Vol. 96, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1980 Pages 203-210

ACTIVATION OF RAT LUNG PARTICULATE GUANYLATE CYCLASE DUE TO FILIPIN INDUCED FLUIDITY CHANGE

Pushkaraj J. Lad*

John M. Dalton Research Center, University of Missouri-Columbia, Columbia, Missouri 65211 U.S.A.

Received July 25,1980

SUMMARY: Involvement of cholesterol in the regulation of rat lung particulate guanylate cyclase was studied with filipin. The enzyme was not activated to a great extent by sodium nitroprusside alone; however, in presence of filipin nitroprusside activated the enzyme about 12-16 fold over the basal. Filipin did not affect the soluble enzyme significantly. The changes induced by filipin did not cause solubilization of proteins or enzyme. The Arrhenius plot of filipin-treated particulate enzyme did not have a "break" which was evident with untreated enzyme. The results suggest that the sequestering of cholesterol by filipin can modulate the activity of membrane-associated guanylate cyclase probably by changing the membrane fluidity.

INTRODUCTION

Guanylate cyclase [GTP pyrophosphate lyase (cyclizing), EC 4.6.1.2] is found in particulate and soluble fractions prepared from homogenates of most mammalian tissues. The particulate enzyme from rat lung differs from the soluble enzyme in physical and kinetic properties (1,2). Because of the latent nature of the particulate activity it is usually assayed in the presence of solubilizing concentrations of nonionic detergents such as Triton X-100 or Lubrol PX (2,3). However, detergents make it difficult to study the regulation of the particulate enzyme in its native surroundings.

Phospholipid perturbing polypeptides, melittin gramicidin S and alamethicin stimulate the particulate guanylate cyclase (4,5). In the present investigation, the possible involvement of cholesterol in the regulation of guanylate cyclase was examined by utilizing an antibiotic, filipin. Filipin, like other polyenes, increases the permeability of cholesterol-containing bilayers to ions, water and small hydrophilic molecules by interacting with membrane cholesterol (6-8).

Present address: The Salk Institute, P. O. Box 85800, San Diego, CA 92138.

0006-291X/80/170203-08$01.00/0

MATERIALS AND METHODS

Filipin (Lot #3-1194) was purchased from Polysciences, Inc. GTP and nystatin were purchased from P. L. Biochemicals, and [α-^{32}P]GTP and [^{3}H]cGMP were obtained from New England Nuclear. Cyclic GMP, creatine phosphate, creatine phosphokinase, amphotericin B and other unspecified biochemicals were from Sigma. Bio-Gel A-5m was obtained from Bio-Rad.

Enzyme Preparation. Male rats weighing in the range of 100-150 g were decapitated and lungs were perfused *in situ* with 10 ml of homogenizing buffer (50 mM Tris-HCl, 0.5 mM Na_2 EDTA, 10 mM 2-mercaptoethanol, pH 7.6) by injecting it in the right ventricle. The lungs were removed and washed twice, blotted, placed in 3 volumes homogenizing buffer (w/v) and homogenized for 10 sec at 10,000 rev/min with a Willems Polytron equipped with a PT 20 ST generator. The homogenate was filtered through a 40-mesh stainless steel wire screen and centrifuged for 10 min at 8000 xg, after which the supernatant solution was centrifuged at 160,900 xg for 40 min. The supernatant was used as soluble enzyme. The pellet (microsomes) was resuspended in 1 ml buffer and chromatographed on a Bio-Gel A-5m column (1.6 X 15 cm). The column was eluted with 50 mM Tris-HCl, pH 7.6 buffer containing 10 mM 2-mercaptoethanol. The void volume fractions were pooled and used as the particulate enzyme.

Assays and Biochemical Determinations. Guanylate cyclase assays were performed by determining the conversion [α-^{32}P]GTP to [α-^{32}P]cGMP. The assay mix contained 1.2 mM GTP, 6 mM $MnCl_2$, 5 mM cGMP, 50 mM Tris-HCl, 15 mM creatine phosphate and 10 units of creatine phosphokinase (9). Enzyme activity was assayed for 5 or 6 min at 37°C with 10-30 µg protein. The [α-^{32}P]cGMP formed was separated from substrate by sequential chromatography on Dowex 50 and neutral aluminum oxide according to White and Karr (10). Proteins were determined by the method of Lowry *et al.* (11) after precipitation with silicotungstic acid (12). Bovine serum albumin was used as the standard. Cholesterol was quantitated by the method of Zlatkis and Zak (13). Specific activities are expressed as picomoles cGMP formed/min/mg protein. All assays were in triplicate, and the activities are given as the mean ± standard error of the mean. Data presented here are representative of at least 2 or 3 experiments.

