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Modification of adenylate cyclase activity in LM cells by manipulation of the membrane phospholipid composition *in vivo**

(3′:5′-cyclic AMP/hormone stimulation/polar head group/fatty acid/fluidity)

VICTOR H. ENGELHARD, JEFFREY D. ESKO, DAN R. STORM, AND MICHAEL GLASER

Department of Biochemistry, School of Chemical Sciences, University of Illinois, Urbana, Ill. 61801

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ABSTRACT **Adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] activities were examined in mouse LM cell (fibroblast) membranes that were supplemented with ethanolamine and/or fatty acids. The supplements were incorporated into the plasma membrane phospholipids in significant amounts. Fatty acid supplementations had distinct effects as compared to polar head group supplementations. All lipid supplementations increased basal adenylate cyclase activity relative to control cells grown in choline-containing medium. Double supplementation with ethanolamine and linoleate increased the specific activity of adenylate cyclase up to 4-fold. Activity in the presence of fluoride was unaffected by ethanolamine supplementation, but was increased by fatty acid supplementation. In contrast, prostaglandin E_1 stimulation was 4.2-fold in controls and ethanolamine and/or elaidate supplements, 6-fold in choline plus linoleate supplements, and 3.1-fold in ethanolamine plus linoleate supplements. Differences in activity could not be ascribed to changes in membrane protein composition in supplemented cells, and could be abolished by detergent solubilization. The fluidity of the supplemented membranes was monitored by fluorescence polarization, and no correlation was observed between membrane viscosity and adenylate cyclase activity or hormone stimulation. These results emphasize the importance of the membrane lipid phase for this enzyme.**

Adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] is associated with the plasma membranes of animal cells (1–7). The enzyme is tightly bound to the membrane, and has been solubilized only by the use of membrane-disruptive agents such as detergents. Several lines of evidence indicate that adenylate cyclase is dependent upon membrane lipids for basal and hormone-stimulated activities. The membrane-bound enzyme has been treated with filipin (3), nonionic detergents (8–10), digitonin (11), phospholipases (11–13), and organic solvents (13). These treatments resulted in changes in either basal activity or hormone stimulation or both. When phospholipids were added back (11, 13–16) there was a restoration of basal activity or hormone stimulation to a degree that was highly dependent upon the nature of the phospholipid polar head group. More recently Houslay *et al.* (17) have fused phospholipids with membranes and observed changes in the activity and temperature dependence of adenylate cyclase.

An approach that circumvents the difficulties associated with reconstitution studies is the modification of the membrane lipid composition *in vivo*. Brivio-Haugland *et al.* showed significant changes in basal and hormone-stimulated adenylate cyclase activity when rats were fed an essential fatty acid deficient diet (18). Techniques for the modification of membrane lipid composition that originated in studies on microorganisms (19) have recently been extended to animal cells growing in tissue culture, so that it is now possible to systematically modify their fatty acid composition (20–24) and polar head group composition (24, 25). These developments now make it possible to carry out enzymatic and physical studies in the membranes of animal cells with biosynthetically modified lipid compositions (22, 23, 26).

Although the mechanism for hormone stimulation of adenylate cyclase has not been elucidated, hormone stimulation may proceed through conformational changes of single or multisubunit structures (27–29), or involve association (30) or dissociation (31) of subunits that diffuse laterally in the membrane. These models suggest that hormone stimulation may be dependent upon membrane viscosity. The effects of varying membrane lipid composition and viscosity on adenylate cyclase and its hormone stimulation in LM cells are reported in this paper.

MATERIALS AND METHODS

Growth and Supplementation of Cells. Mouse LM cells were grown in suspension culture in Higuchi's medium (24) containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.4, 1 g/liter of methylcellulose, and 0.02 g/liter of sodium dextran sulfate. Growth and supplementation were carried out as described (20, 24).

