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Effect of Membrane Phospholipid Compositional Changes on Adenylate Cyclase in LM Cells†

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ABSTRACT: Adenylate cyclase activities were examined in mouse LM cell membranes which had been supplemented with polar head groups and/or fatty acids. Basal, fluoride-, and PGE_1-stimulated activities varied systematically with changes in phospholipid composition, and PGE_1-stimulated activities correlated with the average degree of unsaturation of the phospholipid fatty acids or with the primary amino group character of the phospolipid polar head groups. In addition, the K_m for ATP of basal adenylate cyclase was systematically changed by both polar head group and fatty acid supplementation. Alteration of the membrane lipid composition also changed the temperature dependence of the enzyme and the lag time between addition of PGE_1 and the onset of a change in catalytic rate. However, none of the alterations in the enzyme activity could be correlated with the viscosities of supplemented membranes and, instead, seemed to be characteristic for a specific polar head group or fatty acid composition. The data suggest a specific interaction of the enzyme with phospholipids and indicate that structural features of phospholipids may play a role in regulating adenylate cyclase activity. It is proposed that adenylate cyclase can exist in several different conformations in the membrane depending upon the phospholipid composition.

Adenylate cyclase is associated with the plasma membranes of a number of animal cells (Butcher et al., 1965; Pohl et al., 1971a; Bilezikian & Aurbach, 1974; Engelhard et al., 1976a) and has also been reported to be associated with particulate fractions of many other cell types. Several studies have implicated the importance of membrane lipids for adenylate cyclase activity and hormone stimulation. For example, membranes have been treated with nonionic detergents (Sutherland et al., 1962), digitonin (Pohl et al., 1971b), phospholipases

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(Pohl et al., 1971b; Rubalcava & Rodbell, 1973), and organic solvents (Rethy et al., 1971). These treatments all resulted in changes in basal activity, hormone stimulation, or both. In several studies, readdition of phospholipids to treated membranes has resulted in restoration of basal activity or hormone stimulation to an extent that was dependent upon phospholipid polar head group compositions (Pohl et al., 1971b; Levey, 1971; Rethy et al., 1972). However, the enzyme from different tissues or treated with different agents exhibited varying requirements for phospholipids. This suggested that loss and restoration of activity might result from actions of these phospholipids other than specific interactions with adenylate cyclase. There are also uncertainties regarding the physical state of the added phospholipids and of the enzyme both before and after readdition of phospolipids.

Several other experimental approaches have also been employed to evaluate the importance of membrane lipids for adenylate cyclase activities. For example, Puchwein et al. reported the reversible inhibition of catecholamine stimulation of adenylate cyclase using filipin, which complexes cholesterol (Puchwein et al., 1974). Orly & Schramm have demonstrated that the addition of free unsaturated fatty acids to erythrocyte membranes enhanced isoproterenol stimulation of adenylate cyclase up to 25-fold (Orly & Schramm, 1975). In addition, Houslay et al. fused phospholipid vesicles with membranes and observed changes in both the temperature dependence and activity of adenylate cyclase (Houslay et al., 1976).

A method which avoids some of the difficulties associated with reconstitution studies is the in vivo modification of the membrane lipid composition. Brivio-Haugland et al. observed significant changes in basal and hormone-stimulated adenylate cyclase when rats were fed an essential fatty acid deficient diet (Brivio-Haugland et al., 1976). Techniques have now been developed for the in vivo modification of the fatty acid or polar head group composition of mouse LM cells in tissue culture (Glaser et al., 1974; Williams et al., 1974; Blank et al., 1975; Ferguson et al., 1975; Schroeder et al., 1976). We recently reported the modification of adenylate cyclase activity in LM cells as a result of the manipulation of the polar head group and fatty acid compositions (Engelhard et al., 1976b). In this paper, the influence of membrane lipid compositional changes on adenylate cyclase and its response to PGE_1 are extensively characterized.

Materials and Methods

Materials. All chemicals were reagent grade. Organic solvents were redistilled. Prostaglandin E_1 (PGE_1)[1] was kindly supplied by Dr. John Pike, Upjohn Co.

Growth and Supplementation of Cells. Mouse LM cells were grown in suspension culture in Higuchi's medium (Higuchi, 1970) containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4, 1 g/L methylcellulose, and 0.02 g/L sodium dextran sulfate. Growth and supplementation were carried out as previously described (Glaser et al., 1974; Engelhard et al., 1976b). For polar head group supplementation, cells were centrifuged and resuspended in medium containing 40 μg/mL of the appropriate polar head group in place of choline and were grown for 3 days (2 days for 2-amino-1-butanol) prior to harvest. Fatty acid supplementation was carried out by addition of the fatty acid complexed to bovine serum albumin to the growth medium. The fatty acid supplement was added at the indicated concentration 18 h before harvest.

[1] Abbreviations used: PGE_1, prostaglandin E_1; $NaDodSO_4$, sodium dodecyl sulfate.

Lipid Determinations. Lipids were extracted by the method of Bligh and Dyer as described by Ames (Ames, 1968). Phospholipids were separated by two-dimensional thin-layer chromatography as previously described (Glaser et al., 1974). 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) 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). The $CHCl_3$ phase was used for total phosphate analysis according to the method of Ames (Ames, 1966). 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. Methyl esters were prepared in sodium methoxide–methanol (Applied Science) or concentrated HCl–methanol (1:20) and chromatographed on a 15% SP-2340, Chromosorb PAWDMCS, 100–200 mesh column at 200 °C (Supelco). This column resolved cis and trans fatty acid isomers.

Preparation of Plasma Membranes. Plasma membranes were prepared by a method previously described (Esko et al., 1977). Examination of the adenylate cyclase activities in the different fractions showed a similar purification to the Na^+, K^+-ATPase and indicated the adenylate cyclase was localized in the plasma membrane of the cells (data not shown). The 48 000 × *g* membrane pellet was routinely used to assay adenylate cyclase in cells with different lipid compositions.

Adenylate Cyclase Assay. Adenylate cyclase was assayed according to the method of Salomon et al. in 50 μL containing 1 mM α-[^{32}P]ATP (400 cpm/pmol), 2 mM cAMP, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM β-mercaptoethanol, 5 mM theophylline, 1.1% bovine serum albumin, and 20 mM K_2HPO_4, pH 7.5 (Salomon et al., 1974). 1 mg/mL creatine kinase and 20 mM phosphocreatine were present as an ATP regenerating system. ATP used in assays was purified by DEAE-Sephadex A-25 chromatography followed by Dowex AG-50 chromatography. Each sample contained 90–110 μg of membrane protein. Samples were incubated at 30 °C for 10 min. In some experiments, the concentration of ATP was less than 1 mM. In these cases, the cAMP concentration was maintained at 200% of the ATP concentration, and the incubation time was reduced to 2 min. Concentrations for fluoride and PGE_1 were 15 mM and 4 μM unless otherwise specified. Each data point is the mean of triplicate determinations with a standard deviation of less than 5%. For the kinetic measurements, a 1-mL incubation volume was used. Membranes were preequilibrated for 1 min at assay temperature in the presence of the appropriate hormone solution, and the reaction was initiated by the addition of substrate. The incubation mixture was stirred in a thermostated water bath, and 50-μL aliquots were withdrawn at appropriate intervals and pipetted into 100 μL of stopping solution. These samples were then processed in the normal fashion.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Slab gels (7.5%) were prepared by the method of Laemmli (1970). After staining for 2–3 h in 0.1% Coomassie Blue, 12.5% trichloroacetic acid, 50% methanol, they were destained overnight in 10% acetic acid.

