Biochimica et Biophysica Acta, 482 (1977) 358—369

BBA 68145

THE INFLUENCE OF CHANGES IN THE PHOSPHOLIPID PATTERN OF INTACT FIBROBLASTS ON THE ACTIVITIES OF FOUR MEMBRANE-BOUND ENZYMES

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(Received January 7th, 1977)

Summary

Human skin fibroblasts, grown to confluency in the presence of ^{32}P for random labelling of the phospholipids, showed upon 24 h incubation in the presence of either 8 mM L-serine or 4 mM ethanolamine an increased content of phosphatidylserine (150% of control cells) or phosphatidylethanolamine (116% of control cells), respectively. Concomitantly the phosphatidylcholine correspondingly decreased. Upon cell harvesting and gentle enzyme preparation the base-treated cells demonstrated a significantly higher unstimulated, fluoride- and thyrotropin-stimulated activity of adenylate cyclase. The activities of total ATPase, ouabain-sensitive ATPase, 5′-nucleotidase and γ-glutamyltransferase remained unaltered. When subjecting enzyme preparations from fibroblasts to ultrasonication the activity of adenylate cyclase decreased progressively with the energy applied, whereas the activities of the other enzymes were unaltered ($(K^+ + Na^+)$-ATPase, 5′-nucleotidase) or even increased (Mg^{2+}-ATPase, γ-glutamyltransferase). The results have a bearing upon the regulatory function of the phospholipid microenvironment of membrane-bound enzymes.

Introduction

Many previous studies have firmly established the requirement for phospholipids of the activity of membrane-bound enzymes such as $(K^+ + Na^+)$-ATPase [1—4], Mg^{2+}-ATPase [5—7], and adenylate cyclase [4,8—11]. Both the polar and the non-polar part of the phospholipids seem to be of importance. Theories

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about the phospholipid dependence of enzyme activities have been based to a large extent on in vitro studies in which membranes or subcellular fractions have lost the enzyme activities upon lipid depletion brought about by detergents, extraction with organic solvents or by specific enzyme treatment, followed by restoration of the lost activities upon addition of various phospholipids. The results of these studies have led to speculation as to whether the phospholipid microenvironment of membrane enzymes plays a role in the regulation of their activities [12,13]. However, as pointed out by many authors and most recently by Cuck-Chong [14], the physiological significance of the afore-mentioned results is difficult to evaluate because they were obtained under highly unphysiological conditions.

In the present study we have experimentally increased the relative content of phosphatidylserine (about 150% of control cells) or phosphatidylethanolamine (about 116% of control cells) in the membranes of intact cultured human fibroblasts. Furthermore, the effect of these changes on the activation of adenylate cyclase, (K^+ + Na^+)-ATPase, total ATPase, 5′-nucleotidase and γ-glutamyltransferase have been examined. Care was taken to make a gentle enzyme preparation. The activity of only adenylate cyclase was found to be changed significantly by these moderate and presumably near physiological alterations in the phospholipid pattern of cell membranes.

Materials and Methods

Materials. [^{32}P]Orthophosphate (5 mCi/ml) was purchased from Institutt for Atomenergi, Norway, and [2-^{3}H]ATP, ammonium salt (16 Ci/mmol), and cyclic [8-^{3}]AMP, ammonium salt (26 Ci/mmol), were from The Radiochemical Centre, England. ATP, cyclic-3′,5′-AMP, 5′-AMP and bovine serum albumin were from Sigma. Creatine kinase (cat. No. 15334) was from Boehringer. The various phospholipid standards were from Koch-Light, L-serine, 2′,7′-dichlorofluorescein spray and silica gel H were from Merck, and monoethanolamine from Baker. Minimum essential medium with Hank's salts and fetal calf serum were from Gibco. Plastic culture flasks (30, 270, 840 cm^3) were from Nunc, Denmark. All other chemicals used were of highest available purity.