Lipid Determinations. Lipids were extracted by the method of Bligh and Dyer (32) as described by Ames (33). Phospholipids were separated by two-dimensional thin-layer chromatography (20, 24). Spots were visualized with I_2 vapor, scraped, and eluted with 5 ml of $CHCl_3$:CH_3OH:acetic acid:H_2O (5:5:1:1, vol/vol) followed by 2 ml of CH_3OH. The extracts were combined, evaporated to dryness, and redissolved in $CHCl_3$:CH_3OH:H_2O (2:2:1.8, vol/vol). The $CHCl_3$ phase was used for total phosphate analysis by the method of Ames (34).

Fatty acid compositions were determined after extraction of the phospholipids by the method of Bligh and Dyer and separation of phospholipids from neutral lipids on a short Unisil column (20). Methyl esters were chromatographed on a 15% SP-2340, Chromosorb PAWDMCS, 100/120 mesh column at 200° (Supelco). Fatty acid and polar head group compositions are the mean of triplicate determinations with a standard deviation of 3–5%. Lipid compositions are expressed as percentage of the total recovered.

Preparation of Plasma Membranes. Plasma membranes were prepared by a procedure similar to that of Schimmel *et al.* (35), except that the cells were hypotonically lysed. Differential centrifugation yielded a pellet that was used for adenylate cyclase assays, while purified plasma membranes were obtained after a sucrose density gradient centrifugation. Details of this procedure will appear elsewhere (J. D. Esko, J. R. Gilmore, and M. Glaser, in preparation).

Abbreviations: PGE_1, prostaglandin E_1; 18:2, linoleic acid, *cis,cis*-$\Delta^{9,12}$-18:2; 18:1(*cis*), oleic acid, *cis*-Δ^9-18:1; 18:1(*trans*), elaidic acid, *trans*-Δ^9-18:1; LM cells, mouse fibroblast cells.

* A preliminary report of this work has been presented [Engelhard, V. H., Esko, J. D., Storm, D. R. & Glaser, M. (1976) *Fed. Proc.* 35, 1532].

Table 1. Fatty acid composition of plasma membrane phospholipids of LM cells

Supplement	Fatty acid (% by weight)						
	14:0	16:0	16:1	18:0	18:1(*cis*)	18:2	18:1(*trans*)
Choline (control)	0.8	20.7	6.1	15.4	55.7	—	—
Choline + 18:2	1.0	18.6	6.7	15.5	34.9	23.2	—
Choline + 18:1(*trans*)	0.8	17.0	4.8	10.4	39.9	—	26.7
Ethanolamine	2.3	18.0	11.2	20.1	47.4	—	—
Ethanolamine + 18:2	0.4	16.2	7.3	19.6	36.2	20.0	—
Ethanolamine + 18:1(*trans*)	0.7	11.1	7.6	9.9	40.8	—	30.0

Cells were grown for 3 days in the presence of 40 μg/ml of choline or ethanolamine, and 40 μg/ml of either 18:1 (*trans*) or 18:2 (complexed to bovine-serum albumin) was added where indicated 18 hr prior to harvest. Fatty acid compositions were determined as described in *Materials and Methods*. Fatty acids of chain length greater than 18 represented less than 8% of the total and were not considered in this analysis.

Adenylate Cyclase Assay. Adenylate cyclase was assayed by the method of Salomon *et al.* (36) using [α^{32}P]ATP as a substrate. Each data point is the mean of triplicate determinations with a standard deviation of less than 5%. Protein was determined by the method of Lowry *et al.* (37), using bovine serum albumin as a standard. Detergent-containing samples were first incubated at 100° in 5% sodium dodecyl sulfate to reduce turbidity.

Detergent Solubilization. One milliliter of 1% Brij 56, 1 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris·HCl (pH 7.5) was added to 5 mg of membrane protein and the pellet was dispersed with a 22-gauge syringe. The suspension was incubated on ice for 30 min, with mixing on a Vortex at 5-min intervals. It was then centrifuged at 100,000 × *g* for 1 hr. The supernatant was removed and assayed for adenylate cyclase.