RESULTS

Rat lung homogenate contain both soluble and particulate guanylate cyclase. A vasodilator, sodium nitroprusside, activates soluble guanylate cyclase 20-25 fold (14); however, the particulate enzyme is activated only 2-3 fold (5). As illustrated in Fig. 1, fillipin activated the particulate enzyme 2-4 fold, and the optimum activity appeared at 0.2-0.4 mg/ml filipin where the cholesterol to filipin ratio was about one. A decrease in activity was seen with higher concentrations of filipin. However, the maximum activity was less than that achieved with Lubrol PX, which activated the enzyme 7-9 fold. Sodium nitroprusside plus filipin synergistically activated the enzyme about 24-fold, which was greater than the activity observed with Lubrol PX.

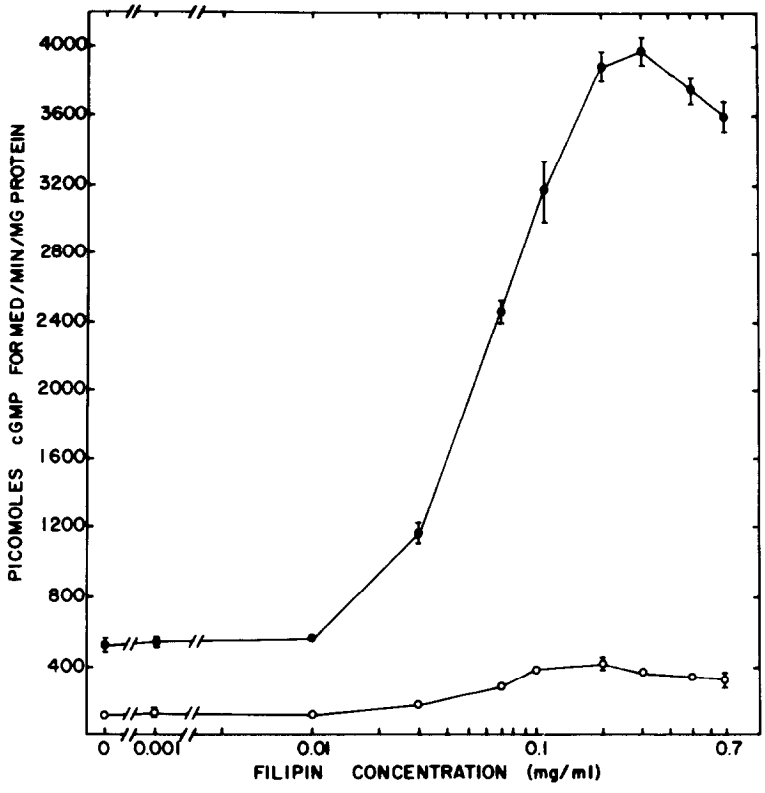

Fig. 1. The effect of filipin on particulate guanylate cyclase. Enzyme aliquots were added to tubes containing various concentrations of filipin and preincubated for 5 min at 37°C, then guanylate cyclase activity was assayed for 6 min at 37°C with (●) and without (O) 1 mM sodium nitroprusside. Activity in presence of 0.9% Lubrol PX was 1127.1 picomoles cGMP formed/min/mg protein.

Filipin in the range of 0.001 - 0.5 mg/ml did not activate the basal soluble guanylate cyclase or potentiated its activation by nitroprusside (not shown). In addition filipin treatment did not express contaminating or trapped soluble enzyme. This was evident by solubilization and column chromatography studies. Furthermore, membrane vesicles disrupted with hypotonic medium remained nitroprusside sensitive (not shown). Other soluble enzyme activators like organic peroxides, lipoxygenase and arachidonic acid did not activate the particulate enzyme treated with or without filipin. When the K_m for GTP was determined, the particulate enzyme treated with filipin plus nitroprusside had a K_m for GTP of about 0.2 mM, which was identical to the particulate enzyme treated with Lubrol PX but about 4 times higher than the soluble enzyme K_m (with or without nitroprusside treatment).

