STIMULATION OF GUANYLATE CYCLASE ACTIVITY BY SEVERAL FATTY ACIDS.

Takeo Asakawa*, Ilene Scheinbaum and Ren-jye Ho

Earl W. Sutherland Research Laboratories
Department of Biochemistry, University of Miami School of Medicine, Miami, Florida 33152.

Received September 1,1976

Summary: Guanylate cyclase of plasma membrane of isolated rat fat cells was activated 7 to 11 fold by oleic acid, linoleic acid, linolenic acid or arachidonic acid. The activation of the enzyme by linoleic acid or oleic acid was influenced by the concentration of enzyme protein and that of the fatty acid. At 158 μg/ml of enzyme protein, 0.6 mM linoleic acid produced maximal activation of 12 fold which was partially reversed by washing. Particulate guanylate cyclase of cerebral cortex and liver was also activated by linoleic acid.

Introduction: Guanylate cyclase has been reported to exist in two forms, soluble and particulate in various tissues (1,2) and demonstrated to be stimulated by detergents and other agents (3-5) including NaN_3 (6). Several investigators have reported an activation of guanylate cyclase by some hormones (7-10), but hormonal activation of this enzyme is not always evident. we have reported a compound designated as "feedback regulator" (FR), isolated from fat cells of rat after stimulation by cyclic AMP raising hormones (11,12). Feedback regulator (FR) has an inhibitory effect on hormonal activation of adenylate cyclase (13), cyclic AMP elevation (11) and phosphodiesterase activity (14) also modulates protein kinase activity (13,15). FR was also demonstrated to elevate cyclic GMP levels in fat cells (16) and to stimulate markedly guanylate cyclase activity of plasma membrane of fat cells (manuscript submitted). Recently, cyclic GMP of isolated fat cells was reported to be elevated by oleic acid (17). In this report we describe the activation of

*Recipient of a Career Investigator Fellowship from the American Heart Association (1973-1975) and of a Special Fellowship from the Florida Heart Association (1975-1976). Present address: Dept. of Pharmacology, Osaka Univ. School of Medicine, Kita-ku, Osaka, 530 Japan.
Abbreviations: cyclic GMP, guanosine 3',5'-monophosphate; cyclic AMP, adenosine 3',5'-monophosphate.

guanylate cyclase of plasma membrane of isolated fat cells by naturally occuring fatty acids.**

Materials and methods: Epididymal fat pads of male Sprague-Dawley rats (200-250 g body weight) were used. Plasma membrane was prepared according to the method of Laudat et al. (18) from isolated cells prepared by the method of Rodbell (19) with modification.
Guanylate cyclase assay: Unless otherwise indicated, the standard reaction mixture contained 40 mM Tris-HCl buffer (pH 8.0), 3 mM $MnCl_2$, 1 mM [α-32p] -GTP (6-10 μCi/umole), 1 mM cyclic GMP, 16 mM phospho-enol-pyruvate, 25 μg of phruvate kinase (115 units/mg protein, Sigma type I) and enzyme in a total volume of 0.25 ml.

The reaction mixture was preincubated for 7 min at 38°C. The reaction was initiated with the simultaneous addition of GTP and cyclic GMP. After 30 min of incubation at 38°C, the reaction was terminated by the addition of 1.75 ml of TCA (5% a final concentration). The radioactive cyclic GMP formed was isolated by sequential chromatography on alumina and Dowex AG 1X8.
Isolation of cyclic GMP: The TCA-supernatant of the reaction mixture was loaded directly on the alumina column. The conditions for chromatography on alumina were described in a previous report (20). The cyclic GMP fraction (2 ml) from the alumina column was drained directly onto a Dowex AG 1X8 column (0.62 x 2 cm, 100-200 mesh, formate form). The Dowex column was washed with 2-3 ml of water and then with 10 ml of 2 N formic acid. Cyclic GMP was subsequently eluted from the column with 5 ml of 5 N formic acid. A 0.5 ml aliquot of this fraction was used to determine the absorbance at 256 nm, which provided a measure of the recovery of cyclic GMP through the entire procedure. The radioactivity of the cyclic GMP fraction (4.5 ml) was measured with 15 ml of scintillation fluid "tT2l" (21). With this sequential chromatography, approximately 55-65% of cyclic GMP was constantly recovered in 5 ml of 5 N formic acid. The blank value of radioactivity was less than 0.004% of added [α -32P] -GTP.