Fluorescence Polarization Measurements. Fluorescent measurements were made on membranes isolated from cells that had been labeled with 1,6-diphenyl-1,3,5-hexatriene (J. D. Esko, J. R. Gilmore, and M. Glaser, in preparation). Viscosity was calculated from the observed anisotropy and lifetime as determined by the relative fluorescence intensity as previously described (38–40). The overall error in the viscosity measurements is estimated at ±5%.

RESULTS

Incorporation of fatty acids and ethanolamine into membranes

The methodology used to achieve alterations of polar head group and fatty acid composition of phospholipids in LM cells grown in monolayers has been reported (20, 24). When applied to LM cells grown in suspension, supplements were also incorporated into plasma membrane phospholipids. In the absence of exogenously supplied fatty acid, oleic acid [18:1 (*cis*)] comprised 56% of the fatty acid present in phospholipids, followed by 21% palmitate (16:0), 15% stearate (18:0), and 6% palmitoleate (16:1) (Table 1). When linoleate (18:2) was added to the growth medium for 18 hr, it comprised approximately 22% of the plasma membrane phospholipid fatty acids. This level was achieved irrespective of the presence of choline or ethanolamine as the predominant phospholipid polar head group. The major decrease accompanying 18:2 incorporation was in the levels of oleate [18:1(*cis*)]. Minor variations were seen in the percentages of other fatty acids. When elaidate [18:1(*trans*)] was added to the growth medium, it was incorporated to about 28%. Accompanying this was a decrease in 18:1(*cis*), which was not as large as that seen during supplementation with 18:2. Instead, there were also changes in the levels of all other major fatty acids, both saturated and unsaturated.

Table 2. Polar head group composition of plasma membrane phospholipids of LM cells

Supplement	Polar head group (mole %)				
	PC	PE	PI/PS	SP	Other
Choline (control)	45.9	31.6	9.7	7.3	5.4
Choline + 18:1(*trans*)	46.3	25.7	6.2	11.0	10.8
Choline + 18:2	51.9	26.1	5.8	6.9	10.0
Ethanolamine	19.9	47.9	6.2	12.4	13.6
Ethanolamine + 18:1(*trans*)	19.7	52.7	8.3	9.4	9.9
Ethanolamine + 18:2	21.3	48.0	8.6	12.0	10.1

Cells were grown as in the legend to Table 1 and polar head group compositions were determined as in *Materials and Methods*. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SP, sphingomyelin. "Other" represents the sum of five minor phospholipid spots.

LM cells grown under normal conditions in medium containing choline have about 46% phosphatidylcholine and 32% phosphatidylethanolamine in their plasma membrane phospholipids (Table 2). When ethanolamine was substituted for choline in the medium for 3 days, phosphatidylethanolamine constituted 48% of the phospholipids, while phosphatidylcholine now constituted only 20%. These values were not changed by further supplementation with fatty acids, although there was some variation in the levels of sphingomyelin and phosphatidylserine plus phosphatidylinositol.

Adenylate cyclase activities in supplemented cells

Control membranes had a basal adenylate cyclase activity of 23 pmol/10 min per mg of protein, which was stimulated 13.5-fold by fluoride ion and 4.2-fold by prostaglandin E_1 (PGE_1) (Table 3). Ethanolamine supplementation resulted in a 2.5-fold elevation of basal activity. Activity in the presence of fluoride was identical to control membranes, so that the degree of fluoride stimulation decreased to 5-fold. PGE_1 stimulation in ethanolamine-supplemented membranes was identical to that in control membranes.

Supplementation with 18:1(*trans*) increased the basal activity by the same amount (12 pmol/10 min per mg) in choline- and ethanolamine-supplemented membranes. The corresponding activities in the presence of fluoride were very close to 400 pmol/10 min per mg in both cases. Stimulation by PGE_1 was unaffected by 18:1(*trans*) supplementation.