Protein. Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Results

Fatty Acid Supplementation of LM Cells. The fatty acid compositions of membrane phospholipids from cells supplemented with several unsaturated fatty acids are reported in Table I. For all cis fatty acids used as supplements, the total percentage of unsaturated fatty acids remained the same.

TABLE I: Fatty Acid Composition of Membrane Phospholipids from LM Cells Supplemented with Fatty Acids and Ethanolamine.[a]

supplement	fatty acid (percent by weight) 14:0	16:0	16:1	18:0	18:1	20:1	18:2	18:3	20:4	18:1 (*t*)	16:1 (*t*)	total unsaturated	double bonds/ 100 fatty acids
choline (control)	1.3	21.8	8.9	10.3	56.0	1.6						66.5	66.5
20 μg/mL 18:2	1.2	20.5	5.8	12.8	42.0	1.4	16.2					65.4	81.6
40 μg/mL 18:2	0.9	18.4	5.6	13.1	31.0	3.3	27.6					67.5	95.1
20 μg/mL 18:3[b]	1.6	20.0	8.6	12.8	35.9			21.1				65.6	107.8
20 μg/mL 20:4[b]	1.3	19.8	7.5	12.1	40.2	1.5			17.5			66.7	119.2
40 μg/mL 18:1 (trans)	0.8	6.7	5.2	4.9	35.8					44.3	2.3	87.6	87.6
ethanolamine	1.2	18.3	12.2	14.7	48.3	5.3						65.8	65.8
ethanolamine + 40 μg/mL 18:2	0.8	19.3	6.3	14.6	34.9	0.9	23.1					65.2	88.3
ethanolamine + 40 μg/mL 18:1 (trans)	0.6	10.7	6.6	7.6	37.7					31.4	0.6	76.3	76.3

[a] 200 mL cells were grown for 2 days in medium containing 40 μg/mL of either choline or ethanolamine and were then supplemented with fatty acids at the indicated concentrations. After 18 h cells were harvested and lysed. The 48 000*g* for 1 h pellet was resuspended in 1 mM Tris-HCl, 7.5, and analyzed for fatty acids as described in Materials and Methods. Fatty acids of chain length greater than 18 represented less than 8% of the total and were not included in this analysis except for 20:1 and on supplementation with 20:4. [b] A level of 40 μg/mL of these fatty acids was toxic.

TABLE II: Polar Head Group Composition of Membrane Phospholipids from LM Cells Supplemented with Polar Head Group Analogues.[a]

supplement	phospholipid composition[b] (mole %) PC	PE	PI + PS	SPH	PDMEA	PMEA	PBA	PPA
choline	46.1	30.5	9.9	13.6				
N,N-dimethylethanolamine	18.9	20.8	8.8	11.6	40.0			
N-methylethanolamine	15.8	17.2	11.3	12.7	5.3	37.7		
ethanolamine	25.9	47.9	13.8	12.3				
3-aminopropanol	25.0	16.8	9.3	11.5				37.4
l-2-amino-1-butanol	30.2	17.6	8.3	15.4			28.5	

[a] One hundred milliliters of cells was grown for 3 days in medium containing 40 μg/mL of the indicated supplement in place of choline, except for *l*-2-amino-1-butanol cells, which were grown for 2 days. Cells were harvested and lysed, and the 48 000*g* pellet was used for phospholipid analysis as described in Materials and Methods. Several minor phospholipids (<5%) were not present in sufficient amounts for accurate determination and were not included in this analysis. [b] PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; PDMEA, phosphatidyldimethylethanolamine; PMEA, phosphatidylmethylethanolamine; PBA, phosphatidylbutanolamine; PPA, phosphatidylpropanolamine.

However, since the added fatty acid primarily replaced 16:1 and 18:1, the number of double bonds per 100 fatty acids increased with the degree of unsaturation of the added fatty acid. In contrast, the incorporation of 18:1 (trans) resulted in an increase in the total percentage of unsaturated fatty acids from 66.5% to 87.6% and almost two-thirds of the saturated fatty acids were replaced by 18:1 (trans). Fatty acid supplementation was also carried out in cells which had been grown in the presence of ethanolamine rather than choline, in which case the polar head group composition of plasma membrane phospholipids was predominantly phosphatidylethanolamine rather than phosphatidylcholine. The total phospholipids from ethanolamine-supplemented cells had a slightly different fatty acid composition from those of the choline-supplemented cells. There were lower levels of 16:0 and 18:1 and higher levels of 16:1 and 18:0.

Polar Head Group Supplementation of LM Cells. The polar head group compositions of membranes prepared from supplemented LM cells are reported in Table II. Incorporation of analogues other than choline and ethanolamine was accompanied by a decrease in the levels of phosphatidylcholine and phosphatidylethanolamine, but no significant change in the levels of sphingomyelin or phosphatidylinositol plus phosphatidylserine. Approximately the same level (37–40%) of incorporation was achieved with *N,N*-dimethylethanolamine, *N*-methylethanolamine, and 3-aminopropanol. *l*-2-Amino-1-butanol was incorporated to only 27%. *N,N*-Dimethylethanolamine and *N*-methylethanolamine appeared to substitute preferentially for choline since the levels of phosphatidylcholine were 41% and 34% of the control levels, respectively, while phosphatidylethanolamine levels were 68% and 56% of the control levels, respectively. In contrast, 3-aminopropanol subsituted equally well for choline or ethanolamine, both levels being reduced to 55% of the control. *l*-2-Amino-1-butanol appeared to substitute more effectively for ethanolamine. The fatty acid composition of membranes supplemented with the various polar head groups described above did not vary significantly and the total percentage of unsaturated fatty acids remained constant.

NaDodSO$_4$-Polyacrylamide Gel Electrophoresis of Supplemented Membranes. It was important to show that no changes in the membrane protein composition occurred as a consequence of lipid supplementation. NaDodSO$_4$-polyacrylamide gel profiles of membranes from supplemented cells are shown in Figure 1. No changes in profiles as a consequence of either fatty acid and/or polar head group analogue incorporation were apparent. It is unlikely, however, that adenylate cyclase would be detectable on these gels.