Cell cultures. Primary culture of human fibroblasts were obtained from the umbilical cord and further cultured in Nunc plastic flasks as previously described [15]. To obtain random ^{32}P labelling of the phospholipids, the cells were grown in a medium (minimal essential medium in Hank's balanced salt solution with 15% fetal calf serum) containing [^{32}P]orthophosphate (0.13–0.15 μCi/ml). The medium was changed every 2–3 days. Only one cell strain (sub-cultured from 6 to 14 times) has been used throughout the study. All experiments were performed in cells grown to confluency.

Homogenate preparation. Cells were washed three times with isotonic saline and then harvested by scraping with a rubber policeman in ice-cold saline and centrifuged. The pellet was resuspended in a slightly hypotonic solution (0.20 M sucrose and 50 mM Tris · HCl buffer, pH 7.6 (25°C)) and disrupted at 0°C by standardized homogenization (six strokes) with a teflon pestle in a Potter-Elvehjem homogenizer. Sonication of the homogenates, when carried out, was done as stated in the legends using a MSE-Ultrasonic disintegrator with a

titanium microprobe. The protein was determined in triplicate by the method of Lowry et al. [16] using bovine albumin as standard.

Assay of the various phospholipids. Homogenates from cells grown in the presence of [^{32}P]orthophosphate were extracted with chloroform/methanol (2 : 1, v/v) according to the method of Folch et al. [17]. The extracts were dried and redissolved in a mixture of chloroform/methanol (4 : 1, v/v) containing appropriate amounts of pure carrier phospholipids. Aliquots of the cell extract corresponding to 0.1—0.2 mg cell protein (about $3 \cdot 10^3$—$5 \cdot 10^3$ cpm) were applied to thin-layer chromatographic plates and the phospholipids separated either by two-dimensional chromatography at room temperature on silica gel H (0.5 mm thickness) using the following solvent systems: Solvent I, chloroform/methanol/1 M ammonia (65 : 35 : 5, by vol.); solvent II, chloroform/acetone/acetic acid/water (10 : 4 : 2 : 1, by vol.), or by one-dimensional chromatography at 4°C on $(NH_4)_2SO_4$ (0.2—0.3%)-impregnated silica gel H (0.5 mm thickness) using chloroform/methanol/acetic acid/water (50 : 25 : 8 : 1, by vol.) as developing system [18]. The plates were activated for 1.5 h at 110°C immediately before use. The spots were visualized by iodine vapour or by spraying with 0.5% 2,7-dichlorofluorescein in ethanol and viewing under ultraviolet light. The areas containing the phospholipids were marked and scraped off into counting vials containing Instagel scintillation liquid (Packard), and the ^{32}P counted in a liquid scintillation counter (Tri-Carb, model 3375, Packard Instrument Co.). The recovery of the radioactivity from the plates was 83—88% (two-dimensional) and 85—92% (one-dimensional). The absolute amounts of ^{32}P-labelled phospholipids could be determined by using the specific activity of the ^{32}P employed in the growth medium.

Incorporation of L-serine and ethanolamine into phospholipids. Cells grown to confluency in the presence of ^{32}P were as a standard procedure incubated at 37°C for 24 h in ^{32}P-containing minimal essential medium without serum in the presence or the absence of either 8 mM L-serine or 4 mM ethanolamine. To the medium was also added Ca^{2+} and phosphate to a final concentration of 3.0 and 2.5 mM, respectively. This was done to ensure the same concentrations of these ions and in the case of ^{32}P the same specific activity as that of the growth medium used. The incubation was terminated by cooling the system on ice and harvesting as described above (see homogenate preparation).