The elevations of basal activity in 18:2-supplemented membranes over the corresponding values for choline- and ethanolamine-supplemented cells were also the same (36

Table 3. Adenylate cyclase activities of LM cells supplemented with ethanolamine or fatty acids

Supplement	Adenylate cyclase (pmol cAMP/10 min·mg) Basal	NaF (15 mM)	PGE_1 (5 μg/ml)
Choline (control)	23	304 (13.5×)	95 (4.2×)
Choline + 18:1(*trans*)	34	379 (11.1×)	144 (4.2×)
Choline + 18:2	56	473 (8.5×)	333 (6.0×)
Ethanolamine	57	303 (5.3×)	250 (4.4×)
Ethanolamine + 18:1-(*trans*)	70	405 (5.8×)	265 (3.8×)
Ethanolamine + 18:2	96	440 (4.6×)	301 (3.1×)

Adenylate cyclase was determined at 30° for 10 min as described in *Materials and Methods*. Each data point is the mean of triplicate samples containing approximately 100 μg of protein with a standard deviation of less than 5%. Cells were grown as described in the legend to Table 1. The numbers in parentheses represent the stimulation over basal activity.

pmol/10 min per mg). Activities in the presence of fluoride averaged 455 pmol/10 min per mg. Interestingly, PGE_1 stimulation in membranes supplemented with choline plus 18:2 was increased from 4.2-fold to 6-fold, while that in membranes supplemented with ethanolamine plus 18:2 was down to 3.1-fold.

Detergent solubilization of adenylate cyclase

It is possible that the differences in adenylate cyclase activities reflect changes in the level of adenylate cyclase or of other membrane proteins rather than changes in the membrane environment of the enzyme. Sodium dodecyl sulfate-polyacrylamide gels of control and lipid-supplemented membranes showed no detectable differences in the polypeptide gel profiles (data not shown).

Adenylate cyclase was solubilized from control and lipid-supplemented membranes in order to determine more directly if the membrane lipid environment was responsible for changes in specific activity observed in supplemented cells. The increased basal activity in supplemented membranes was lost upon solubilization (Table 4). In all cases, except for ethanolamine and ethanolamine plus 18:1(*trans*), the solubilized basal activities were equivalent to the activity solubilized from control membranes. In these two cases, basal activity was 50% of the control activity. Fluoride stimulation was about 8- to 10-fold after solubilization, but variation in this number was seen in several experiments. PGE_1 stimulation was essentially abolished by solubilization, so that conclusions regarding the changes in stimulation seen in membranes cannot be made. The amount of protein solubilized from the various membrane preparations was uniformly 16–19%, and sodium dodecyl sulfate-polyacrylamide gels of material solubilized from control and supplemented membranes were identical, demonstrating that proteins were solubilized from the membranes in the same amounts regardless of membrane lipid composition (data not shown).

Table 4. Activity of adenylate cyclase from supplemented cells after detergent solubilization

Supplement	Basal adenylate cyclase (pmol cAMP/10 min·mg)
Exp. I	
Choline (control)	17.2
Choline + 18:1(*trans*)	14.4
Choline + 18:2	15.2
Exp. II	
Choline (control)	22.9
Ethanolamine	10.5
Ethanolamine + 18:1(*trans*)	11.1
Ethanolamine + 18:2	23.3

Cells were grown as outlined in Table 1. Adenylate cyclase was assayed at 30° for 10 min. Each point is the mean of triplicate samples containing 20 μg of protein, and with a standard deviation of less than 5%.

Table 5. Viscosity of LM cell plasma membranes

Supplement	Viscosity (centipoise) 10°	25°	37°
Choline (control)	693	358	246
Choline + 18:2	707	388	249
Choline + 18:1(*trans*)	1047	459	264
Ethanolamine	1158	499	315
Ethanolamine + 18:2	1015	491	306
Ethanolamine + 18:1(*trans*)	1054	462	272

Viscosities were determined as described in *Materials and Methods* on plasma membranes purified from cells that had been prelabeled with diphenylhexatriene. Cells were grown as described in Table 1.