This assay method for guanylate cyclase is reliable with more than 98% of the radioactive product, isolated by the procedures described above, identified as cyclic GMP by phosphodiesterase digestion followed by chromatography on Dowex AG 1X8 (0.62 x 10 cm) and TLC on cellulose (2 M formic acid:2 M LiCl, 1:1 v/v).
Preparation of fatty acid solution: Fatty acid was dissolved in a small volume of ethyl alcohol and suspended at a concentration of 10 mM in 10 mM Tris-HCl buffer, pH 8.0 (containing final 1-1.5% alcohol). pH was adjusted by adding NaOH. The fatty acid suspension was prepared immediately before use. The effect of ethyl alcohol contained in the fatty acid suspension on guanylate cyclase was non-detectable.
Other: Maximal activity of guanylate cyclase was observed at 3 mM $MnCl_2$. The activity-GTP concentration curve was sigmoidal. Half-maximal activity was obtained at 0.57 mM GTP.

Results: Guanylate cyclase activity of purified plasma membrane of isolated fat cells increased markedly in the presence of linoleic acid and other unsaturated fatty acids. Guanylate cyclase activity was determined in the presence of a fatty acid in the reaction mixture. As shown in Fig. 1, all

**During the course of these studies, Ira Pastan and his co-workers observed a stimulation of guanylate cyclase of plasma membrane of a type of tissue cultured cell by oleic acid.

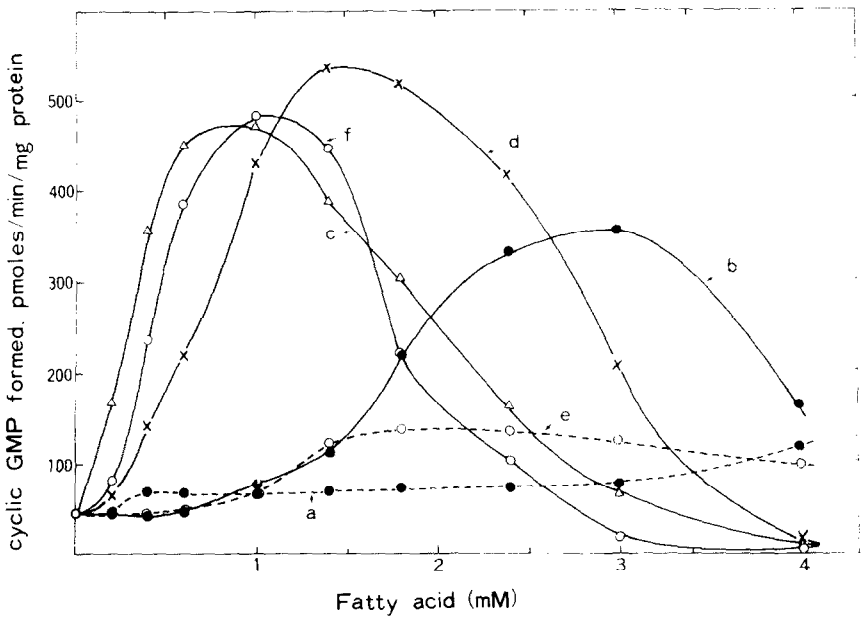

FIGURE 1. Effect of fatty acids on guanylate cyclase activity of plasma membrane of isolated fat cells. Plasma membrane (79 μg of protein) was incubated with the fatty acid indicated in the standard reaction mixture. a, ●-----●, stearic acid; b, ● ——— ●, oleic acid; c, △——— △, linoleic acid; d, X ——— X, linolenic acid; e, 0-----0, arachidic acid; f, 0———0, arachidonic acid.

of the unsaturated fatty acids examined stimulated guanylate cyclase activity 7 to 10 fold. Each fatty acid had a different potency on the activation of the enzyme. The peak levels of activation by unsaturated fatty acids and their concentrations for maximal activation were as follows: oleic acid, 7.2 fold at 3.0 mM; linoleic acid, 9.9 fold at 0.9 mM; linolenic acid, 11.2 fold at 1.4 mM and arachidonic acid, 10.0 fold at 1.0 mM. Linoleic acid was the most effective fatty acid at the concentration range of less than 1.0 mM.

