Proc. Natl. Acad. Sci. USA
Vol. 73, No. 5, pp. 1586–1590, May 1976
Biochemistry

Regulation of guanylate and adenylate cyclase activities by lysolecithin

(membrane preparations/surfactant/3T3 and SV3T3 cells)

W. THOMAS SHIER*, JULIA H. BALDWIN†, MARIT NILSEN-HAMILTON†, RICHARD T. HAMILTON†, AND NATALIE M. THANASSI†

* Cell Biology Laboratory and † Molecular Biology Laboratory, The Salk Institute, Post Office Box 1809, San Diego, California 92112

Communicated by Robert W. Holley, March 5, 1976

ABSTRACT **The guanylate cyclase activity [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] in membrane preparations from 3T3 mouse fibroblasts is stimulated approximately 5-fold by lysolecithin at concentrations of 100 μg/ml and above. The stimulation of the adenylate cyclase activity in these preparations by sodium fluoride is inhibited up to 95% by lysolecithin over a similar concentration range. The regulatory properties of lysolecithin appear to result from the surfactant properties of the phospholipid, since (*i*) the activity cannot be attributed to any single substructure within the molecule, and (*ii*) lysolecithin affects the subcellular distribution of guanylate cyclase in rat heart homogenates in a manner similar to that reported for the non-ionic detergent Triton X-100. Stimulation of guanylate cyclase by lysolecithin was observed with membrane preparations from both 3T3 cells and simian virus 40 transformed 3T3 cells (SV3T3). These results suggest a possible role for lysolecithin in the coordinate regulation of the intracellular levels of both cyclic nucleotides, and in the control of the responsiveness of target tissues to hormone or mitogen stimulation.**

Studies on cultured mouse cells (3T3) have contributed a body of evidence indicating a role for cyclic nucleotides in the regulation of the proliferation of nontransformed cells (1, 2). Stimulation of 3T3 cell growth by insulin induces an approximately 75% drop in intracellular cyclic AMP concentration (3) at 5 min after treatment. In contrast, insulin induces a concentration-dependent increase in the intracellular concentration of cyclic GMP ranging from 10- to 40-fold (1, 2). These results and the results of several additional studies of the effects of various drugs and hormones on the levels of cyclic nucleotides in their target tissues have been interpreted by Goldberg and his associates (1, 4) in terms of a hypothesis that cyclic AMP and cyclic GMP regulate biological systems through opposing influences. The cytoplasmic calcium ion concentration and cyclic nucleotide phosphodiesterases probably play important but not yet fully understood roles in this regulatory process (1). If the activities of nucleotide cyclases determine the intracellular levels of cyclic nucleotides, any agent capable of regulating the activity of these enzymes could also play a role in the regulation of intracellular cyclic nucleotide levels.

White and Lad (5) have observed that both soluble and insoluble guanylate cyclase activities [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] in rat lung are stimulated by lysolecithin and phospholipase A. A similar stimulation of guanylate cyclase in guinea pig tracheal muscle homogenates by phospholipase A was observed by Fujimoto and Okabayashi (6). Ishikawa *et al.* (7) have reported that guanylate cyclase is stimulated and adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] inhibited by the same concentration of the synthetic nonionic detergent Triton X-100 in homogenates of rat small intestines. The general similarity of the surfactant properties (8) of Triton X-100 and lysolecithin, a normal minor component of biological membranes [e.g., 1.8% of total membrane lipids in rat liver plasma membranes (9)], combined with recent observations that lysolecithin stimulates membrane-associated sialyltransferase (10) and galactosyltransferase activities (11–14) in a manner similar to Triton X-100, prompted us to investigate the effects of lysolecithin on membrane-associated guanylate and adenylate cyclase activities.

Abbreviations: SEM, standard error of the mean; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

MATERIALS AND METHODS

Unless otherwise indicated, egg yolk lysolecithin was used in all experiments. All nonradioactive phospholipids were obtained from Sigma except stearoyl, myristoyl, and oleoyl lysolecithins and lysodimethylphosphatidylethanolamine, which were prepared from the corresponding diesterified phospholipids (Sigma) by treatment with phospholipase A_2 (*Crotalus adamanteus* venom, Worthington) according to the method of Wells and Hanahan (15). Lipopolysaccharide and lipid A were the gifts of Dr. J. Watson.