Enzyme assays. Adenylate cyclase (EC 4.6.1.1) activity was determined by assaying the formation of cyclic AMP from ATP at 37°C. The assay solution (final volume of 0.2 ml) contained: 50 mM Tris · HCl, pH 7.6 (25°C), 5 mM $MgCl_2$, 50, 100, 250 or 500 μM [2-^{3}H]ATP (0.03—0.05 μCi per sample), 10 mM theophyllamine, 10 mM creatine phosphate, 0.2 mg/ml creatine kinase (EC 2.7.3.2), 0.2 mg/ml bovine serum albumin, and when added: 10 mM NaF or 0.004 I.U./ml thyrotropin (Actyron, Ferring, 2 I.U./ml). The reaction was started by the addition of homogenate (0.1—0.2 mg cell protein), and ended after 20 min of incubation (linear reaction curve) by the addition of 0.1 ml of a solution of cyclic AMP (50 mM) and ATP (75 mM), immediately followed by heating to 100°C for 1 min. After cooling the cyclic AMP was isolated in one step [19] by the addition of 0.2 ml 0.25 mM $ZnSO_4$ and 0.2 ml 0.25 mM $Ba(OH)_2$. The radioactivity remaining in the supernatant was counted (at least 10 000 counts) in the Packard counter 24 h after the addition of 10 ml

scintillation solution. The precipitation of labelled ATP was quantitative, and the recovery of cyclic [8-^{3}H] AMP added to the incubation mixture was close to 100% when applying the same isolation procedure. About 0.5—1% non-precipitable impurities were present in the [2-^{3}H] ATP preparations, making the blank value under the most unfavourable conditions (lowest enzyme activities and highest substrate concentration (500 μM)) up to 80% of the total counting values. Usually, however, the blank values represented from 10 to 25%.

The within-series precision (CV%) as calculated from duplicate determinations were found to be 10% (50 μM ATP), 12% (100 μM ATP), 20% (250 μM ATP), and 29% (500 μM ATP).

Total and ouabain-insensitive (Mg^{2+}-ATPase) ATPase (EC 3.6.1.3) activity was assayed by measuring the release of inorganic phosphate from ATP during 30 min of incubation at 37°C (linear reaction curve) [20]. Phosphate was determined according to Morin and Prox [21]. The within-series precision as calculated from duplicate determinations was with 1 mM ouabain 17% (CV) and without ouabain 10% (CV). Ouabain-sensitive, (Na^+ + K^+)-ATPase was estimated by subtracting the ouabain-insensitive ATPase activity from the total ATPase activity.

5'-Nucleotidase (EC 8.1.3.5) activity was assayed by measuring the release of inorganic phosphate [21] from 5'-AMP during 30 min of incubation at 37°C (linear reaction curve) [20]. In repeated experiments we found no significant phosphate formation with 2'(3')-AMP as substrate, i.e. the unspecific phosphatase activity was negligible under the conditions used. The within-series precision as calculated from duplicate experiments was 5.5% (CV).

γ-Glutamyltransferase (EC 2.3.2.2) activity was measured in a LKB 8600 reaction rate analyzer by a procedure previously described for serum [22]. The time of incubation was, however, increased to 4 min and the volume fraction of enzyme preparation was doubled. The within-series reproducibility as calculated from duplicate experiments was 12% (CV).

To increase the precision of the assays all enzymes were determined in duplicate.

Statistical treatment of the data was done by paired comparison using Student's *t*-test or by analysis of variance.

Results

Fig. 1 shows the separation of the various phospholipids in an extract from cultured human fibroblasts as obtained by the one-dimensional thin-layer chromatography used. Six different compounds could be clearly separated: sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine and diphosphatidylglycerol. Table I (two left columns) shows the relative amounts of these phospholipids in cultured human fibroblasts. There was a good agreement between the two-dimensional and the one-dimensional procedure, apart from the somewhat higher amount of sphingomyelin indicated by the latter. This was found to arise from lysophosphatidylcholine (which represented about 1.5% of the total phospholipid) co-chromatographing with sphingomyelin in the one-dimensional system. In both cases the reproducibility of the results was satisfactory (Table I).