In contrast to unsaturated fatty acids, the effect of saturated fatty acids on guanylate cyclase was much smaller. In the presence of stearic acid (0.4-4 mM) or arachidic acid (1.8-2.0 mM) in the reaction mixture, guanylate cyclase was enhanced 1.5 fold and 2.8 fold respectively (Fig. 1). Myristic acid (1.8-2.0 mM) and palmitic acid (1.0-4.0 mM) were also observed

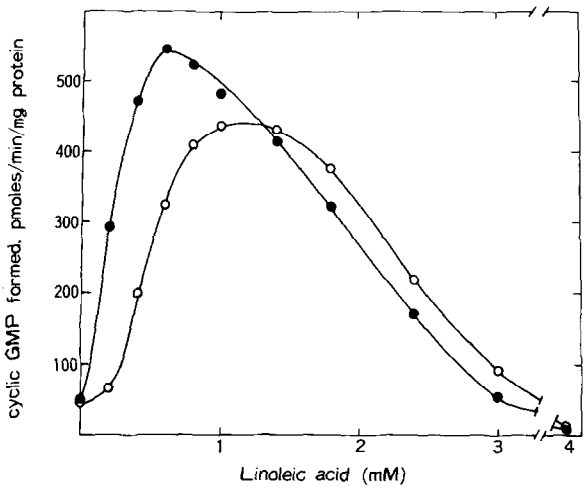

FIGURE 2. Effect of linoleic acid and protein concentration on the activation of guanylate cyclase. Plasma membrane (●——●, 40 μg of protein; O——O, 160 μg of protein) was incubated in a standard reaction mixture with different concentrations of linoleic acid.

to stimulate guanylate cyclase 2.5 fold and 1.5 fold respectively (data not shown). The presence of capric acid (0-4 mM) however, gave no effect on the enzyme activity (data not shown).

The stimulatory effect of unsaturated fatty acids on guanylate cyclase was influenced by the concentrations of enzyme protein and fatty acid. The effect of linoleic acid on guanylate cyclase activity was examined with two different concentrations of enzyme protein (Fig. 2). The concentration of linoleic acid required for maximal activity depended on the concentration of enzyme protein in the reaction mixture. The linoleic acid concentration-activity curve shifted to the left with a decrease in enzyme concentration, but the peak level of activation was enhanced (Fig. 2). At 633 μg/ml of enzyme protein, 1.1-1.2 mM linoleic acid produced a maximal activation of 9.6 fold, and at 158 μg/ml of enzyme protein, 0.6 mM linoleic acid gave a peak level of activation of 11.9 fold.

The activation of guanylate cyclase by oleic acid was also observed to be dependent on the concentration of enzyme protein. Optimal concentration

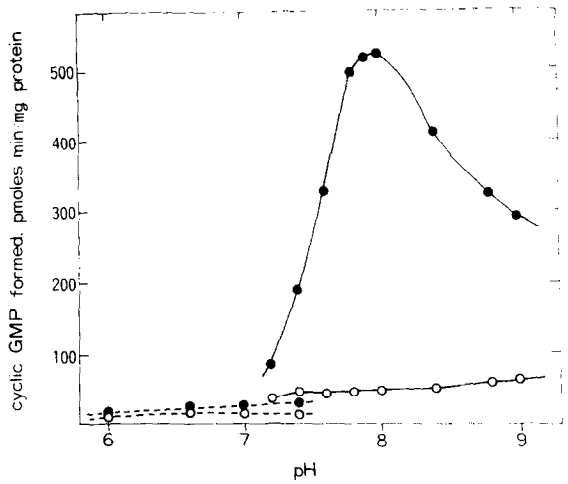

FIGURE 3. Effect of pH on guanylate cyclase activity. Plasma membrane (79 μg of protein) was incubated with (●) or without (○) 1 mM linoleic acid in the standard reaction mixture, except that the buffer was replaced by 50 mM Na-phosphate buffer (----) or 50 mM Tris-HCl buffer (———) with pH values indicated. Linoleic acid was added at the concentration (1.0 mM) which produced the maximal activation at enzyme protein concentration used.

of oleic acid which produced the maximal activation decreased approximately one half with a one fourth decrease in enzyme protein concentration.