Balb/3T3 cells were obtained from Dr. W. Eckhart, 3T3-4a cells from Dr. R. W. Holley, and 3T3 cells transformed by simian virus 40 (SV3T3 cells) were recloned from a line obtained from Dr. P. Rudland. Cells were grown to confluence in Dulbecco's modification of Eagle's medium containing 10% calf serum.

Preparation of Membrane Fractions. A modification of the methods of Wallach and Kamat (16) and Graham (17) was used. Balb/3T3 cells were harvested by removing the medium, rinsing the cell monolayers, first at 37°, then at 4° with Tris-buffered saline, and scraping the cells with a rubber policeman into 0.01 M potassium phosphate (pH 7.4), 0.15 M NaCl at 4°. All subsequent operations were at 4°. Cells were collected by centrifugation at 260 × *g* for 10 min, resuspended to a 10% (vol/vol) suspension in 0.25 M sucrose, 5 mM Tris, 0.2 mM $MgSO_4$ and disrupted by nitrogen cavitation at 600 pounds/inch^2 (4.1 MPa) in an Artisan Pressure Homogenizer (0.068 cm diameter orifice), 5 min being allowed before pressure release. The homogenate was collected dropwise. Immediately after homogenization 0.1 M Na_2EDTA (pH 7.0) was added to give a final concentration of 1 mM. The resultant suspension was centrifuged at 2400 × *g* for 5 min. The postnuclear supernatant (3–4 ml per gradient) was then loaded onto a 32 ml discontinuous 10–60% (wt/wt) sucrose gradient in 0.005 M Tris·HCl (pH 7.4), 1 mM Na_2EDTA, prepared by forming a 10 ml linear 60–30% sucrose gradient on top of which was layered 8 ml of 30% sucrose. The gradient was completed by forming a 14 ml 30–10% linear sucrose gradient on the barrier. The combined gradients were centrifuged at 81,000 × *g* for 90 min at 4°, and collected by pumping 35% sucrose, 45% NaI at 3 ml/min into the bottom of the gradient. Fractions (1.2 ml) collected from

the top were monitored continuously for absorbance at 280 nm in an Isco fraction collector at 25°. Fractions were then stored at 4° before being assayed. The fractions of the sucrose gradient that contained membrane, as indicated by 5′-nucleotidase activity, were pooled and dialyzed overnight against 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (Sigma Chemical), pH 7.3, or against 0.25 M sucrose containing 5 mM Tris and 0.2 mM $MgSO_4$. The membranes were then centrifuged for 90 min at 100,000 × *g* and resuspended in the same buffer to a final concentration of 1–2 mg of membrane protein per ml.

Enzyme Assays. Enzyme activity was determined by conversion of the nucleoside triphosphate to the cyclic nucleotide (18, 19), which was then measured by a radioimmune assay (20). For *guanylate cyclase* reaction mixtures (150 μl) usually contained: 40 mM Hepes–Na (pH 7.6), 2 mM $MnCl_2$, 0.2 mM GTP, 0.5 mM Na_4EDTA, 0.5 mM dithiothreitol, 0.25 M sucrose, 10 mM theophylline, 20 mM caffeine, 31.25 μg of crystalline bovine serum albumin, and the lipids and enzyme preparations to be tested. For *adenylate cyclase*, 6 mM $MgCl_2$ replaced $MnCl_2$; 2 mM ATP, 15 mM creatine phosphate, and 5 μg of creatine kinase replaced GTP. Fluoride-stimulated adenylate cyclase activity was determined under the same conditions with 10 mM NaF added. Reaction mixtures were incubated for 10–15 min at 37°, and the reactions were terminated by dilution with 150 μl of 0.05 M sodium acetate (pH 6.2) and boiling for 3 min. The extracts were then centrifuged at 1300 × *g* for 30 min and the supernatants were stored at −20°. Triplicate samples were assayed for the cyclic nucleotides, using the radioimmune assay of Steiner *et al.* (20) (Schwarz/Mann radioimmune assay kits). Results were recorded as pmol of cyclic nucleotide synthesized per min/mg of protein.

