EFFECTS OF LIPIDS ON CYCLIC-NUCLEOTIDE PHOSPHODIESTERASES

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SUMMARY

Several naturally-occurring lipids but not n-propanol, guanidine-HCl or a variety of synthetic detergents stimulate the 3',5'-cyclic AMP-phosphodiesterase activities of a supernatant fraction of brain at 1.25×10^{-7} M cAMP. The time courses of the reaction are linear in the presence and absence of lipid. On the other hand, lipid has different effects on various phosphodiesterase activities in fractions obtained after gel filtration of the crude extract. It stimulates the phosphodiesterase activities measured at 1.25×10^{-7} M and 10^{-4} M 3',5'-cyclic-AMP and 1.25×10^{-7} M 3',5'-cyclic GMP in two of the fractions partially retained in the gel. However, lipid has little effect on the enzymatic hydrolysis of low concentrations of cAMP or cGMP and markedly inhibits the hydrolysis of high concentrations of cAMP by the fraction excluded from the gel.

It is now firmly established that 3',5'-cyclic AMP (cAMP) plays an important role in regulating many metabolic processes. The concentration of cAMP appears to be determined largely, if not entirely, by the relative activities of adenyl cyclase which converts ATP into cAMP and a phosphodiesterase which hydrolyzes cAMP to 5'-AMP. Although adenyl cyclase is known to be under metabolic regulation, little is known about a possible control of the phosphodiesterase. However, many puzzling observations of mammalian phosphodiesterases suggest that this enzyme also may be under regulation. For example, brain and heart contain a low molecular weight protein which activates partially purified enzyme (1,2) and more than one phosphodiesterase with different substrate specificities and kinetic properties may be separated physically from a variety of tissues (3-9). Generally phosphodiesterases are stable to storage but difficult to purify. The difficulties in purifying the enzyme especially using DEAE-cellulose (10)

as well as the particulate nature of much of the enzyme (11) suggested that lipids may affect the activity or stability of the enzyme. This report describes some affects of lipids on the activities on phosphodiesterases from brain.

METHODS AND MATERIALS

Phosphatidyl ethanolamine, 8-(C^{14})-adenosine, (H^3)-cAMP and (H^3)-3',5'-cyclic GMP [(H^3)-cGMP] were obtained from Schwartz-Mann; (H^3)-cAMP was purified by chromatography before use (3). Paper chromatography in (2-propanol:water:NH_4OH) (7:2:1) (3) revealed that 99% of the radioactivity in (H^3)-cGMP was present as c-GMP. The R_f values in this system were: 5'-GMP, 0.10; cGMP, 0.35; cAMP, 0.52. Bovine lecithin, cAMP, cGMP, 5'-GMP and c. Atrox venom were supplied by Sigma. Other lipids were obtained from the following sources: lysolecithin, Nutritional Biochemicals, Co.; phosphatidyl inositol, General Biochemicals; sphingomyelin and phosphatidyl serine, Light and Co. The number of components in the lipid samples were determined by the chromatographic method of Rouser, et al. (12) to be: lysolecithin, 1 major and 1 minor component; sphingomyelin, 2 major components; bovine lecithin, 1 component; phosphatidyl ethanolamine, 1 component; phosphatidyl serine, 1 major and 2 minor components; phosphatidyl inositol, 5 major components. The concentrations of the phospholipids were estimated from their content of phosphate (13). The lipids were suspended in 0.02 M Tris-HCl, pH 8.0 and sonicated in a Branson Sonifier Cell Distruptor (setting 3) for at least 5 min. or until further sonication produced no additional change in the appearance of the suspension. Orthophosphate was determined by the method of Gomori (14).

Preparation of enzyme. A modification of the method of Thompson and Appleman (3) was used. Fresh rat brains were homogenized in a loose glass-Teflon homogenizer in 8 vol of 10.9% sucrose for 2 min. The homogenate was then adjusted to pH 6.0 with 0.1 N acetic acid and clarified by centrifugation

for 1 hr. at 105,000 g. The supernatant fluid was frozen in separate tubes and used as a source of enzyme unless otherwise stated.