Figure 3 shows guanylate cyclase activity with varying pH in the presence and absence of linoleic acid. The maximal activity in the presence of linoleic acid was observed at pH 7.9-8.0, in contrast to the basal activity which was enhanced with an increase in pH value. At pH 7.4, activities with and without linoleic acid were much higher with Tris-HCl buffer than with Na-phosphate buffer.

The reversibility of the activation of guanylate cyclase by linoleic acid was examined by washing with centrifugation, the incubated reaction mixture (Table 1). Much of the enhanced activity persisted after washing. The control sample (incubated without linoleic acid) showed about a 2 fold increase in specific activity after the washing procedure. Readdition of linoleic acid at 1 mM to the assay mixture for both samples after washing, resulted in an increase in the activity of each to the level attained in the

Incubation	Guanylate cyclase activity (p-moles/min/mg protein) Before washing	After washing	Readdition of 1 mM linoleic acid	Recovery Activity* (1 mM linoleic acid)	Protein
linoleic acid absent	(fold) 49.0 (1.0)	(fold) 119.4 (2.4)	(fold) 530.0 (10.8)	65.7%	54.0%
linoleic acid present	472.3 (9.6)	427.1 (8.7)	538.9 (11.0)	67.5%	59.0%

Table 1. Partial reversibility of the activation of guanylate cyclase by linoleic acid.

Plasma membrane (377 μg protein/ml) was incubated for 8 min with and without 1.2 mM linoleic acid in the standard reaction mixture with GTP and cyclic GMP absent. After aliquots were removed for assay of guanylate cyclase, the incubated mixtures were diluted with 10 volumes of 10 mM Tris-HCl (pH 7.4) and centrifuged at 9,000 x g for 20 min. The pellets were suspended in 10 mM Tris-HCl (pH 7.4) and centrifuged. The final pellets were assayed for guanylate cyclase in the presence and absence of 1 mM linoleic acid in the standard reaction mixture. Linoleic acid was added at the concentration which gave maximal activation at the enzyme protein concentration used.

*Values represent % of total activity assayed in the presence of 1 mM linoleic acid before washing (472.3 p-moles/min/mg protein).

original incubation with linoleic acid. The specific activities after washing and readdition of linoleic acid were higher than that of the original incubation with linoleic acid, and was probably due to loss of protein from the membrane by washing. The recoveries of protein after incubation and washing procedures were almost the same for both samples, incubated with and without linoleic acid. The recoveries of total activity assayed in the presence of linoleic acid were slightly higher than the recoveries of protein.

Thus, the stimulatory effect of linoleic acid on guanylate cyclase does not seem to be due to solubilization of the enzyme bound to membrane. The activation by linoleic acid may be partially reversed (20%).

Guanylate cyclase activity
(p-moles/min/mg protein)

	linoleic acid		
	---	1 mM	2 mM
Cerebral cortex	(%)	(%)	(%)
particulate	21.1 (100)	47.1 (223)	32.9 (156)
soluble	75.5 (100)	22.1 (29)	---
Liver			
particulate	6.0 (100)	26.7 (445)	43.8 (730)
soluble	22.8 (100)	15.8 (69)	---

Table 2. Effect of linoleic acid on particulate and soluble guanylate cyclase of cerebral cortex and liver.

Rat cerebral cortex and liver were each homogenized with 10 volumes of 0.32 M sucrose containing 5 mM Tris-HCl (pH 7.4) and 0.5 mM EDTA. After centrifugation at 1,000 X g for 15 minutes, this supernatant was recentrifuged at 100,000 X g for 60 minutes. The supernatant (soluble enzyme) and the pellets (particulate enzyme) obtained were assayed for guanylate cyclase in the presence and absence of linoleic acid in the standard reaction mixture containing 7.8 mM theophylline. Enzyme protein used for assay was as follows: cerebral cortex, 8.1 μg of soluble and 71 μg of particulate; liver, 26 μg of soluble and 106 μg of particulate.