Membrane fluidity measurements

Bulk membrane viscosity was measured with diphenylhexatriene (Table 5). Ethanolamine-supplemented membranes showed a pronounced increase in viscosity relative to the control membranes at all temperatures. Addition of 18:2 did not significantly affect the viscosity of either choline- or ethanolamine-supplemented membranes. Addition of 18:1(*trans*) resulted in a large increase in the viscosity of choline-supplemented membranes and a small decrease in the viscosity of ethanolamine-supplemented membranes, such that the viscosity of 18:1(*trans*)-supplemented membranes was identical regardless of polar head group composition. A comparison of basal, fluoride-stimulated, and PGE_1-stimulated adenylate cyclase activities with the viscosities of the respective membranes revealed no simple correlation between these parameters.

Temperature dependence of adenylate cyclase activity

It has been reported in a number of systems that the activity of membrane-bound enzymes depends upon the physical state of membrane lipids (41–44). Therefore, the activity of adenylate cyclase in control and supplemented membranes was measured from 15° to 40°. Adenylate cyclase in control membranes exhibited a striking temperature dependence (Fig. 1B). At 25°, basal activity showed a sudden downward break, which may be indicative of thermal denaturation. Activity decreased regularly to 33° and then rose again. In contrast, the downward break at 25° was not seen in the presence of PGE_1. Arrhenius plots of both basal and PGE_1-stimulated activity in all supplemented membranes showed no breaks of any kind in this temperature range (Fig. 1A). Lipid supplementations increased the activation energies of the enzyme catalyzed reaction up to 3-fold (data not shown) but did not correlate with changes in viscosity.