Guanylate cyclase and fluoride-stimulated adenylate cyclase activities in the presence and absence of lysolecithin (1 mg/ml) were linear with respect to time for at least 20 min, and linear with respect to total enzyme protein in the concentration range 8 μg/ml to 65 μg/ml for guanylate cyclase and 0.125 mg/ml to 1 mg/ml for fluoride-stimulated adenylate cyclase. The cyclic AMP synthesized by the preparation was 69% hydrolyzed by beef heart phosphodiesterase (Sigma) and the cyclic GMP was 83% hydrolyzed by the same phosphodiesterase during a 15 min incubation under the conditions described by Thompson and Appleman (21). The GTPase activity in the membrane preparation (0.81 nmol of phosphate released per min/mg of protein) measured as phosphate (22) released during 60 min under the conditions of the guanylate cyclase assay was sufficient to hydrolyze approximately 1.2% of the GTP during the course of a 10 min assay. Addition of lysolecithin (1 mg/ml) stimulated GTPase activity of one preparation 73%.

5′-Nucleotidase (EC 3.1.3.5) was assayed by the method of Avruch and Wallach (23). Cyclic AMP and cyclic GMP phosphodiesterases (EC 3.1.4.17) were assayed by the method of Thompson and Appleman (21). Protein was measured by tryptophan fluorescence (24), using bovine serum albumin in the same buffer as standard.

RESULTS

As shown in Fig. 1A, egg yolk lysolecithin stimulated guanylate cyclase activity in Balb/3T3 membranes approximately 5-fold at concentrations above 1 mg/ml, with suboptimal stimulation from 0.03 to 1 mg/ml. Fluoride-stimulated adenylate cyclase was approximately 95% inhibited by added lysolecithin at concentrations above 1 mg/ml (see Fig. 1B), with suboptimal inhibition in a range similar to that observed for guanylate cyclase stimulation.

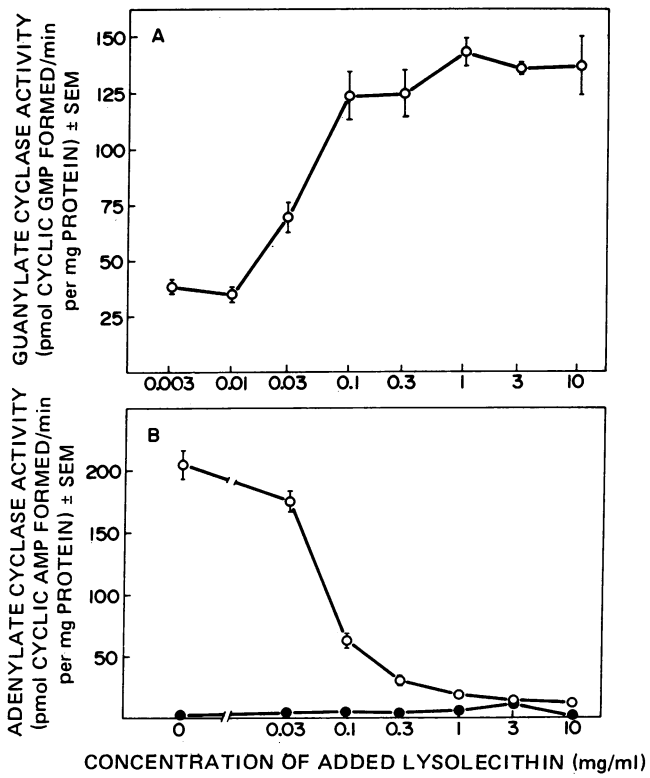

FIG. 1. (A) Effect of egg yolk lysolecithin on guanylate cyclase activity in Balb/3T3 membranes. (B) Effect of egg yolk lysolecithin on fluoride-stimulated (—○—) and unstimulated (—●—) adenylate cyclase activity in Balb/3T3 membranes.

Lysolecithin preparations from three different natural sources (egg yolk, bovine liver, and soybean) stimulated guanylate cyclase activity to a similar degree (see Table 1). A series of synthetic lysolecithins with various fatty acid chain lengths all stimulated guanylate cyclase, and maximum stimulation was achieved with myristoyl lysolecithin. Three other phosphoglycerides (lysophosphatidylethanolamine, lysodimethylphosphatidylethanolamine, and lecithin) did not stimulate guanylate cyclase activity. Similar results were observed for the inhibition of fluoride-stimulated adenylate cyclase by these lipids. *Escherichia coli* lipopolysaccharide and lipid A, which induce elevated levels of cyclic GMP in mouse lymphocytes (25), did not stimulate guanylate cyclase in this system.