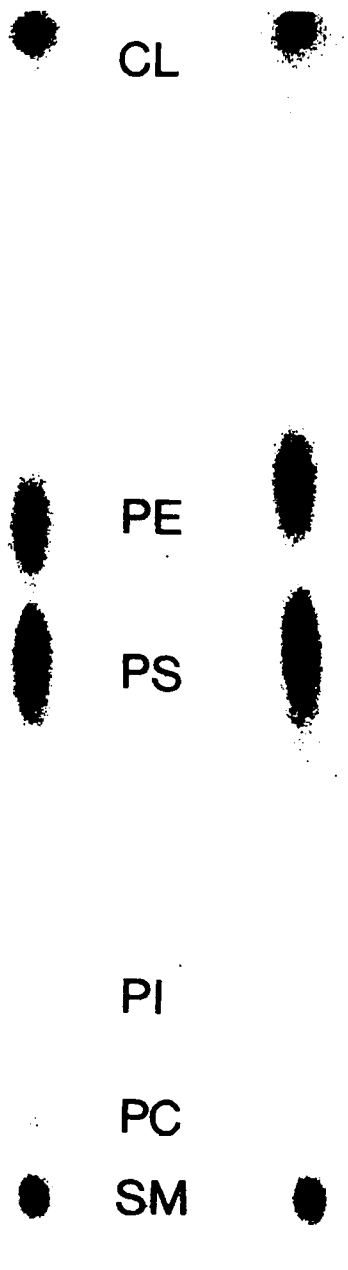

Fig. 1. Separation of phospholipids in a fibroblast extract by one-dimensional thin-layer chromatography as described in the text. Standards had been added to the extract for better visualization by iodine vapour. Abbreviations: SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; CL, diphosphatidylglycerol.

When fibroblasts were incubated in the presence of L-serine or of ethanolamine, gradual increases in the respective phosphatidylserine and phosphatidylethanolamine contents of the cells occurred (Fig. 2). These increases occurred at the expense of phosphatidylcholine (see below). The exchange reactions apparently involved and which required the presence of Ca^{2+} (results not

TABLE I

PHOSPHOLIPID PATTERNS IN CULTURED HUMAN FIBROBLASTS

The relative amounts of the various phospholipids of fibroblasts were determined upon ^{32}P labelling as described in Materials and Methods. The cells cultured to confluency were incubated at 37°C for 24 h in the absence (controls) and in the presence of 8 mM serine (cells + serine) or 4 mM ethanolamine (cells + ethanolamine). The amount of ^{32}P incorporated ranged from 146 to 281 μmol/g cell protein. The number of experiments performed are given in parentheses, and the relative amounts (percent of total) are expressed as mean ± S.E. The difference from control cells by paired comparison significant at <5% *, <1% **, and <0.1% *** level.

Phosphilipids	Two-dimensional chromatography Controls (5)	One-dimensional chromatography		
		Controls (10)	Cells + serine (3)	Cells + ethanolamine (3)
Sphingomyelin	4.1 ± 0.1	5.7 ± 0.1	6.7 ± 0.4	6.2 ± 0.2
Phosphatidylcholine	53.4 ± 0.6	52.1 ± 0.4	49.1 ± 0.8 **	46.3 ± 0.3 **
Phosphatidylinositol	9.0 ± 0.1	8.0 ± 0.2	8.2 ± 0.4	8.8 ± 0.3
Phosphatidylserine	5.9 ± 0.3	6.0 ± 0.2	9.1 ± 0.05 ***	6.3 ± 0.2
Phosphatidylethanolamine	25.0 ± 0.4	25.3 ± 0.5	23.9 ± 1.1	29.5 ± 0.4 *
Diphosphatidylglycerol	2.6 ± 0.2	2.8 ± 0.2	3.3 ± 0.1	2.9 ± 0.2

shown), were dependent upon the time of incubation (Fig. 2, left) and the base concentration (Fig. 2, right). Based on these results the following incubation conditions for altering the phospholipid pattern of fibroblasts were chosen as