Alterations in Adenylate Cyclase Activity in Fatty Acid Supplemented Cells. The influence of unsaturated fatty acid supplements on adenylate cyclase activity is summarized in Table III. All unsaturated fatty acid supplements produced

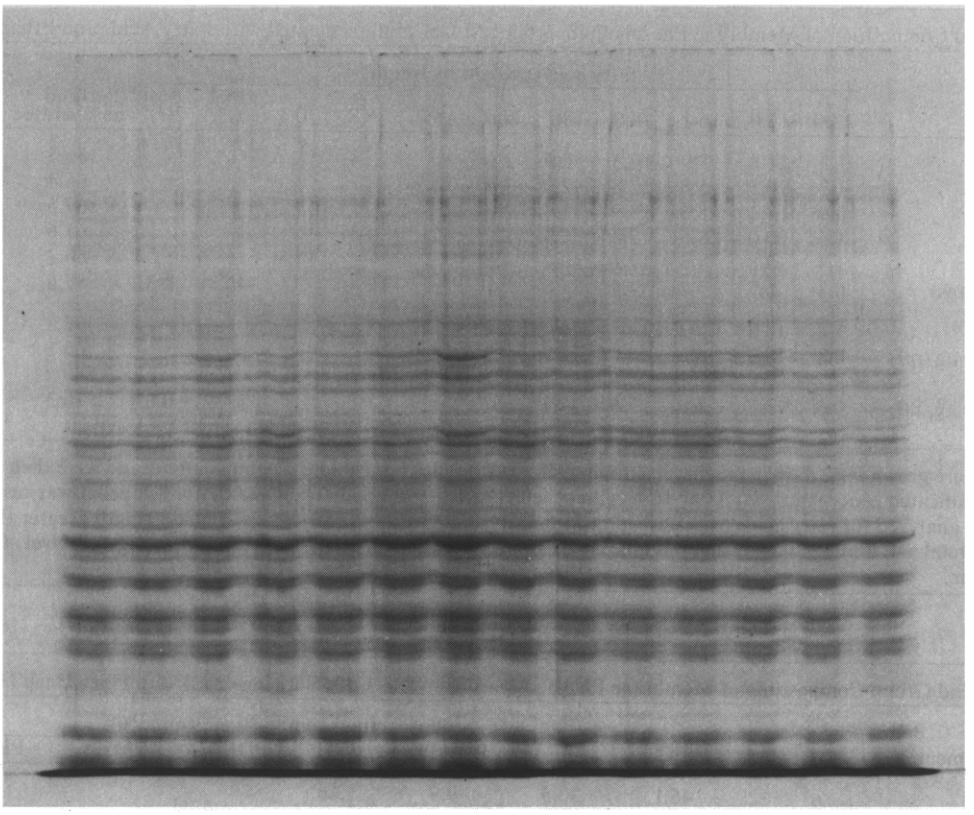

FIGURE 1: $NaDodSO_4$–polyacrylamide gel of membranes from cells given different fatty acid and/or polar head group supplements. Slab gels (7.5%) containing 25 μg of membrane protein per lane. From left to right: choline, choline + elaidate, choline + linoleate, ethanolamine, ethanolamine + elaidate, choline, *N,N*-dimethylethanolamine, *N*-methylethanolamine, ethanolamine, *l*-2-amino-1-butanol, 3-aminopropanol, choline, choline, and choline.

TABLE III: Comparison of Adenylate Cyclase Activities in Membranes from Cells Supplemented with Fatty Acids.[a]

supplement	adenylate cyclase activity (pmol of cAMP/(10 min mg))			
	basal	15 mM NaF	14 μM PGE_1	14 μM PGE_1 + 0.5 mM GTP
choline	12.3	153 (12.5×)	29.2 (2.4×)	338 (27.5×)
choline + 18:1 (trans)	18.8	206 (11.0×)	44.2 (2.4×)	361 (19.2×)
choline + 18:2	30.5	259 (8.5×)	102 (3.3×)	619 (20.3×)
choline + 18:3	28.6	259 (9.1×)	128 (4.5×)	672 (23.5×)
choline + 20:4	29.4	274 (9.3×)	154 (5.2×)	712 (24.2×)
ethanolamine	31.3	166 (5.3×)	72.0 (2.3×)	516 (16.5×)
ethanolamine + 18:1 (trans)	38.0	220 (5.8×)	76.3 (2.0×)	699 (18.4×)
ethanolamine + 18:2	52.4	241 (4.6×)	86.7 (1.7×)	634 (12.1×)

[a] Cells were grown in the presence of 40 μg/mL of either choline or ethanolamine for 3 days and 40 μg/mL of 18:1 (trans) or 18:2 or 20 μg/mL 18:3 or 20:4 added 18 h prior to harvest. Cell harvest and membrane (48 000*g* pellet) preparation were as described in Materials and Methods. Adenylate cyclase was assayed as described in Materials and Methods. Numbers in parentheses refer to degree of stimulation over basal activity. The fatty acid and phospholipid compositions of membranes obtained from lipid supplemented cells are reported in Tables I and II.

increases in basal, fluoride-, and PGE_1-stimulated activities, whether choline or ethanolamine was present as a polar head group supplement. In the presence of choline, incorporation of 18:1 (trans) was accompanied by increases of 53% and 52% in basal and PGE_1-stimulated activities. Because of the parallel changes, the degree of stimulation by PGE_1 remained unchanged at 2.4-fold. Activity in the presence of fluoride, in contrast, increased only 35%. Consequently, the degree of stimulation by fluoride dropped from 12.4- to 11-fold.

When any of the cis unsaturated fatty acids were incorporated into choline-supplemented cells, an increase in basal activity to about the same level of 29 to 30 pmol of cAMP per 10 min per mg of protein was observed. This represented a 140% increase in basal activity over that of the control cells. As with 18:1 (trans) supplemented cells, the change in fluoride-stimulated activity was smaller than the change in basal activity, resulting in a drop in the degree of fluoride stimulation from 12.4- to 9-fold.