The effect of linoleic acid on guanylate cyclase from rat cerebral cortex and liver was examined. Particulate guanylate cyclase of cerebral cortex and liver was observed to be enhanced in the presence of linoleic acid. The enzyme activity of liver was increased 7.3 fold at 2 mM linoleic and that of cerebral cortex enhanced 2.2 fold at 1 mM linoleic acid (Table 2). Soluble guanylate cyclase however, of both cerebral cortex and liver was inhibited by 1 mM linoleic acid (Table 2).

Discussion: Fatty acid is an endogenous substance occuring naturally in various tissues. Unsaturated fatty acids effectively stimulated guanylate cyclase whereas saturated fatty acids have a small stimulatory effect. The mechanism of action of fatty acid on guanylate cyclase is not yet known. Possible

mechanisms might include a lipid peroxidation reaction on the membrane or a detergent-like effect of fatty acid on the membrane.

The physiological significance of the stimulatory effect of fatty acid on membrane guanylate cyclase remains to be clarified. The stimulatory effect of linoleic acid was also observed on particulate guanylate cyclase of cerebral cortex and liver. The phenomenon of activation by fatty acid could be a useful tool to examine the regulation of guanylate cyclase activity of membrane.

References:

1. Hardman, J.G., Beavo, J.A., Gray, J.P., Chrisman, T.D., Patterson, W.D. and Sutherland, E.W. (1971) Ann. N.Y. Acad. Sci., 185, 27-35.
2. Kimura, H. and Murad, F. (1974) J. Biol. Chem., 249, 6910-16.
3. Limbird, L.E. and Lefkowitz, R.J. (1975) Biochim. Biophys. Acta, 377, 186-96.
4. Fujimoto,M. and Okabayashi, T. (1975) Biochem. Biophys. Res. Comm., 67, 1332-36.
5. Rudland, P.S., Gospodarowicz, D. and Seifert, W. (1974) Nature, 250, 741-42, 773-74.
6. Kimura, H., Mittal, C.K. and Murad, F. (1975) J. Biol. Chem., 250, 8016-22.
7. Thompson, W.J., Williams, R.H. and Little, S.A. (1973) Biochim. Biophys. Acta, 302, 329-37.
8. White, L.E., Ignarro, L.H. and George, W.J. (1973) The Pharmacologist, 15, 157.
9. Amer, M.S. (1974) Gastroenterology, 67, 333-37.
10. Howell, S. and Montague, W. (1974) Biochem. J., 142, 379-84.
11. Ho, R.J. and Sutherland, E.W. (1971) J. Biol. Chem., 246, 6822-27.
12. Ho, R.J., Russell, T.R., Asakawa, T. and Sutherland, E.W. (1975) proc. Nat. Acad. Sci. USA, 72, 4739-43.
13. Ho, R.J. and Sutherland, E.W. (1975) Proc. Nat. Acad. Sci. USA, 72, 1773-77.
14. Ho, R.J., Russell, T.R., Asakawa, T. and Hucks, M.W. (1975) J. Cyclic Nucleotide Res. 1, 81-88.
15. Ho, R.J., Russell, T.R., Asakawa, T. and Snyder, R.P. (1975) J. Cyclic Nucleotide Res. 1, 349-58.
16. Asakawa, T., Ruiz, J., Snyder, R., Scheinbaum, I., Russell, T.R. and Ho, R.J. (1975) Fed. Proc., 34, 616.
17. Fain, J.N. and Butcher, F.R. (1976) J. Cyclic Nucleotide Res. 2, 71-78.
18. Laudat, M.H., Pairault, J., Bayer, P., Martin, M and Laudat, Ph. (1972) Biochim. Biophys. Acta, 205, 1005-08.
19. Rodbell, M. (1964) J. Biol. Chem., 239, 375-80.
20. Asakawa, T., Russell, T.R. and Ho, R.J. (1976) Biochem. Biophys. Res. Comm., 68, 682-90.
21. Patterson, M.S. and Greene, R.C. (1965) Anal. Chem., 37,854-57.