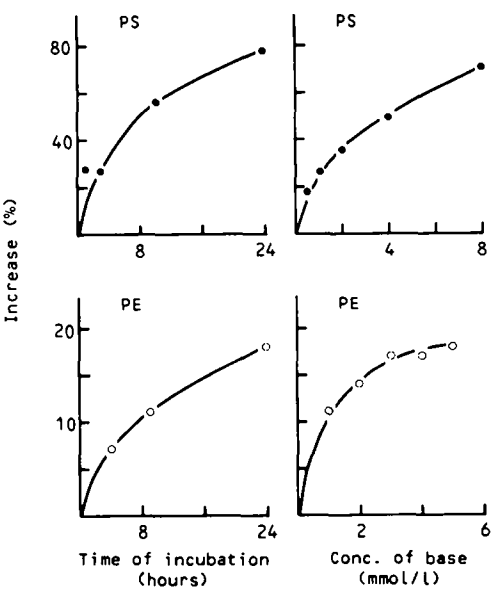

Fig. 2. The formation of phosphatidylserine and phosphatidylethanolamine in non-proliferating fibroblasts as a function of incubation time (left-hand figures) and of concentration of the respective bases in the incubation medium (right figures). The final concentration of L-serine was 8 mM and of ethanolamine 4 mM in the time experiments. The amounts of phosphatidylserine and phosphatidylethanolamine found in the base-treated cells are expressed as percentage increases relative to the amounts present in control cells run in parallel but without base being added to the medium (see Materials and Methods).

standard procedure in the further study: 24 h of incubation at 37°C in the presence of either 8 mM L-serine or 4 mM ethanolamine. Table I shows that under these conditions the presence of L-serine led to a significant and selective increase in phosphatidylserine (about 50%), and the presence of ethanolamine to a significant increase in phosphatidylethanolamine (about 16%). In both cases the amount of phosphatidylcholine decreased correspondingly, whereas the proportions of other phospholipids remained essentially unaltered (Table I). An extra addition of choline did not lead to any detectable change in the phospholipid profile (results not shown).

The effect of altering the cell phospholipid pattern on membrane-bound enzymes was studied by assaying the following enzymes: adenylate cyclase, total and ouabain-sensitive ATPase, 5'-nucleotidase and γ-glutamyltransferase.

Fig. 3 and Fig. 5 (left) show that a moderate increase of unstimulated, fluoride-stimulated as well as thyrotropin-stimulated adenylate cyclase activity was found in the homogenates from serine-treated cells as compared to those from untreated control cells. A similar increase of the adenylate cyclase activity was found in the ethanolamine-treated cells (Fig. 4 and Fig. 5, right). By analyses of variance the differences between control cells and the base-treated cells were found to be highly significant ($P < 0.001$) for the unstimulated and the fluoride-stimulated activities (Fig. 3 and Fig. 4), and the P values for the increase in the thyrotropin-stimulated activities were <0.01 and <0.05 for the serine-treated (Fig. 5, left) and the ethanolamine-treated cells (Fig. 5, right), respectively. We were unable to stimulate the adenylate cyclase activity by adrenaline, glucagon and adrenocorticotropin. Possibly, the preparation

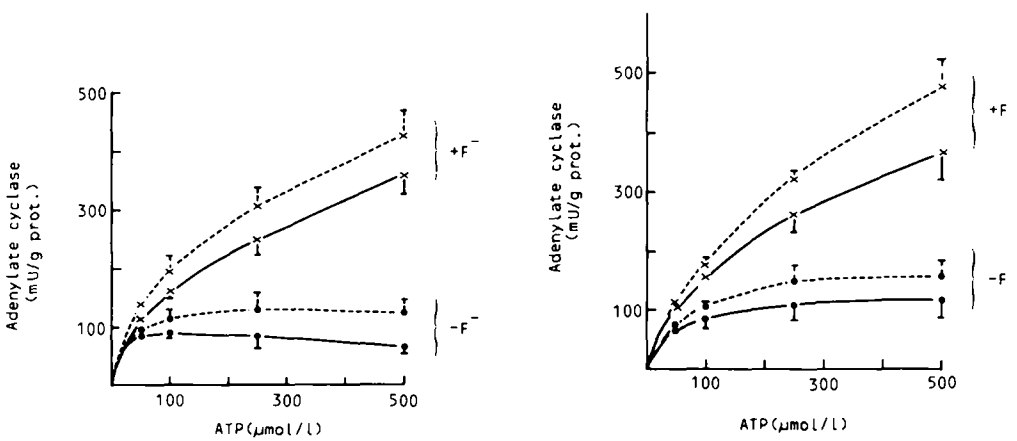

Fig. 3. Effect of increased cell content of phosphatidylserine on the activity of fibroblast adenylate cyclase. Fibroblasts, cultured to confluency, were incubated at 37°C for 24 h with (dashed curves) and without (solid curves) 8 mM L-serine present in the medium (see Materials and Methods). The adenylate cyclase activities were assayed at four concentrations of ATP in the presence (+F) or absence (—F) of F^-. The results are expressed as mean ± S.E. of three different experiments.