In contrast to the relatively equal basal or fluoride-stimulated activities expressed by all cis fatty acid-supplemented cells, PGE_1-stimulated activity increased steadily with the degree of unsaturation of the incorporated supplement (Figure 2). The degree of unsaturation of the added fatty acid correlated with an increase in the number of double bonds per 100 fatty acids of the membrane phospholipids, while leaving the total percentage of unsaturated fatty acids unchanged. There was a strong correlation between PGE_1-stimulated activity and the number of double bonds per 100 fatty acids. It is also no-

TABLE IV: Comparison of Adenylate Cyclase Activities in Membranes from Cells Supplemented with Polar Head Group Analogues.[a]

supplement	adenylate cyclase activity (pmol of cAMP/(10 min mg))			
	basal	15 mM NaF	14 μM PGE_1	14 μM PGE_1 + 0.5 mM GTP
choline	12.3	153 (12.4×)	29.2 (2.4×)	338 (27.5×)
dimethylethanolamine	16.7	154 (9.2×)	39.1 (2.3×)	437 (26.2×)
methylethanolamine	17.2	139 (8.1×)	56.1 (3.3×)	352 (20.4×)
ethanolamine	31.3	166 (5.3×)	72.0 (2.3×)	516 (16.5×)
3-aminopropanol	28.4	185 (6.5×)	72.9 (2.6×)	485 (17.1×)
l-2-amino-1-butanol	51.3	224 (4.4×)	81.8 (1.6×)	687 (13.4×)

[a] Cells were grown in the presence of 40 μg/mL of the appropriate polar head group for 3 days (except aminobutanol for 2 days) prior to harvest. Cell harvest, membrane (48 000*g* pellet) preparation and adenylate cyclase assay were as described in Materials and Methods. Numbers in parentheses refer to degree of stimulation over basal activity. The polar head group compositions of supplemented membranes are reported in Table II.

table that the activity of 18:1 (trans) supplemented cells did not fall on this line whether the number of double bonds was calculated on the basis of cis plus trans double bonds or only cis double bonds. Although (PGE_1 + GTP)-stimulated activities also increased with the number of double bonds per 100 fatty acids, the increase was less pronounced. It is not clear why PGE_1 and (PGE_1 + GTP)-stimulated activities differed in their sensitivity to fatty acid supplementation. However, this may suggest that the GTP concentration dependence was affected by changes in the fatty acid composition.

Fatty acid supplementation of ethanolamine-supplemented cells also showed several interesting trends (Table III). As in choline-supplemented cells, addition of either 18:2 or 18:1 (trans) led to an increase in basal activity. The increases in activity were a smaller percentage of the control (ethanolamine-supplemented) activity than in the corresponding choline-supplemented cells. 18:1 (trans) supplementation gave only a 21% increase in basal activity, and 18:2 supplementation caused only a 67% increase. However, the differences in activity were quite similar: 18:1 (trans) increased basal activity by 6.5 and 6.7 pmol of cAMP/(10 min mg) in choline- and ethanolamine-supplemented cells, respectively; 18:2 increased basal activity 18.1 and 21.1 pmol of cAMP/(10 min mg) in choline- and ethanolamine-supplemented cells, respectively. Also, the increases in fluoride-stimulated activities were similar, regardless of polar head group composition. Because of this and because ethanolamine by itself gave no increase in fluoride-stimulated activities, the degree of fluoride stimulation was lower in cells supplemented with fatty acids + ethanolamine than in the corresponding choline-supplemented cells.

PGE_1-stimulated activities increased only slightly in cells supplemented with ethanolamine and fatty acids, 6% in 18:1 (trans) cells and 20% in 18:2-supplemented cells. Because these increases are low compared with the increases in basal activity, the degree of PGE_1 stimulation dropped from 2.3- to 1.7-fold. In contrast to basal and fluoride-stimulated activities, the differences between PGE_1-stimulated activities in fatty acid supplemented and control cells were not independent of polar head group. While the activity of ethanolamine + 18:2-supplemented cells increased 14.7 pmol of cAMP/(10 min mg), that of choline + 18:2-supplemented cells increased 72.8 pmol of cAMP/(10 min mg). A similar effect was observed with 18:1 (trans) supplemented cells.

Alterations in Adenylate Cyclase Activity in Polar Head Group Supplemented Membranes. The changes in adenylate cyclase activity which occurred upon polar head group supplementation are reported in Table IV. The series choline, *N,N*-dimethylethanolamine, *N*-methylethanolamine, to ethanolamine shows the effect of successive removal of methyl groups from the choline nitrogen atom on adenylate cyclase activity. Removal of the first methyl group led to a 36% increase in basal and a 35% increase in PGE_1-stimulated activities. Thus, no change in the degree of hormone stimulation was observed. Removal of the second methyl group resulted in no change in basal activity, but did cause an increase in PGE_1-stimulated activity, such that an increase in hormone stimulation from 2.3- to 3.3-fold was observed. Removal of the last methyl group resulted in a doubling of basal activity with only a small increase in PGE_1-stimulated activity. Consequently, the degree of hormone stimulation dropped back to the control level. Stimulation by PGE_1 + GTP systematically decreased from 27.5- to 16.5-fold with successive removal of methyl groups from choline. Fluoride-stimulated activity was relatively unaffected by changes in polar head group composition, and, as a result, the degree of fluoride stimulation dropped from 12.4-fold in choline cells to 5.3-fold in ethanolamine-supplemented cells.

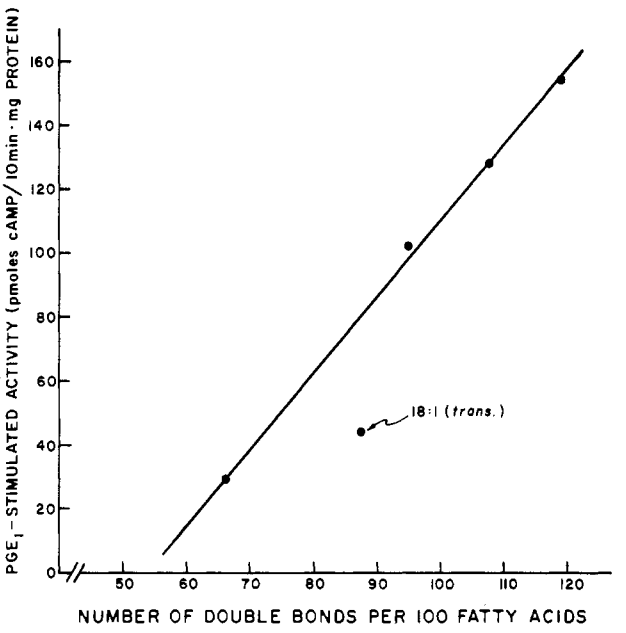

FIGURE 2: Correlation between PGE_1-stimulated activity and number of double bonds/100 fatty acids. Data were taken from Tables I and III. The line was obtained by a linear least-squares fit.

The second series of polar head group analogues included ethanolamine, 3-aminopropanol, and *l*-2-amino-1-butanol, which all contain primary amino groups but modified alkyl chains. 3-Aminopropanol-supplemented cells were very similar

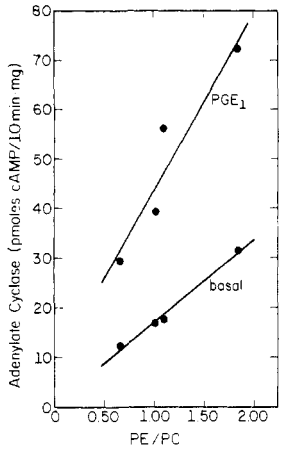

FIGURE 3: Correlation between adenylate cyclase activity and the ratio of phosphatidylethanolamine to phosphatidylcholine. Data were taken from Tables II and IV. Lines were obtained by linear least-squares fit.