The stimulation of guanylate cyclase by lysolecithin was observed in all membrane preparations examined. Guanylate cyclase activity was stimulated by lysolecithin and Triton X-100 in preparations from Balb/3T3 cells, from two clones of Swiss 3T3 cells, and from SV40-virus-transformed Swiss 3T3 cells (Table 2). The stimulatory effect was similar in preparations from normal and transformed cells. The effect of lysolecithin on nucleotide cyclase activities was also examined in rat heart subcellular fractions prepared by the method of White (26) (see Table 3). As observed by White (5) in his studies using the nonionic detergent Triton X-100, the majority of the guanylate cyclase activity is in the supernatant fraction in the absence of added surfactant, and addition of the surfactant stimulates activity in all fractions with the greatest stimulation being observed in the microsomal fraction. In agreement with White (26), adenylate cyclase is observed with the highest specific activity in the microsomal fraction and the activity in every fraction can be stimulated with sodium fluoride, with the largest degree of stimulation being observed in the microsomal fraction. Lysolecithin inhibits adenylate cyclase in every fraction.

Table 1. Effect of various lipids on guanylate cyclase and fluoride-stimulated adenylate cyclase activities* in Balb/3T3 membrane preparations

Lipid	Concentration (μg/ml)	Guanylate cyclase activity ± SEM (pmol of cyclic GMP formed per min/mg of protein)	Sodium fluoride (10 mM) stimulated adenylate cyclase activity† ± SEM (pmol of cyclic AMP formed per min/mg of protein)
None	—	54 ± 12	40.3 ± 4.8
Lysolecithin (egg yolk)	30	195 ± 44	53.9 ± 0.2
	100	455 ± 86	8.9 ± 0.7
	1,000	361 ± 81	3.8 ± 0.6
Lysolecithin (bovine liver)	30	127 ± 41	36.3 ± 6.2
	100	266 ± 39	9.7 ± 1.9
	1,000	490 ± 35	2.2 ± 6.2
Lysolecithin (soybean)	30	25 ± 4	52.7 ± 6.3
	100	166 ± 33	8.5 ± 0.6
	1,000	527 ± 96	6.1 ± 0.5
Stearoyl lysolecithin	30	113 ± 22	55.6 ± 4.6
	100	338 ± 51	26.4 ± 3.8
	1,000	263 ± 52	4.8 ± 1.3
Palmitoyl lysolecithin	30	106 ± 25	43.6 ± 5.3
	100	482 ± 83	8.1 ± 0.8
	1,000	429 ± 0	2.3 ± 0.2
Myristoyl lysolecithin	30	18 ± 10	47.6 ± 0.8
	100	562 ± 130	13.6 ± 3.2
	1,000	405 ± 40	2.5 ± 0.4
Lauroyl lysolecithin	30	46 ± 9	46.7 ± 10.9
	100	117 ± 37	50.2 ± 5.4
	1,000	403 ± 94	2.1 ± 0.3
Oleoyl lysolecithin	30	22 ± 11	55.5 ± 10.8
	100	163 ± 36	24.6 ± 3.0
	1,000	349 ± 32	4.9 ± 0.5
Lysophosphatidyl-ethanolamine (egg yolk)	30	30 ± 9	24.3 ± 4.2
	100	56 ± 24	22.0 ± 1.3
	1,000	66 ± 9	18.8 ± 1.3
Lysodimethylphospha-tidylethanolamine	30	69 ± 23	19.6 ± 0.5
	100	125 ± 10	37.6 ± 1.2
	1,000	78 ± 59	21.1 ± 1.9
Lecithin (egg yolk)	100	22 ± 9	25.0 ± 1.6
	1,000	20 ± 7	26.8 ± 1.0
	10,000	42 ± 19	11.9 ± 1.2
Lipopolysaccharide (*E. coli* 0111:B4)	30	86 ± 23	ND
	100	74 ± 9	ND
	1,000	72 ± 8	ND
Lipid A (*E. coli* 9111:B4)	30	69 ± 2	ND
	100	43 ± 9	ND
	1,000	62 ± 10	ND

ND, not done.

* Assays here and in the following tables were performed in triplicate.

† The basal adenylate cyclase activity was 0.26 ± 0.60 pmol of cyclic AMP formed per min/mg of protein.

The observed stimulation of guanylate cyclase by lysolecithin does not represent inhibition of cyclic GMP phosphodiesterase activity, since none of the preparations examined contained detectable cyclic GMP phosphodiesterase activity measured by the method of Thompson and Appleman (21). Similarly, the inhibition of adenylate cyclase activity cannot be explained by stimulation of cyclic AMP phosphodiesterase activity in the presence of lysolecithin. The basal level of cyclic AMP phosphodiesterase activity in membrane preparations was always less than 10 pmol of cyclic AMP hydrolyzed per min/mg of protein with 0.1 mM cyclic AMP as substrate. Addition of lysolecithin at 1.0 mg/ml or at 0.1 mg/ml stimulated the cAMP phosphodiesterase activity of one preparation by a factor of 1.9, which is not large enough to account for the observed results.

