinase-catalysed phosphorylation activates acylation or deactivates phospholipase A2, and that a phosphatase activates phospholipase A2 or deactivates acylation. Cyclic AMP has been shown to activate a protein phosphatase in toad bladder (De Lorenzo & Greengard, 1973). The elimination of the effects of pre-incubation of synaptic membranes with ATP by adenosine or ADP is consistent with adenosine and ADP inhibiting adenylate cyclase or cyclic nucleotide-activated protein kinase.

The lower values of unstimulated hydrolysis by phospholipase A2 in membranes which had been incubated in phosphate buffer, compared with those which had been incubated in Tris-HCl or water (Table 6), and the predisposition of membranes in sodium phosphate to stimulation of acylation (Fig. 1), correlate with the inhibition of cerebral protein phosphatase by phosphate (Maeno & Greengard, 1972). Interpretations of the effects of phosphate must be made with caution, however, since phosphate could decrease pools of Ca^{2+} and Mg^{2+} , as discussed previously (Gullis & Rowe, 1975b). Fluoride, which predisposed the membrane to stimulation of acylation, also inhibits protein phosphatase (Maeno & Greengard, 1972), besides stimulating adenylate cyclase (Perkins & Moore, 1971).

The transfer of oleate from oleoyl-CoA to membrane lysophosphatidylcholine had the characteristics of a kinase-mediated reaction insofar that ATP was required for the maximum rate of reaction (Fig. 10b; Gullis & Rowe, 1975b) and that the transfer was inhibited by adenosine, ADP and 5'-AMP.

Synaptic membranes contain relatively high concentrations of adenylate cyclase (De Robertis *et al.*, 1967), phosphodiesterase (De Robertis *et al.*, 1967; Cheung & Salganicoff, 1967; Florendo *et al.*, 1971), cyclic AMP-activated protein kinase (Maeno *et al.*, 1971; Gaballah & Popoff, 1971), substrate for protein kinase (Johnson *et al.*, 1971; Ueda *et al.*, 1973) and protein phosphatase (Maeno & Greengard, 1972). Moreover, in rat brain, phosphodiesterase is concentrated close to the post-synaptic side of post-synaptic membranes (Florendo *et al.*, 1971). On the basis of these observations it has been suggested that increased phosphorylation of protein catalysed by cyclic nucleotide-stimulated protein kinase causes the changes in permeability in membranes after synaptic transmission (Greengard & McAfee, 1972). Changes in the phospholipase A2-acylation system may be the mechanism whereby changes preceding, accompanying or following alterations in permeability to cations are brought about. Moreover, control of this system by phosphorylation-dephosphorylation and by Ca^{2+} may be similar to that of glycogen phosphorylase and other cyclic AMP-activated systems.

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