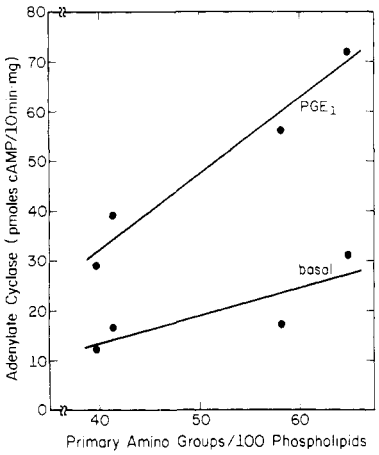

FIGURE 4: Correlation between adenylate cyclase activity and primary amino group character of membrane phospholipids. Data were taken from Tables II and IV. Lines were obtained by linear least-squares fit.

to those given ethanolamine, showing similar increases in all adenylate cyclase activities. However, *l*-2-amino-1-butanol-supplemented cells were strikingly different. Basal activity increased 4-fold over the control value. Fluoride-, PGE_1-, and (PGE_1 + GTP)-stimulated activities were also higher than the corresponding values for ethanolamine or 3-aminopropanol-supplemented cells, but, due to the larger increase in basal activity, the degree of stimulation by all effectors dropped to low levels.

Attempts were made to correlate these activity changes with simple compositional parameters. 3-Aminopropanol- and *l*-2-amino-1-butanol-supplemented cells were not included in this analysis because of the variation introduced by modification of the alkyl chains. Instead, an attempt was made to define the degree of "choline-like" or "ethanolamine-like" properties present in the dimethyl- and monoethylethanolamine analogues, and to what extent primary or quaternary ammonium groups influenced activity. The increases in basal or PGE_1-stimulated activity correlated poorly with the percentage of phosphatidylethanolamine in the membranes (correlation coefficients of 0.78 and 0.55, respectively). However, a stronger correlation was observed between basal or PGE_1-stimulated activities and the ratio of phosphatidylethanolamine to phosphatidylcholine (Figure 3). The correlation coefficients between this ratio and basal or PGE_1 activities were 0.994 and 0.943, respectively. PGE_1-stimulated

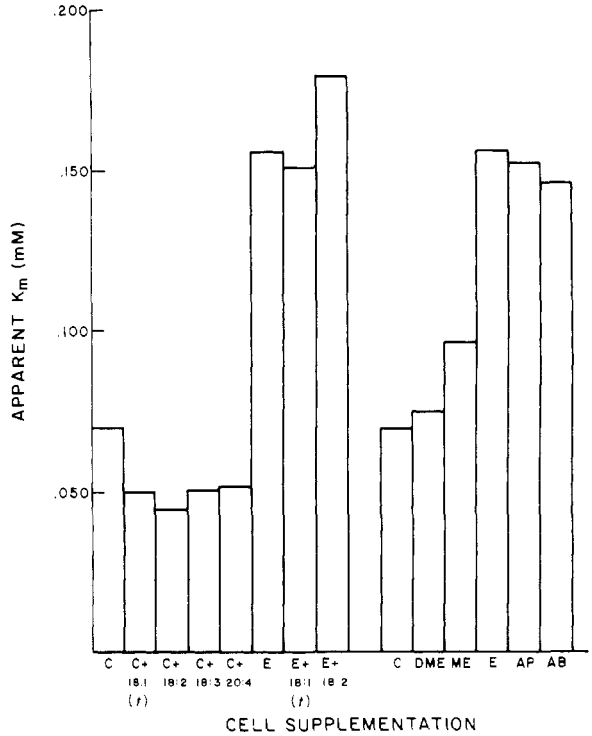

FIGURE 5: K_m for ATP of basal adenylate cyclase activity from supplemented LM cells. K_ms were determined from linear Eadie-Hofstee plots of the kinetic data. The slopes and intercepts of the lines were computed by linear regression analysis, and the coefficients of determination (r) were in all cases greater than 0.98. C, choline; E, ethanolamine; DME, dimethylethanolamine; AP, 3-aminopropanol; AB, *l*-2-amino-1-butanol.

activity showed the highest correlation with the relative primary amino group character of the membrane phospholipids, calculated by arbitrarily assigning ethanolamine a value of 1, *N*-methylethanolamine = $^2/_3$, *N,N*-dimethylethanolamine = $^1/_3$, and choline = 0, thereby weighting the percentage composition (Figure 4). The correlation coefficients between this parameter and basal or PGE_1-stimulated activities were 0.987 and 0.976, respectively.

Alterations in Apparent K_m for ATP of Adenylate Cyclase in Lipid-Supplemented Membranes. Since there were substantial effects on the specific activity of adenylate cyclase in fatty acid and polar head group supplemented cells, the effect of supplementation on the apparent K_m for ATP of the enzyme was examined. The kinetic data were analyzed by Eadie-Hofstee plots and no deviations from linearity indicative of cooperativity or site heterogeneity were observed over two orders of magnitude in ATP concentration (0.02-2 mM). The K_m data obtained for all supplemented cell types are reported in Figure 5.

Supplementation with unsaturated fatty acids in the presence of choline led to a uniform reduction in the apparent K_m from 70 μM to about 50 μM. The constancy of the K_m in the presence of cis or trans unsaturated fatty acids is striking. In contrast, incorporation of fatty acids into ethanolamine-supplemented cells had no significant effect on the K_m.

In contrast to fatty acid supplemented cells, supplementation with polar head group analogues led to a progressive increase in the apparent K_m. While *N,N*-dimethylethanolamine-supplemented cells had the same K_m as choline-supplemented cells, *N*-methylethanolamine supplementation resulted in a 37% increase in the K_m. The K_m of ethanolamine-supplemented cells was over twice as high as the control value. In addition, 3-aminopropanol- and *l*-2-amino-1-butanol-supplemented membranes exhibited K_ms equal to the ethanol-

TABLE V: Activation Energies of Adenylate Cyclase and Membrane Microviscosity from Supplemented LM Cells.[a]

supplement	E_a (kcal/mol) basal	PGE_1	$\bar{\eta}$
choline (control)	8.0, 13.1	8.4, 12.6	6.7
choline + 18:2	17.1	21.9	6.7
choline + 18:1 (trans)	20.3	22.6	8.9
ethanoline	12.7	13.5	8.4
ethanolamine + 18:2	16.3	12.6	7.7
ethanolamine + 18:1 (trans)	13.8	13.5	8.7

[a] Activation energies were computed from linear least-squares analysis of data plotted according to ln (specific activity or viscosity) $= -(E_a/RT) +$ constant. In each case all possible two-line fits of the data were compared with the single-line fit of the data by linear regression analysis. Coefficients of determination were greater than 0.95. The two numbers in each column for control cells give activation energies below and above the break temperatures, respectively. The activation energy for the membrane microviscosity, $\bar{\eta}$, was calculated from measurements of the microviscosity using the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene as determined previously (Engelhard et al., 1976b).