DISCUSSION

In a number of different biological systems Goldberg and his associates (1, 4) have observed that those hormones and mitogens that promote the cellular accumulation of cyclic GMP also produce cellular responses antagonistic to those responses characterized by high intracellular concentrations of cyclic AMP. On the basis of these observations they have proposed that in some biological systems cyclic AMP and cyclic GMP have opposing or antagonistic regulatory influences (1, 4) (the so-called "Yin Yang hypothesis"). The observation that lysolecithin added to 3T3 membrane preparations stimulates guanylate cyclase and inhibits fluoride-stimulated adenylate cyclase over a similar concentration range suggests a model in which alterations of cyclic nucleotide levels in opposite directions could

Table 2. Stimulation of guanylate cyclase activity in membrane preparations from cultured mouse cell lines

Cell line	Addition	Guanylate cyclase activity ± SEM (pmol of cyclic GMP formed per min/mg of protein)
Swiss 3T3 (clone 6)	None	7 ± 3
	Egg yolk lysolecithin (0.1 mg/ml)	177 ± 18
	Triton X-100 (1.0 mg/ml)	304 ± 23
Balb/3T3 (clone 9)	None	27 ± 4
	Egg yolk lysolecithin (0.1 mg/ml)	143 ± 56
	Triton X-100 (1.0 mg/ml)	357 ± 27
Swiss 3T3-4a	None	44 ± 12
	Egg yolk lysolecithin (0.1 mg/ml)	397 ± 37
	Triton X-100 (1.0 mg/ml)	418 ± 70
Swiss SV3T3-4a	None	34 ± 5
	Egg yolk lysolecithin (0.1 mg/ml)	185 ± 38
	Triton X-100 (1.0 mg/ml)	395 ± 60

be achieved by the action of a single agent at the surface of a cell. In this model a hormone or mitogen could alter the intracellular levels of cyclic nucleotides by altering the amount of lysolecithin in the cell membranes. Restoration of the original level of guanylate cyclase activity after a transient increase in lysolecithin levels could be achieved by mechanisms such as rapid turnover of the enzyme after stimulation by lysolecithin, or by a membrane dissociation-reassociation cycle for the activation and inactivation of the enzyme. Restoration of the original levels of either nucleotide cyclase activity could result from reversible modification of enzyme activity by lysolecithin.

The amount of lysolecithin in cell membranes is determined by the action of the two opposing enzyme activities of the phosphoglyceride deacylation–reacylation cycle (27). The activities of both the degradative (phospholipase A_2) and synthetic (acylCoA:lysophosphoglyceride acyltransferase) enzymes participating in this cycle have been reported to respond to external stimuli under certain conditions. Phospholipase A_2, which degrades lecithin [19.3% of rat liver plasma membranes (9)] to lysolecithin, has been detected in virtually every organism, tissue, and subcellular fraction that has been investigated (27). Gullis and Rowe (28) have reported that net phospholipase A_2 activity of synaptic membranes of the cortex of guinea pig brain is stimulated by Ca^{2+}, noradrenaline, acetylcholine, carbamoylcholine, and serotonin, but not glucagon. These effects on phospholipase A_2 parallel the effects of these agents on intracellular cyclic GMP levels in other systems (4). Acyltransferases, which can catalyze the transfer of fatty acids from coenzyme A esters to lysolecithin, converting the lysolecithin back to lecithin, are most abundant in the endoplasmic reticulum of rat liver (27), but they have also been found in the mitochondrial outer membranes and in plasma membranes. The levels of acyltransferase activity in membranes may also be regulated, since the acylation of lysophosphoglycerides by rabbit granulocytes and alveolar macrophages was increased 3-fold in the presence of ingestible particles (29), and the acyltransferase activity of rabbit lymphocytes was stimulated 2- to 3-fold in the plasma membrane fraction after stimulation with phytohemagglutinin (30).