Fig. 4. Effect of increased cell content of phosphatidylethanolamine on the activity of fibroblast adenylate cyclase. Fibroblasts, cultured to confluency, were incubated at 37°C for 24 h with (dashed curves) and without (solid curves) 4 mM ethanolamine present in the medium (see Materials and Methods). Conditions were otherwise as stated in legend to Fig. 3.

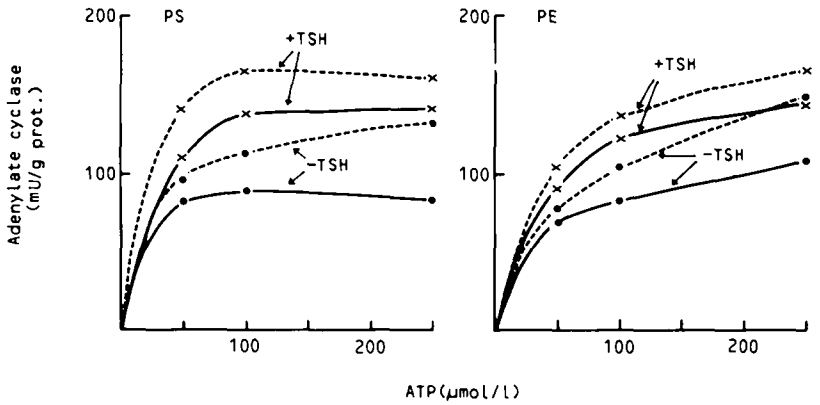

Fig. 5. Effect of increased cell content of phosphatidylserine (PS) or of -ethanolamine (PE) on thyrotropin-stimulated activity of fibroblast adenylate cyclase. The enzyme assays were performed with thyrotropin (+TSH) or without (—TSH) 4 I.U./l of thyrotropin present in the assay mixture. The conditions were otherwise as stated in legend to Fig. 3 (for the left figure) and legend to Fig. 4 (for the right figure).

procedures used resulted in destruction of essential binding sites or structures for signal transfer [11].

The activities of the other membrane-bound enzymes, namely γ-glutamyltransferase, 5'-nucleotidase and total and ouabain-sensitive ATPase were not significantly affected by the present changes in the phospholipid profile of fibroblasts (Table II).

It is now well established that the activities of various membrane-bound enzymes may be influenced by the particular preparation procedures used. Consequently, the effect of sonication in the present system was examined (Fig. 6). It is apparent that sonication of the fibroblast homogenate reduced the unstimulated as well as the stimulated activity of adenylate cyclase, and that the degree of inactivation was increased by increasing the sonication

TABLE II

EFFECT OF INCREASED CONTENT OF PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE ON THE ACTIVITY OF γ-GLUTAMYLTRANSFERASE, 5'-NUCLEOTIDASE AND TOTAL AND OUABAIN-SENSITIVE ATPase

Fibroblasts cultured to confluency were incubated at 37°C for 24 h in the absence (control cells) and in the presence of 8 mM serine (cells + serine) or of 4 mM ethanolamine (cells + ethanolamine), for details see Materials and Methods. The enzyme activities of homogenates (1—2 g protein/l) were assayed in duplicate according to standard conditions. The enzyme activities (munits/g cell protein) are expressed as mean ± S.E. of three experiments.