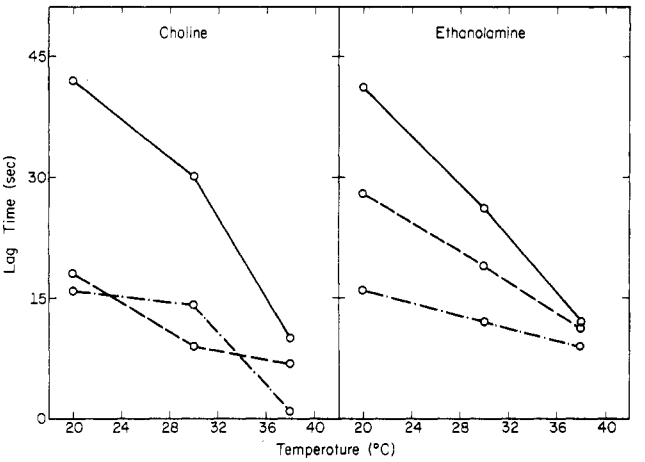

FIGURE 6: Hormone lag time values as a function of temperature and cell supplement. Hormone lag times were determined as described in Methods. (O—O) No fatty acid supplement; (O- - -O) 18:1 (trans) supplemented; (O···O) 18:2 supplemented.

amine value, consistent with the fact that the primary amino group concentration of the membrane phospholipids was comparable in all three membranes. A similar kinetic analysis was made for PGE_1- and (PGE_1 + GTP)-stimulated adenylate cyclase activities. These K_m values did not vary significantly with lipid supplementation and there were no obvious correlations between the observed K_m values and membrane compositional parameters.

Temperature Dependence of Adenylate Cyclase Activity. It has been reported that there were discontinuities in Arrhenius plots of adenylate cyclase activities in choline-supplemented membranes which were abolished by fatty acid or polar head group supplementation (Engelhard et al., 1976b). In addition, no discontinuities were observed in the Arrhenius plots of the microviscosity of LM cell membranes measured by 1,6-diphenyl-1,3,5-hexatriene fluorescence polarization (Esko et al., 1977). If lateral diffusion is the rate-limiting step in hormone stimulation, it is possible that the temperature dependence of the membrane viscosity would correlate with the temperature dependence of the enzyme.

Table V summarizes the activation energies of adenylate cyclase in several supplemented membranes. (Choline + fatty acid)-supplemented membranes showed much higher activation energies for both basal and PGE_1-stimulated activities than the corresponding control membranes. The activation energy of PGE_1-stimulated activity was significantly higher than that of basal activity for choline + 18:2-supplemented cells, which indicates that the degree of PGE_1 stimulation increased with temperature. Ethanolamine-supplemented cells had an activation energy for both basal and PGE_1-stimulated activity which was comparable to that seen in choline-supplemented cells above 32 °C. This activation energy was not significantly changed by the addition of 18:1 (trans). In addition, no change in the activation energy of PGE_1-stimulated activity in ethanolamine + 18:2 supplemented cells was observed, while that of basal activity increased markedly. As a result, the degree of PGE_1 stimulation in these membranes decreased with increasing temperature. It may be significant that differences between basal and PGE_1-stimulated activation energies occurred only in 18:2-supplemented membranes, and that these were consistent with the changes in the degree of PGE_1 stimulation observed in these membranes. Thus, an increase in hormone stimulation from 2.3- to 3.3-fold in choline- and (choline + 18:2)supplemented cells correlated with ΔE_a [E_a(PGE_1-stimulated) − E_a(basal)] of +4.8 kcal/mol, while a decrease in hormone stimulation from 2.3 to 1.7 in ethanolamine- and (ethanolamine + 18:2)-supplemented cells correlated with ΔE_a of −3.7 kcal/mol.

A comparison between the activation energies for adenylate cyclase activities and membrane microviscosities measured by 1,6-diphenyl-1,3,5-hexatriene fluorescence polarization revealed no correlation between these two parameters. Although increases in enzyme activation energy, as a result of the incorporation of 18:2 and 18:1 (trans), occurred in choline-supplemented membranes, only (choline + 18:1 (trans))-supplemented cells showed a change in membrane microviscosity (Engelhard et al., 1976b). Ethanolamine- or (ethanolamine + fatty acid)-supplemented membranes maintained an enzyme activation energy equal to that seen at high temperatures in control membranes, and these membranes had approximately equal microviscosities. One can also compare the activation energies for adenylate cyclase activity with those for membrane microviscosity measured by fluorescence polarization (Table V). However, again there was no simple correlation between these parameters.

Kinetics of Hormone Stimulation of Adenylate Cyclase. Several reports have appeared concerning the observation of a lag time between hormone addition and the onset of the hormone-stimulated rate increase (Bockaert et al., 1973; Nakahara & Birnbaumer, 1974). This phenomenon has been analyzed in some detail, and it has been proposed to reflect a slow conformational transition from a low activity state to a high activity state. However, the nature of these states is unknown. Cuatrecasas has suggested that the lag time might reflect the time required for separate hormone receptor and catalytic subunits to diffuse laterally in the plane of the membrane and interact subsequent to hormone binding (Cuatrecasas, 1974). If this model is correct, then changes in membrane viscosity might be expected to affect the lag time for hormone stimulation.

The lag times for PGE_1 stimulation of adenylate cyclase in choline-supplemented membranes were determined as a function of temperature. Hormone lag times at 20, 30, and 38 °C were 42, 30, and 10 s, respectively. A comparison of lag times obtained at three temperatures for six different cell types is summarized in Figure 6. The lag times were shorter in membranes supplemented with either 18:1 (trans) or 18:2 than

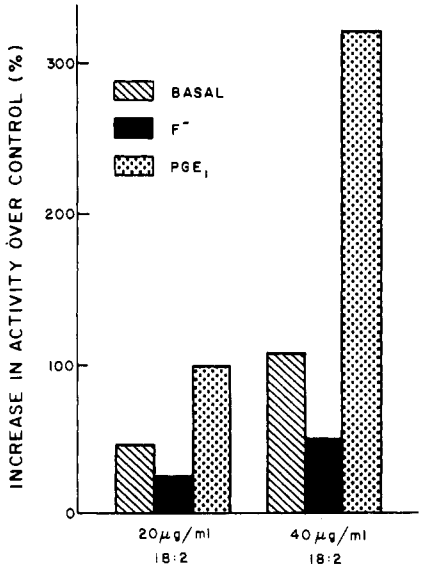

FIGURE 7: Relative sensitivity of different adenylate cyclase activities to fatty acid incorporation in cells given different concentrations of 18:2. Cells were supplemented with either 20 or 40 μg/mL of 18:2 for 18 h. Harvest, membrane preparation, and adenylate cyclase assays were carried out as outlined in Materials and Methods.