Table I. Effects of filipin and detergents on the rat lung particulate guanylate cyclase

Treatment	Concentration (mg/ml)	Guanylate Cyclase Activity	
		- SNP	+ SNP
None	-	105.0 ± 1.6	413.6 ± 8.6
Tween 20	4.0	385.0 ± 9.8	408.3 ± 7.7
Triton X-100	4.0	836.8 ± 2.6	859.8 ± 8.6
Lubrol PX	1.2	737.0 ± 12.5	714.4 ± 8.9
Filipin	0.4	302.3 ± 10.3	1684.2 ± 22.5

Particulate fraction (30 µg) was treated with various detergents or filipin and assayed for guanylate cyclase activity with and without 1 mM sodium nitroprusside (SNP).

Treatment of particulate guanylate cyclase preparation with its known activators such as non-ionic detergents and lysolecithin results in solubilization of guanylate cyclase activity (15). In contrast to filipin, detergents did not potentiate nitroprusside activation of the particulate guanylate cyclase (Table I). When solubilization studies were done, majority of protein and enzyme activity sedimented into the pellet upon high speed centrifugation of filipin treated particulate fraction (Table II), and there was no significant non-sedimentable enzyme activity. Also there was no significant non-sedimentable enzyme activity in control, suggesting that the particulate preparations used were free from soluble enzyme contamination. When microsomes treated with filipin were chromatographed on a Bio-Gel A-5m column, nearly all of the activity eluted in the void volume. Only a trace amount of protein and enzyme was found in the fractions where the soluble enzyme is normally eluted. These observations support that filipin treatment did not solubilize guanylate cyclase activity. The particulate guanylate cyclase rechromatographed after filipin treatment was activated by sodium nitroprusside better than Lubrol PX which suggest that filipin caused an irreversible change.

Effect of temperature on guanylate cyclase activity in particulate preparation treated with filipin (microsomes chromatographed on Bio-Gel A-5m

Table II. Solubilization of particulate guanylate cyclase by filipin

Fractions	Protein	Enzyme Activity in Presence of					
		Water		1.0% Lubrol PX		1 mM SNP	
	(mg)	TA	SA	TA	SA	TA	SA
A. CONTROL							
Original	1.96	260	132 ± 6	2239	1137 ± 15	1046	531 ± 13
Pellet	2.6	196	75 ± 1	2234	898 ± 26	627	241 ± 4
Supernatant	0.1	22	204 ± 60	33	312 ± 20	45	428 ± 56
B. FILIPIN TREATED							
Original	2.23	465	208 ± 12	2222	996 ± 13	2449	1098 ± 2
Pellet	2.7	339	125 ± 8	2161	795 ± 29	1405	517 ± 13
Supernatant	0.1	15	140 ± 64	16	152 ± 80	98	920 ± 76

About 1.8 ml of a particulate enzyme preparation was mixed with 0.2 ml buffer (control) of 5 mg/ml filipin (filipin treated). About 0.25 ml from each tube was removed (original) and assayed, remaining suspensions were centrifuged at 139,000 xg for 60 min. Pellets and supernatants were also assayed for guanylate cyclase activity in presence of various treatments. TA = total activity, picomoles cGMP formed/min in each fraction, SA = specific activity, picomoles cGMP formed/min/mg protein.

column after filipin treatment) and untreated (microsomes chromatographed without filipin treatment) is shown in Fig. 2. The Arrhenius plot of the control enzyme showed a break around 22-25°C, but such a break was not evident for particulate enzyme treated with filipin.

DISCUSSION

These studies demonstrate that the cholesterol binding antibiotic, filipin, activates rat lung particulate guanylate cyclase and potentiates its activation by nitroprusside. The Arrhenius plots suggest that filipin causes membrane fluidity changes. The activity of several membrane bound enzymes has been shown to be dependent upon the fluidity of the phospholipid environment (16-18). Kimelberg and Paphadjopoulos (18) have introduced the term "viscotropic," to describe the effects of membrane fluidity on enzyme activity.