Assay for phosphodiesterase activity. The conditions of incubation of phosphodiesterase with cAMP or cGMP are those described by Thompson and Appleman (3). Unless otherwise noted, the concentration of cAMP was 1.25×10^{-7} M. The tubes were incubated with all components except substrate for 10 min. at 30°. Substrate was then added and after an incubation for 10 min. at 30° the tubes were immersed in a boiling water bath for 2 min. To the cooled tubes, 0.1 ml of 1 mg/ml c. Atrox venom was added and the tubes incubated for another 10 min. at 30°. Then 1.0 ml of a suspension of 25 g Dowex 2 fluoride per 100 ml ethanol was added. The tubes were shaken vigorously for 10 min. and the incubation mixture clarified by centrifugation. The radioactivity in 0.5 ml of the supernatant fluid was determined by liquid scintillation counting. Under the conditions described, there is no adsorption of 8-(C^{14})-adenosine to the resin and about 5-8% of the radioactivity present in (H^3)-cAMP is not removed by the resin. This assay procedure is a modification of the methods of Brooker, et al. (15) and Monard, et al. (16).

RESULTS AND DISCUSSION

Specificity of stimulation of phosphodiesterase. The data in Table 1 show that several naturally-occurring lipids stimulate the phosphodiesterase in the brain supernatant fraction. There is an optimal concentration for at least three of the lipids tested. There is some variation in the extent of the stimulation by any one lipid from experiment to experiment. For example, lysolecithin stimulates the same enzyme preparation from about 2-fold to 4-fold in different experiments. However, the shape of the lysolecithin concentration vs. activity curve is quite reproducible. There also is no difficulty in showing the susceptibility to stimulation by lipids of different phosphodiesterase preparations. Stimulation by the lipids tested suggested that detergents or other agents which perturb protein

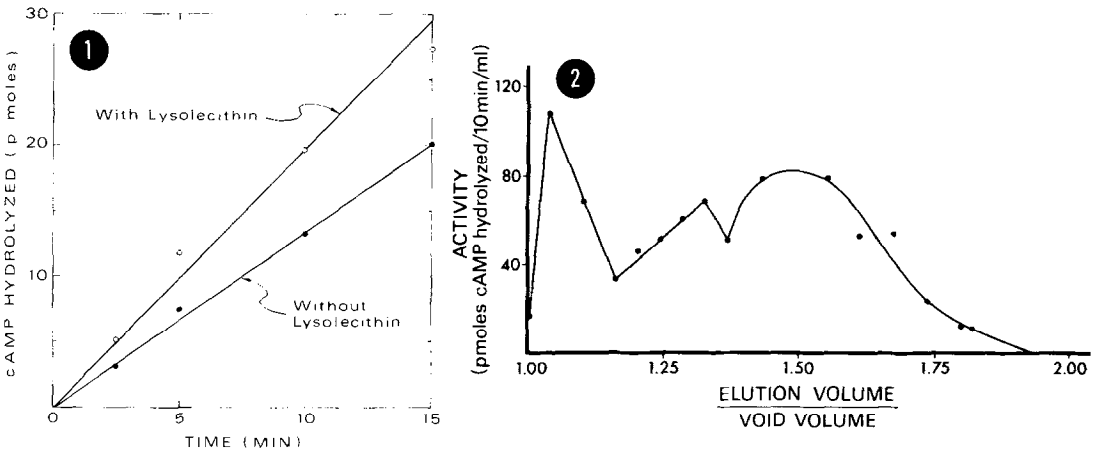

Fig. I. The effect of lysolecithin on the time course of the reaction. The conditions are those described in the text. Lysolecithin (0.21 μM) was added where indicated.