TABLE VI: Activation Energies for Lag Time Process upon Addition of PGE_1.[a]

supplement	E_a (kcal/mol)
choline	14.0
ethanolamine	12.1
C + 18:1 (trans)	9.6
E + 18:2	9.3
C + 18:2	*b*
E + 18:2	5.8

[a] Activation energies were calculated from the data of Figure 7 replotted as ln (lag time) vs. $1/T$. The slope of the line was computed by linear regression analysis, and the coefficients of determination (r) were always greater than 0.93. The slope of the line equals E_a/R. [b] Data did not fit a straight line.

in the corresponding unsupplemented membranes. However, changing the polar head group supplement from choline to ethanolamine had no effect on the lag time. A comparison of membranes supplemented with the same fatty acid but different polar head groups shows that 18:2-supplemented cells exhibited similar lag times at 20 and 30 °C, but the lag at 38 °C in choline + 18:2 cells was essentially nonexistent. In contrast, (choline + 18:1 (trans))-supplemented membranes exhibited lag times shorter than membranes supplemented with ethanolamine + 18:1. Finally, all of the hormone lag time differences noted became smaller at higher temperatures. Activation energies for hormone lag times process were calculated and are reported in Table VI. The values ranged from 14 kcal/mol in choline cells to 5.8 kcal/mol in ethanolamine + 18:2 cells. Activation energies were approximately equal for cells given different polar head group but the same fatty acid. Examination of this data shows that no correlation can be drawn between lag times and microviscosities measured by fluoresence polarization or between lag time activation energies and microviscosity activation energies. Thus, the hormone lag times do not appear to depend upon bulk membrane viscosity. Rather, they seem dependent only upon the fatty acid composition of the membrane.

Correlation between Changes in Adenylate Cyclase Activity and the Level of Incorporation of 18:2. It was of interest to determine how the amount of fatty acid incorporation correlated with changes in adenylate cyclase activity. For this purpose, 18:2 supplementation was examined in more detail. The increases in adenylate cyclase activities exhibited by cells supplemented with either 20 or 40 μg/mL of 18:2 were examined (Figure 7). Adenylate cyclase activities in membranes supplemented with 20 μg/mL were intermediate between those of control cells and those given 40 μg/mL. In addition, the degree of stimulation by fluoride or PGE_1 in 20 μg/mL supplemented membranes was also intermediate between those of the controls and those of the 40 μg/mL supplements. These data indicate that fluoride-stimulated activity was less responsive to 18:2 supplementation than basal activity. The increases in fluoride-stimulated activity were 26% and 49% of the control values in 20 and 40 μg/mL supplemented cells, respectively, while the corresponding changes in basal activity were 40% and 113%. It is also clear that PGE_1-stimulated activity was most responsive to increases in 18:2 levels, increasing by 96% and 319% in cells supplemented with 20 and 40 μg/mL, respectively.

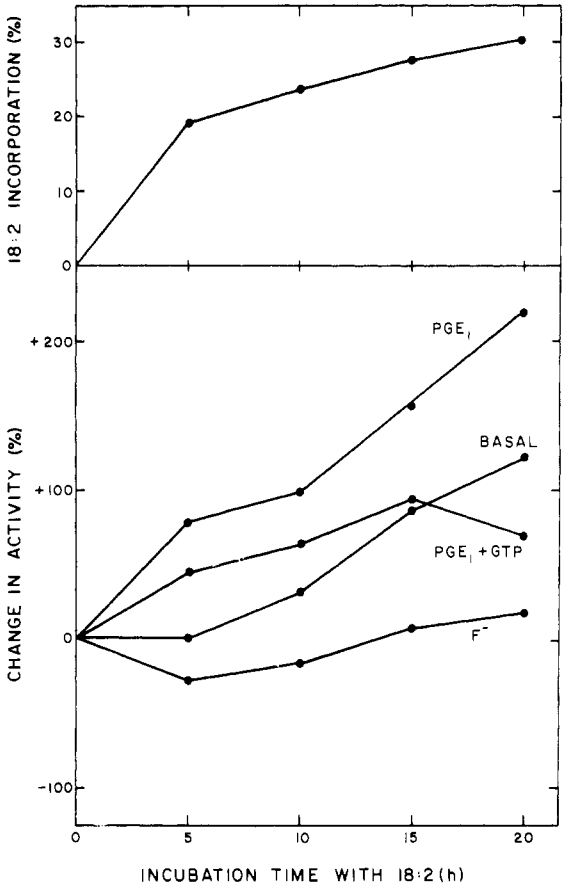

FIGURE 8: Time course of incorporation of 18:2 into membrane phospholipids and response of adenylate cyclase. Cells were given 40 μg/mL 18:2 and allowed to incubate for the indicated times. All cells were harvested simultaneously, membranes (48 000g pellet) were prepared, and adenylate cyclase was assayed according to procedures given in Materials and Methods. When present GTP was at a concentration of 0.5 mM.

The kinetics for incorporation of 18:2 into membrane fractions were compared with the changes in adenylate cyclase activities in order to further characterize the relationship between enzyme activity and phospholipid fatty acid composition (Figure 8). Two-thirds of the final level of 18:2 incorporation was achieved in the first 5 h of incubation, while the major portion of the adenylate cyclase activity changes occurred after

longer periods of time. Basal activity remained constant over the first 5-h interval, while PGE_1- and (PGE_1 + GTP)-stimulated activities expressed only 36 and 48% of their maximal activities, respectively. Fluoride-stimulated activity actually dropped to 73% of its initial value, and remained below the zero-time activity until 13 h of incubation with 18:2. The observation that changes in adenylate cyclase activities occurred slower than the rate of incorporation of 18:2 is quite interesting and may reflect either of two possibilities. This phenomenon could be due to a layer of lipid tightly bound to the enzyme which exchanged slowly with the newly synthesized phospholipids. Alternatively, adenylate cyclase is associated with the plasma membrane and there may be a measurable lag period for transport of newly synthesized phospholipids from the endoplasmic reticulum to the plasma membrane.

Discussion

The objectives of this study were to examine the influence of membrane phospholipid composition on adenylate cyclase activity and its response to hormones in intact membranes. A number of models have been proposed for hormonal stimulation of adenylate cyclase which involve either protein conformational changes or lateral diffusion of protein subunits within the membrane (Robison et al., 1967; Storm & Chase, 1975; Levey et al., 1974; Cuatrecasas, 1974). Therefore, it was important to determine if membrane viscosity was rate limiting and if the lipid composition modulates adenylate cyclase activity or hormone stimulation. The LM cell system was utilized in this study because significant changes in membrane phospholipid composition were obtainable in vivo, with intact membranes.