In membranes, above the transition temperature, cholesterol condenses the average area per molecule and reduced the motion of hydrocarbon chains of phospholipid bilayer (19-20). During the guanylate cyclase activity determination at 37°C, membrane matrix is in a liquid crystalline state thus filipin

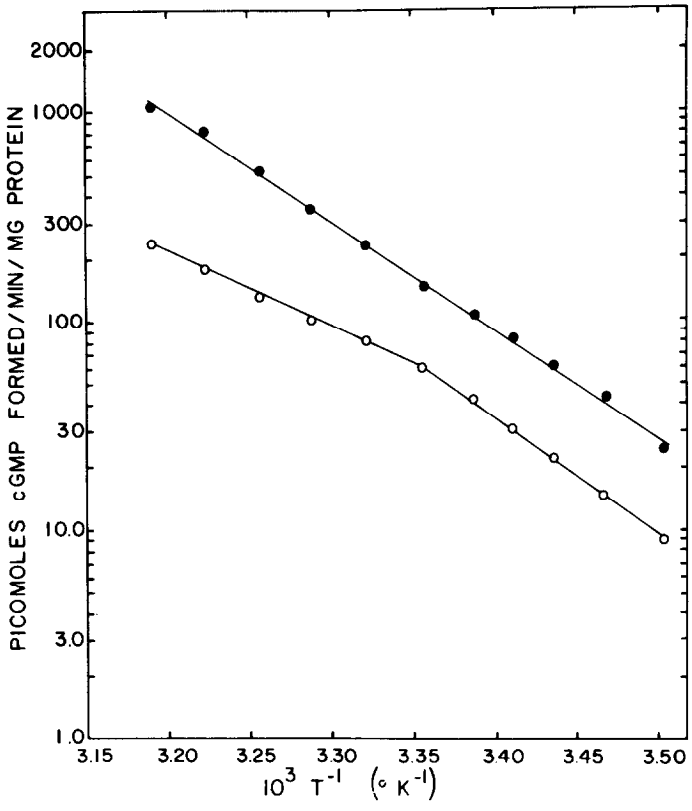

Fig. 2. The effect of temperature on the particulate guanylate cyclase. Microsomal preparation treated with 0.3 mg/ml filipin (●) and buffer (○) were chromatographed on Bio-Gel A-5m column. The enzymes were preincubated for 2 min at various temperatures and activity was assayed for 4 min. The lines were determined by the method of least squares.

probably increases membrane fluidity by sequestering cholesterol. This is supported by spin label studies which demonstrated that filipin increased fluidity in the hydrophobic core of a cholesterol containing membrane (21). However, another polyene amphotericin B did not affect fluidity (21). Interestingly, other polyenes studied, amphotericin B, candicidin and nystatin did not activate particulate guanylate cyclase or potentiated its activation by nitroprusside (not shown). A reason for the difference is that filipin interacts with cholesterol more effectively than other polyenes and is neutral in nature, whereas other polyenes used possess amphoteric properties because of an aminosugar attached to the macrolide ring (22-23).

Alcohols alter fluidity of membranes of various origin (24-25). Ethanol altered "break" in Arrhenius plot of rat lung particulate guanylate cyclase,

however, it did not potentiate guanylate cyclase activation by nitroprusside (Lad, P. J., paper in preparation). This difference may be due to independent mechanisms of action. It is probable that filipin by sequestering cholesterol induces disorganization of fatty acyl chains around guanylate cyclase, in turn exposing site where nitroprusside or its metabolite interacts. Possibility of such hydrophobic site(s) in nitroprusside activation of soluble guanylate cyclase is suggested (Lad, P. J., Liebel, M. A. and White, A. A., paper submitted [26]). This may also explain why detergent solubilized particulate enzyme is not activated by nitroprusside, because in solubilized complex hydrophobic regions of membrane proteins are occupied by detergent molecules (27).

It is also possible that modulation of guanylate cyclase activity results from an increased freedom of motion or a conformational change induced by altered membrane fluidity. Increased diffusion (freedom of motion) due to fluidity changes is implicated in adenylate cyclase activation by catecholamine-bound adrenergic receptor (28,29). A conformational change has been observed with cytochrome b_5 due to the change in lipid fluidity (30). It has been postulated that the rate-limiting step for an enzyme-catalyzed reaction can be a conformational change in the enzyme (31).

ACKNOWLEDGEMENT

This work was supported in part by a USPHS Grant HL 15002, from the National Institutes of Health, and by the John M. Dalton Research Center. I am grateful to Dr. Arnold A. White, in whose laboratory this work was done, for his generous support, advice and criticism.