The normal level of lysolecithin in tissue has been calculated (13) to be approximately 1.5 mg/ml, using values obtained for rat liver by Ray *et al.* (9) and considering the lysolecithin to be uniformly distributed throughout the tissue. Since approximately 80% of the maximal effect of lysolecithin on nucleotide cyclases *in vitro* is achieved with 0.1 mg/ml of additional lysolecithin, it is conceivable that an alteration of this magnitude could be achieved *in vivo* by modifying the activity of either of the enzymes in the phosphoglyceride deacylation-reacylation cycle. Perturbation of the cycle could be achieved by direct stimulation of hormone-sensitive plasma-membrane-associated

Table 3. Distribution of nucleotide cyclases in subcellular fractions from rat heart

Fraction	Protein, %	Guanylate cyclase				Adenylate cyclase					
		Basal		+Lysolecithin (1 mg/ml)		Basal		+Sodium fluoride (10 mM)		+Lysolecithin (1 mg/ml) and sodium fluoride (10 mM)	
		SA*	%	SA	%	SA	%	SA	%	SA	%
Homogenate	100	3.9 ± 0.2	100	6.9 ± 2.6	100	3.3 ± 0.4	100	9.8 ± 1.1	100	5.0 ± 0.4	100
Nuclear	56.8	1.4 ± 0.3	20.8	3.6 ± 0.2	29.7	2.3 ± 0.4	41.5	14.6 ± 2.3	84.3	7.4 ± 1.4	83.9
Mitochondrial	9.6	0.9 ± 0.0	2.1	3.7 ± 0.2	5.1	2.6 ± 0.2	7.9	14.9 ± 3.0	14.5	5.3 ± 0.7	10.1
Microsomal	6.2	0.8 ± 0.2	1.3	4.3 ± 0.1	3.9	4.8 ± 0.4	9.3	40.5 ± 1.8	25.5	11.6 ± 0.7	14.2
Supernatant	27.4	11.7 ± 0.8	80.4	18.3 ± 2.8	72.1	1.7 ± 0.4	14.8	2.0 ± 0.1	5.5	1.8 ± 0.2	9.6
Recovery	98.8		104.6		110.9		73.5		129.9		109.3

* SA, specific activity in pmol of cyclic nucleotide formed per min/mg of protein.

phospholipase A_2 enzymes, as has been described in guinea pig cerebral cortex synaptosomes by Gullis and Rowe (28), or by processes that increase the intracellular concentration of calcium ions, since the phospholipase A_2 enzyme activity associated with the endoplasmic reticulum is stimulated by calcium ions in all tissues that have been examined (27). Lysolecithin generated at one part of the cell could function as a membrane transducer by diffusing rapidly through the lipid portions of the cellular membranes to modify the activity of membrane-associated enzymes in other parts of the cell, as well as possibly altering general properties of the membrane such as fluidity and permeability. This model is presently being evaluated.

The observation (Table 1) that the nucleotide cyclase activities are modified by all lysolecithin preparations tested but not by other phospholipids indicates that the stimulatory activity observed with lysolecithin is not due to the esterified fatty acid or the choline moieties in the molecule (they are present in lecithin), nor to the L-α-lysophosphatidyl moiety (it is present in lysophosphatidylethanolamine and lysodimethylphosphatidylethanolamine). These results are consistent with the suggestion that lysolecithins significantly modify the activities of nucleotide cyclases because they have the appropriate hydrophile-lipophile balance (8). Limbrid and Lefkowitz (31) reported that the soluble but not the particulate myocardial guanylate cyclase from guinea pig is stimulated 1.4- to 4.3-fold by phosphatidylserine.

The possible involvement of lysolecithin in the control of the biosynthesis of some species of phosphoglycerides (27) and in coordinate regulation of glycosyltransferases (10) has been considered previously. However, the observed effects of lysolecithin on nucleotide cyclases suggest a mechanism whereby lysolecithin could play a much broader role in regulation. For example, lysolecithin may participate in (*a*) the regulation of normal cell proliferation, (*b*) the regulation of the responsiveness of target tissues to hormones, and (*c*) in the etiology of hypertension (32) and other disease states that have been reported to involve abnormal cyclic nucleotide ratios (33).

This work was supported by the Theodore Gildred Foundation; U.S. Public Health Service Grants CA16123, CA14195, and CA11176 from the National Cancer Institute; American Cancer Society Grant BC-30; and National Science Foundation Grant GMS72-01899. We gratefully acknowledge the support and encouragement of Dr. R. W. Holley and the technical assistance of Mr. A. Oesterle and Mr. J. T. Trotter, III.

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