Enzyme preparation	γ-Glutamyl transferase	5'-Nucleotidase	ATPase	
			Total	Ouabain-sensitive
Control cells	9.7 ± 0.8	541 ± 98	27 ± 6.9	10 ± 2.3
Cells + serine	10 ± 1.1	471 ± 48	28 ± 5.8	12 ± 4.4
Control cells	6.4 ± 0.5	452 ± 3.1	21 ± 0.9	6 ± 0.6
Cells + ethanolamine	6.8 ± 0.5	443 ± 5.4	22 ± 0.3	6 ± 0.7

TABLE III

EFFECT OF SONICATION ON THE ACTIVITY OF γ-GLUTAMYLTRANSFERASE, 5′-NUCLEOTIDASE AND TOTAL AND OUABAIN-SENSITIVE ATPase

The fibroblasts cultured to confluency were collected and homogenized by standard procedure (homogenate). When kept on ice on sample (3—4 ml) of the homogenate was sonicated for 10 s at an indicated amplitude of 14 μm (Sonicate I), and another sample (3—4 ml) at three 14-μm bursts of 10 s, each separated by a cooling period of 50 s (Sonicate II). The protein concentration of the enzyme preparations ranged from 1.24—1.94 g/l. The enzyme activities (munits/g protein) represent the mean of four parallels. 5′-Nucleotidase is analyzed in one experiment. γ-glutamyltransferase in two and ATPase in three experiments. The difference from homogenate by paired comparison significant at <5% and <1% ** level.

Enzyme preparation	γ-Glutamyl transferase		5′-Nucleotidase	ATPase			
				Total		Ouagain-sensitive	
Homogenate	4.3			22		7	
		4.4	433	28	27	7	6.3
	4.5			32		5	
Sonicate I	6.1			35		3	
		6.3 *	459	39	39 **	5	5.0
	6.6			44		7	
Sonicate II	6.4			44		5	
		6.5 *	444	43	43 **	5	5.0
	6.6			41		5	

energy applied. In contrast, the activities of γ-glutamyltransferase and total ATPase were significantly increased by sonication, whereas the activities of ouabain-sensitive ATPase and 5′-nucleotidase remained essentially unaltered (Table III).

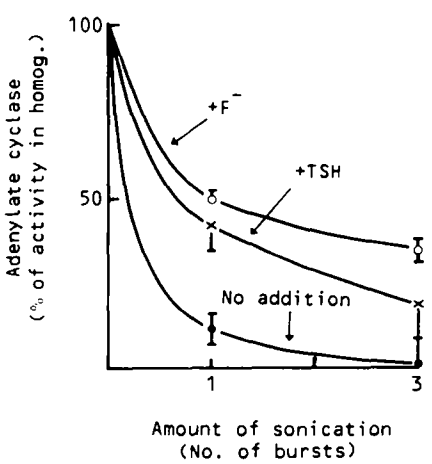

Fig. 6. The effect of sonication of fibroblast homogenate (prepared according to standard conditions) on its activity of adenylate cyclase. The procedure for enzyme preparation was the same as that stated in Table III. Adenylate cyclase activity was assayed at a concentration of 250 μM ATP with the addition of F^- (+F) or of thyrotropin (+TSH) or without any stimulator added (no addition). The abscissa represents the number of 14-μm bursts applied to the samples of the cell homogenates.

Discussion

Phosphatidylethanolamine and phosphatidylcholine were found to be the two quantitatively dominating phosphatidyl derivatives of cultured human fibroblasts, making up about 3/4 of the total phospholipids. This is in good agreement with earlier reports [23,24]. The serine- or ethanolamine-induced increase in phosphatidylserine and phosphatidylethanolamine of fibroblasts probably involves simple exchange reactions analogous to the Ca^{2+}-stimulated reactions reported for microsomes from liver [25] and brain [26,27]. However, whereas the latter studies suggest that phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine may all serve as substrates for the incorporation of serine and ethanolamine into microsomes, the present study with intact fibroblasts indicates phosphatidylcholine to be the only acceptor substrate in these reactions. This conclusion is based on the observation that increases in phosphatidylserine or phosphatidylethanolamine content of fibroblasts were, in the present work, always accompanied by equivalent decreases in the phosphatidylcholine level with no detectable effect being observed on the remaining phospholipids. Furthermore, no discernable alterations in the phospholipid profile of the fibroblasts resulted from the addition of choline to the medium.