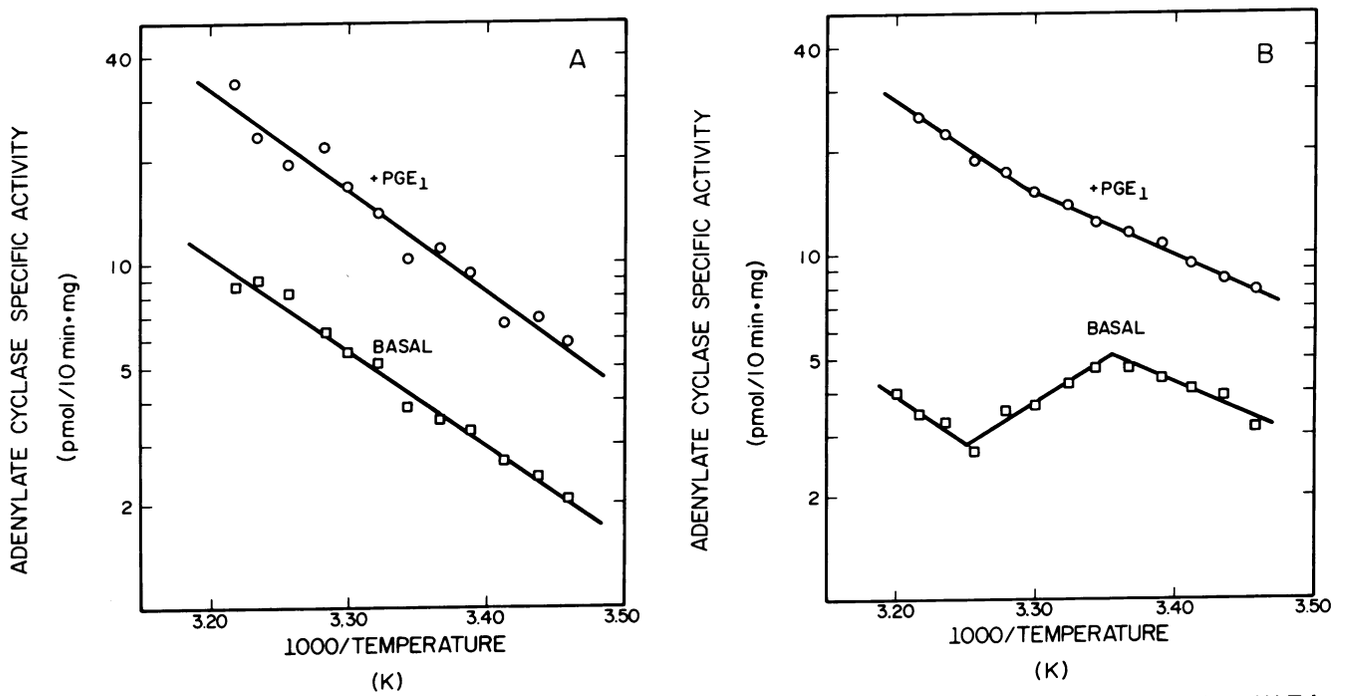

FIG. 1. Arrhenius plots of adenylate cyclase activity over the range 15°–40°. Each point is the mean of triplicate determinations. (A) Ethanolamine-supplemented membranes; (B) choline (control) membranes. Although drawn as straight lines with defined breaks, data from control cells may also be fit by a smooth curve.

DISCUSSION

A major goal of research on membrane-bound enzymes is to define the role that the lipid phase of the membrane plays in modulating enzymatic activity. One approach to this problem is to modify the lipid composition *in vivo*. The difficulties involved in lipid reconstitution experiments are avoided by the presence of the enzyme in a biosynthesized membrane structure. Adenylate cyclase is particularly interesting because hormone stimulation appears to be a transmembrane phenomenon. The catalytic site on the enzyme, which is responsible for the formation of cyclic AMP from ATP, is thought to face the inside of the intact cell, while the receptor site, which interacts with specific circulating hormones, faces the external medium. This study was prompted by the possibility that the membrane lipid phase might exert some control over the activity and hormone stimulation of adenylate cyclase.

Modifications of membrane fatty acid or polar head group composition affected the basal activity of the enzyme, as well as its response to fluoride and PGE_1. The differences between the basal activities of different supplemented membranes were abolished upon solubilization of the enzyme. This indicates that the differences seen in supplemented membranes were a consequence of changes in the lipid/protein interactions, which are disrupted by the detergent, rather than changes in the amount of the enzyme. These data support the hypothesis that adenylate cyclase is an integral membrane protein that can exist in two or more conformations having different catalytic activity depending upon the lipid environment.

Arrhenius plots of adenylate cyclase activity have been reported previously from rat liver (45) and Ehrlich ascites tumor cells (46). Although there is no reason to expect the enzyme from different tissues to behave similarly, the discontinuities in the Arrhenius plots for these enzymes corresponded closely to those which we observed in control membranes. In contrast to the results obtained by Wisnieski *et al.* (47) using nitroxide spin labels, our results with diphenylhexatriene showed no well-defined phase transition of the membrane phase (J. D. Esko, J. R. Gilmore, and M. Glaser, in preparation).

Although the activity in the presence of PGE_1 was increased by supplementation, the extent of hormone stimulation was remarkably constant except in the presence of 18:2. The observation that 18:2 increased PGE_1 stimulation to 6-fold in the presence of choline and decreased it to 3.1-fold in the presence of ethanolamine suggests that both polar head groups and fatty acids can affect the conformation of the enzyme and its response to PGE_1. This is different from the behavior of the enzyme in the presence of fluoride ion, where fatty acid supplementation increased this activity and polar head group supplementation had no effect. This provides evidence for a role of membrane lipids in determining enzyme conformation, and also indicates that the states induced by fatty acids and polar head groups are qualitatively distinct.

Specific models have been proposed in which diffusion of subunits may be rate limiting and which therefore would lead to a direct dependence of hormone stimulation on membrane viscosity (30). Although no simple correlation was observed between viscosity and specific activity or hormone stimulation, our data cannot rigorously exclude these models, since measurements of specific activity were fixed time determinations and measured hormone stimulation after a steady state had been reached.

In summary, we have demonstrated that the incorporation of fatty acids or polar head groups into plasma membranes has effects on the activity, hormone stimulation, and temperature dependence of adenylate cyclase. The LM cell system provides a unique approach to investigation of the effect of lipids on structure-function relationships in animal cell membranes.

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