Fig. II. Agarose A1.5 filtrations. Six ml of brain supernatant fluid was filtered over a column (2.5 x 100 cm) of Agarose A1.5 (BioRad) equilibrated with 0.05 M Tris acetate, pH 6, 3.75 mM mercaptoethanol. The flow rate was 12.7 ml/hr. and fractions were collected every 15 min. The recovery of enzyme was 74%. The void volume determined with Blue Dextran = 175 ml.

structure might also stimulate the enzyme. A wide range of concentrations of several synthetic detergents (cetyl pyridinium chloride, Triton X-100, Triton N-101, Tween 20, sodium docedyl sulfate, Lubrol WX, BRIJ 58), two bile salts (cholate and deoxycholate), n-propanol and guanidine-HCl failed to stimulate the enzyme. Each of these compounds was tested up to concentrations which were inhibitory. In these tests it was essential to show that the agents were inhibiting the phosphodiesterase and not the 5'-nucleotidase in the venom. This was done in one of two ways. Inhibitory concentrations of reagents which did not interfer with the determination of P_i were shown directly not to affect the ability of the venom to release P_i from 5'-AMP. On the other hand, the 5'-nucleotidase was shown to be present in excess when each of the reagents interfering with the P_i determination was used. Thus only naturally-occurring lipids stimulate the enzyme.

The effect of lysolecithin on the time course of the reaction. The data in Fig. I show that the rates of hydrolysis of cAMP are linear for at least 15 min. both in the presence and absence of lysolecithin.

TABLE I

Effect of Various Lipids on the Activity of Crude cAMP-Phosphodiesterase

The conditions are described in the text.

Additions		
Concentration μM		cAMP Hydrolyzed pmoles/10 min.
	None	2.40
0.021	Bovine Lecithin	2.97
0.063	Bovine Lecithin	3.08
0.21	Bovine Lecithin	3.72
0.0042	Lysolecithin	2.69
0.0084	Lysolecithin	4.76
0.0168	Lysolecithin	4.59
0.042	Lysolecithin	6.09
0.126	Lysolecithin	8.75
0.42	Lysolecithin	8.72
0.84	Lysolecithin	7.50
0.050	Phosphatidyl Inositol	6.68
0.15	Phosphatidyl Inositol	8.14
0.50	Phosphatidyl Inositol	6.38
0.21	Sphingomyelin	3.04
0.63	Sphingomyelin	3.07
0.048	Phosphatidyl Ethanolamine	3.44
0.114	Phosphatidyl Ethanolamine	4.42
0.48	Phosphatidyl Ethanolamine	5.66
0.059	Phosphatidyl Serine	3.69
0.168	Phosphatidyl Serine	5.14
0.59	Phosphatidyl Serine	3.30

Effects of lysolecithin on phosphodiesterases as separated by gel filtration. Since crude brain extracts contain more than one cyclic nucleotide phosphodiesterase (3,4,7) it was of interest to test the effects of lipid on some of the separated enzymes. Therefore, the supernatant fraction was subjected to gel filtration (4). Fig.1l shows the elution profile which was observed. The second peak probably contains some of the higher and lower molecular weight enzymes as well as the enzyme of intermediate molecular weight. As seen in Table II lipid has little effect on

TABLE II

Effect of Lysolecithin on the Enzymatic Hydrolysis of cAMP and cGMP in Selected Agarose A1.5 Fractions

The conditions are described in the text. The units in the table are pmoles substrate hydrolyzed/10 min./0.14 ml enzyme. The numbers in parentheses are the ratios of the enzymatic activity with lipid/enzymatic activity wothout lipid.