There are several plausible explanations for the variation of adenylate cyclase activities in response to membrane lipid compositional changes. It might be argued that the absolute amount of adenylate cyclase in the membranes varied as a function of lipid compositional changes. This possibility cannot be rigorously excluded since it is presently not possible to accurately quantitate the amount of adenylate cyclase present in membrane preparations. However, several observations suggest that the changes in adenylate cyclase activity seen with lipid supplemented membranes were not due to variations in the amount of the protein present. $NaDodSO_4$ gels of control and lipid supplemented membranes revealed approximately 30 to 40 polypeptide bands. The $NaDodSO_4$ gels for control and all lipid supplemented membranes were identical, indicating that neither the synthesis nor incorporation of proteins into membranes was affected by in vivo lipid supplementation. Furthermore, activity differences between various lipid-supplemented membranes were abolished by detergent solubilization (Engelhard et al., 1976b). The activation energy and K_m for adenylate cyclase were also significantly affected by lipid compositional changes. Neither of these parameters should vary as a function of the amount of adenylate cyclase present in the membrane.

It is also possible that lipid supplementation may have indirectly affected adenylate cyclase activities by altering the metabolic state of the cells. For example, phospholipid compositional changes may affect membrane bound or cytoplasmic activities other than adenylate cyclase. However, as mentioned above, detergent solubilization abolished differences in adenylate cyclase activity in a variety of lipid supplemented membranes. It is more likely that adenylate cyclase is sensitive to its membrane lipid environment and that the observed activity differences reflect different conformational states of the enzyme in the membrane.

Supplementation with cis unsaturated fatty acids enhanced basal adenylate cyclase activity to comparable levels in all supplemented membranes. An analogous effect was observed with fluoride-stimulated activity. However, PGE_1- and (PGE_1 + GTP)-stimulated activities were much more sensitive to the degree of unsaturation of membrane phospholipids. As a result, stimulation by PGE_1 was directly proportional to the number of fatty acid double bonds present in the membrane. In contrast to membranes containing phosphatidylcholine as the major phospholipid, PGE_1-stimulated activity was essentially unaffected by fatty acids in ethanolamine-supplemented membranes. This indicates that modulation of PGE_1-stimulated activity by fatty acids is subject to polar head group composition and suggests that phosphatidylethanolamine and phosphatidylcholine may interact with adenylate cyclase quite differently.

The changes in adenylate cyclase activities caused by 18:2 supplementation were dependent upon the levels of the fatty acid incorporated. However, the initial rate of 18:2 incorporation was significantly faster than the changes in enzyme activity. This observation suggests that either adenylate cyclase is surrounded by slowly exchanging boundary lipid, or that the transport of newly synthesized phospholipid from the endoplasmic reticulum to the plasma membrane is relatively slow. These kinetic studies also illustrated the greater sensitivity of PGE_1-stimulated adenylate cyclase activity to fatty acid compositional changes compared to basal activity. The latter was unaffected following 5 h of incubation with 18:2, whereas PGE_1-stimulated activity had doubled during the same period.

The data discussed above indicate that unsaturated fatty acids may play an important role in modulating hormone sensitivity of adenylate cyclase. This question has not been thoroughly examined in the literature, although it has been reported that basal, fluoride-, glucagon-, and (glucagon + GTP)-stimulated adenylate cyclase activities of rat liver decreased in rats fed on diets deficient in 18:2 and 20:4 (Brivio-Haugland et al., 1976). In agreement with the results reported here, stimulations of fluoride or glucagon + GTP were lower in rats fed higher levels of unsaturated fatty acids, but in contrast, the degree of stimulations by hormone alone was also lower. It should be noted that the changes in membrane fatty acid composition upon modification of the rat's diets were more complex than those reported in this study, and included a large increase in the level of 20:3 with rats fed a diet deficient in 18:2 and 20:4.

Supplementation with choline polar head group analogues also increased basal, PGE_1- and (PGE_1 + GTP)-stimulated activities, while fluoride-stimulated activity was affected only by *l*-2-amino-1-butanol supplementation. These data indicated that the degree of alkyl substitution of the phospholipid amino groups may be an important determinant of adenylate cyclase activity. For example, the K_m for ATP increased progressively with systematic removal of choline methyl groups. It is interesting that PGE_1-stimulated activity was more sensitive to the polar head group structural differences within the series choline, dimethylethanolamine, methylethanolamine, ethanolamine than basal activity. Again, it is apparent that stimulation by PGE_1 is dependent upon the phospholipid polar head group composition as well as the degree of fatty acid unsaturation.

It is difficult to compare these results with those obtained by other investigators who have added back phospholipids to solubilized preparations or membranes partially depleted of phospholipid. The available data indicate that phosphatidylserine influenced glucagon stimulation in cat heart (Levey, 1971) and rat liver (Rethy et al., 1972), phosphatidylinositol affected norepinephrine stimulation in cat heart (Levey, 1971)

and basal or glucagon-stimulated activity in rat liver (Rethy et al., 1972; Pohl et al., 1971a,b) while phosphatidylethanolamine influenced histamine stimulation of cat heart (Levey & Klein, 1972) and glucagon stimulation in rat liver (Pohl et al., 1971b). Significantly, phosphatidylcholine had no activating effect on any of these preparations. In most of the studies referred to above, phospholipids of varying purity were added to preparations containing residual phospholipids. It has been demonstrated in lipid reconstitution studies with the Na^+ K^+-ATPase that the presence of residual phospholipids can affect the apparent specificity for phospholipids observed for reconstitution of adenylate cyclase activity depended upon the treatment used to remove phospholipids. In some cases, phospholipase digestion has generated inhibitory end products (Rethy et al., 1971; Azhar et al., 1976) and residual organic solvents or detergents might be expected to have similar effects. Finally, the fatty acid composition of phospholipids used in reconstitution studies was usually not determined.

The data presented in this study indicated that adenylate cyclase activities appeared to vary systematically with several phospholipid structural parameters including the primary amino group concentration and degree of fatty acid unsaturation. However, it was not clear whether this reflected interactions between adenylate cyclase and specific structural elements of the phospholipids or a general dependence upon physical properties of the membranes, notably membrane viscosity. However, adenylate cyclase activity, stimulation by fluoride or PGE_1, and hormone lag times did not vary systematically with membrane microviscosity measured by fluoresence polarization. Nor was there any apparent correlation between activation energies for enzyme activity, hormone lag times and the activation energies for membrane microviscosity, or the membrane viscosity that may be predicted from the phospholipid composition. The absence of any correlation between enzyme activity and membrane fluidity measured by 1,6-diphenyl-1,3,5-hexatriene fluorescence polarization must be interpreted with some caution since the use of this dye to determine the absolute value of the membrane viscosity is subject to a number of uncertainties (Dale et al., 1977; Kawato et al., 1977; Pessin et al., 1978). However, it seems reasonable to compare the relative changes in the rotational relaxation times of 1,6-diphenyl-1,3,5-hexatriene, or the microviscosities, for similar membrane preparations. Thus in conclusion, it appears that the variation of adenylate cyclase activity and hormone stimulation with the membrane phospholipid composition is due to direct or indirect interactions with phospholipids rather than a general sensitivity to bulk membrane viscosity. Presumably, adenylate cyclase in native membranes can exist in several distinct conformations differing in catalytic activity which are dependent upon the lipid environment.

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