The activities of several membrane-bound enzymes have been shown to be dependent upon phospholipids (see Introduction). Thus adenylate cyclase, as a typical example of such enzymes, is markedly or totally inactivated by exposing the membranes to phospholipid-depleting or enzyme-solubilizing procedures. Simultaneously the activity response to hormones and even to fluoride is reduced or lost [28]. The activity as well as the hormone responsiveness may be restored by phosphatidylserine or phosphatidylinositol [5,11,28], the other phospholipids being less efficient [29] or ineffective [9]. We found, using cultured fibroblasts, that an experimentally induced increase in the cell content of phosphatidylserine to about 150% or of phosphatidylethanolamine to about 116% of control cells with a corresponding decrease in phosphatidylcholine led in both cases to a significant and similarly pronounced rise in the activity of adenylate cyclase. These results should be compared cautiously with those of the above cited studies. Obviously, the changes in phospholipids here induced in intact cells are far less dramatic. They may on the other hand be considered to mimic more closely changes which occur in vivo.

The active site of adenylate cyclase is located towards the cytoplasmic internal leaflet of the plasma membrane [13] i.e. to the part that is quantitatively dominated by the phosphatidyl derivatives phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol [30]. The external leaflet of the plasma membrane are dominated by phosphatidylcholine and sphingomyelin [30], and it is on this side that the hormone receptor is located [13]. Consequently, one might expect the experimentally induced increase in phosphatidylserine and phosphatidylethanolamine to affect preferentially the internal leaflet. This would explain why the catalytic activity was altered whereas the hormone responsiveness remained essentially unaltered.

In contrast to adenylate cyclase, total ATPase, ouabain-sensitive ATPase, 5′-nucleotidase and γ-glutamyltransferase did not show altered activities in

cells with increased content of phosphatidylserine or phosphatidylethanolamine. Of these membrane-bound enzymes the ATPase has been studied most extensively with respect to the phospholipid requirement. $(Na^+ + K^+)$-ATPase is shown to be strongly lipid dependent, and phosphatidylserine appears to be the phospholipid that most effectively restores the activity of lipid-depleted ATPase [3,7,31]. However, Roelofsen and van Deenen [7] have presented evidence that loss in activity occurs only when the last 13% of the endogenous phosphatidylserine is removed. Moreover, the treatment of kidney $(Na^+ + K^+)$-ATPase with a mild, non-ionic detergent (Lubrol) did not cause the loss of ATPase activity [32]. Perhaps ATPase, as a penetrating integral protein, belongs to those membrane proteins which are surrounded by a shell of phospholipids that is removed less easily than the phospholipids present in the bulk of the membrane [33]. If this is correct, it may explain why a moderate change in the phospholipid profile of the cells did not alter the activity of ATPase.

One may speculate whether the membrane contains three major groups of enzymes. One group consists of phospholipid-dependent enzymes that are closely surrounded by phospholipids continuously undergoing dynamic alterations. These alterations may in turn influence the activity of the enzymes and by this mode exert a regulatory function (e.g. adenylate cyclase). Another group could consist of phospholipid-dependent enzymes that have a shell microenvironment of phospholipids less liable to undergo dynamic changes and thereby affect the enzyme activities (e.g. $(Na^+ + K^+)$-ATPase). The third group could then comprise enzymes which are located in the membrane, but that are not influenced by phospholipids or in this state even show diminished activities (e.g. 5′-nucleotidase, Mg^{2+}-ATPase and γ-glutamyltransferase). The release of the latter enzymes from the membrane may cause an increase in their activities (e.g. Mg^{2+}-ATPase and γ-glutamyltransferase). The striking correlation revealed between the sensitivity of the enzymes to sonication and the degree of alteration in their activities upon moderate changes of the phospholipid pattern of the membranes is in accordance with this hypothesis. If these different ways of interaction between enzymes and phospholipids in membranes do indeed occur, they may represent a precise and selective mechanism of enzyme regulation. Moreover, the present study offer evidence that such changes may be obtained by moderate alterations in the external milieu of the cells.

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