	Substrate Added					
	50 pmoles cAMP		4,000 pmoles cAMP		50 pmoles cGMP	
Agarose Fraction	Without Lysolecithin	With 0.21 μM Lysolecithin	Without Lysolecithin	With 0.21 μM Lysolecithin	Without Lysolecithin	With 0.21 μM Lysolecithin
Elution Vol./Void Vol.						
1.04	11.7	13.4 (1.15)	1,380	305 (0.22)	5.5	5.7 (1.04)
1.33	8.0	13.1 (1.64)	1,180	2,570 (2.22)	10.5	39.9 (3.80)
1.65	7.7	17.7 (2.30)	900	5,260 (5.85)	50.9*	97.4*(1.91)

*50 μl enzyme used in the assay. The values in the table are calculated for 0.14 ml enzyme.

the ability of the highest molecular weight fraction to hydrolyze either cGMP or low concentrations of cAMP; however, it inhibits the hydrolysis of high concentrations of cAMP by this fraction. On the other hand, lipid stimulates all three activities in both of the other peaks. The excellent ability of the smallest fraction tested to hydrolyze cGMP was unexpected, since Thompson and Appleman found no such activity in their comparable fractions. Possibly this discrepancy may be attributed to differences in preparing the crude, supernatant enzyme. Thompson and Appleman sonicated their homogenate which was then centrifuged at relatively low speeds. We omitted the sonication and centrifuged vigorously in order to maximize the possibility of eliciting an effect of lipids on the soluble enzymes.

The significance of the abilities of lipids to modify the activities of brain cyclic nucleotide phosphodiesterases is obscure at present. It would be of interest to test the possible effect of lipids on the electrophoretic or gel filtration profiles of the enzyme, the sensitivities of the enzyme to 5'-GMP (2), cGMP (17,18) calcium (19) insulin (20) and various inhibitors and the interaction of the partially purified enzyme with the protein activator. Furthermore, the further purification of these enzymes may be facilitated by the addition of lipids to the assay system or by stabilization of the enzyme by lipids during fractionation procedures.

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REFERENCES

1. Cheung, W.Y., J. Biol. Chem., 246, 2859 (1971).
2. Goren, E.N. and Rosen, O.M., Arch. Biochem. Biophys., 142, 720 (1971).
3. Thompson, W.J. and Appleman, M.M., Biochemistry, 10, 311 (1971).
4. Thompson, W.J. and Appleman, M.M., J. Biol. Chem., 246, 3145 (1971).
5. Jard, S. and Bernard, M., Biochim. Biophys Acta, 41, 781 (1970).
6. Goren, E.N., Hirsch, A.H. and Rosen, O.M., Anal. Biochem., 43, 156 (1971).
7. Uzunov, P. and Weiss, B., Biochim. Biophys. Acta, 284, 220 (1972).

8. Monn, E. and Christiansen, R.O., Science, 173, 540 (1971).
9. Hrapschak, R.J. and Rasmussen, H., Biochemistry, 11, 4458 (1972).
10. Butcher, R.W. and Sutherland, E.W., J. Biol. Chem., 237, 1244 (1962).
11. De Robertis, E., Arnaiz, G.R.D.L., Alberichi, M., Butcher, R.W. and Sutherland, E.W., J. Biol. Chem., 242, 3487 (1967).
12. Rouser, G., Galli, C., Lieker, E., Blauk, M.L., and Privett, O.S., J. Amer. Oil Chem. Soc., 41, 836 (1964).
13. Marinetti, G.V., J. Lipid Res., 3, 1 (1962).
14. Gomori, G., J. Lab. and Clin. Med., 27, 955 (1942).
15. Brooker, G., Thomas, L.J., and Appleman, M.M., Biochemistry, 7, 4177 (1968).
16. Monard, D., Janecek, J., and Rickenberg, H.V., Biochem. Biophys. Res. Commun., 35, 584 (1969).
17. Beavo, J.A., Hardman, J.G. and Sutherland, E.W., J. Biol. Chem., 246, 3845 (1971).
18. Franks, D.J. and MacManus, J.P., Biochem. Biophys. Res. Commun., 42, 844 (1971).
19. Kakiuchi, S. and Yamazaki, R., Biochem. Biophys. Res. Commun., 41, 1104 (1970).
20. House, P.D.R., Poulis, P. and Weideman, M.J., Eur. J. Biochem., 24, 429 (1972).