REFERENCES

1. Chrisman, T. D., Garbers, D. L., Parks, M. A., and Hardman, J. G. (1975) *J. Biol. Chem. 250,* 274-381.
2. White, A. A. (1975) *Advances in Cyclic Nucleotide Research* (Drummond, G. I., Greengard, P., and Robison, G. A., eds.) Vol. 5, pp. 353-373, Raven Press, New York.
3. Ishikawa, E., Ishikawa, S., Davis, J. W., and Sutherland, E. W. (1969) *J. Biol. Chem. 244,* 6371-6376.
4. Lad, P. J., and Shier, W. T. (1978) *Biochem. Biophys. Res. Commun. 89,* 315-321.
5. Lad, P. J., and White, A. A. (1979) *Biochim. Biophys. Acta 570,* 198-209.
6. Babock, D. F., First, N. L., and Lardy, H. A. (1975) *J. Biol. Chem. 250,* 6488-6495.

7. De Kruijff, B., Gerritsen, W. J., Oerlemans, A., Van Dijck, P. W. M., Demel, R. A., and Van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 339,* 44-56.
8. Norman, A. W., Demel, R. A., De Kruijff, B., and Van Deenen, L. L. M. (1972) *J. Biol. Chem. 247,* 1918-1929,
9. White, A. A., Crawford, K. M., Patt, C. S., and Lad, P. J. (1976) *J. Biol. Chem. 251,* 7304-7312.
10. White, A. A. and Karr, D. B. (1978) *Anal. Biochem. 85,* 451-460.
11. Lowry, O. H., Rosebrough, N. H., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193,* 265-275.
12. White, A. A., Northup, S. J., and Zenser, T. V. (1972) in *Methods in Cyclic Nucleotide Research* (Chasin, M., ed.) pp. 125-167, Marcell Dekker, New York.
13. Zlatkis, A., and Zak, B. (1969) *Anal. Biochem. 29,* 143-148.
14. Liebel, M. A., Lad, P. J., and White, A. A. (1978) *Fed. Proc. 37,* 1537.
15. Lad, P. J., and White, A. A. (1979) *Arch. Biochem. Biophys. 197,* 244-252.
16. Engelhard, V. H., Esko, J. D., Strom, D. R., and Glaser, M. (1976) *Proc. Natl. Acad. Sci. USA 73,* 4482-4486.
17. Dipple, I., and Houslay, M. D. (1978) *Biochem. J. 174,* 179-190.
18. Kimelberg, H. K., and Papahadjopoulos, D. (1972) *Biochim. Biophys. Acta 282,* 277-292.
19. Rothman, J. E., and Engelman, D. M. (1972) *Nature New Biol. 237,* 42-44.
20. Oldfield, E., and Chapman, D. (1972) *FEBS Letts. 23,* 285-297.
21. Oski, K., Kozawa, Y., and Ohnishi, S. (1979) *Biochim. Biophys. Acta 554,* 39-50.
22. Norman, A. W., Demel, R. A., De Kruyff, B., Geurts Van Kessel, W. S. M., and Van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta 290,* 1-14.
23. De Kruijff, B., Gerritsen, W. J., Oerlemans, A., Demel, R. A., and Van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 339,* 30-43.
24. Chin, J. H., and Golstein, D. B. (1977) *Mol. Pharmacol. 13,* 435-441.
25. Johnson, D. A., Lee, N. M., Cooke, G., and Loh, M. M. (1979) *Mol. Pharmacol. 15,* 739-746.
26. Struck, G. J., and Glossman, H. (1978) *Naunyn-Schmiedeberg's Arch. Pharmacol. 304,* 51-61.
27. Helenius, A., and Simons, K. (1975) *Biochim. Biophys. Acta 415,* 29-79.
28. Rimon, G., Hanski, E., Braun, S., and Levitzki, A. (1978) *Nature 276,* 394-396.
29. Hanski, E., Rimon, G., and Levitzki, A. (1979) *Biochemistry 18,* 846-853.
30. Dufoureq, J., Faucon, J. F., Lussan, D., and Bernon, R. (1975) *FEBS Letts. 57,* 112-116.
31. Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology,* pp. 308-312, McGraw-Hill, New York.