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INCREASE OF Na^+/K^+ ATPase ACTIVITY IN BHK 21 CELLS TREATED WITH PHOSPHATIDYLSERINE VESICLES

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BHK 21 cultured cells, incubated with phosphatidylserine vesicles, showed a significant increase of the phosphatidylserine content in the plasma membrane. At the same time the membrane-bound Mg^{2+} dependent, Na^+/K^+ activated ATPase activity was increased by 85%. The cellular viability was not affected by the phosphatidylserine incorporation.

The possibility to use phospholipid liposomes as carriers for introducing biologically active materials into cells has been extensively studied in the latest years (1). Much of the work, primarily concerning the mechanism by which the vesicles are incorporated by the cells, indicates that fusion of the vesicles with plasma membranes is an important pathway for liposomes uptake. This event is restricted to vesicles bearing a net (positive or negative) surface charge and composed of "fluid" phospholipids (2).

Among the negatively-charged phospholipids, phosphatidylserine is particularly active in delivering macromolecules into cultured cells (3,4). Furthermore phosphatidylserine vesicles show some pharmacological properties, e.g. they increase acetylcholine output from rat cerebral cortex (5), as well as antigen-mediated histamine release from isolated rat peritoneal mast-cells (6) and they influence the carbohydrate metabolism in mice (7).

We have previously demonstrated that phosphatidylserine vesicles markedly increase Na^+/K^+ ATPase activity in rat synaptosomes (8). Since a direct effect of phospholipid envelope could be unwanted when the phospholipid liposomes are used as drug carriers, we tried to ascertain whether phosphatidylserine vesicles are metabolically active also in intact cell systems.

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For this purpose the effect of these vesicles on Na^+/K^+ ATPase was studied in BHK 21 cultured cells.

Materials and Methods

Phosphatidylserine was a kind gift from Fidia Laboratories (Abano, Italy). All other reagents were of analitical grade.

1) Cells.

Cultures of established pseudodiploid BHK 21 Syrian hamster fibroblast line adapted to growth in suspension were grown in Eagle's minimal essential medium supplemented with 10% calf serum (MEM). Prior to use, cell suspensions in MEM were centrifuged for 5 min a 1000 g and the supernatant was aspired from the sedimented cells.
The cells were washed twice with Earle balanced salt solution prewarmed to avoid any thermic shock and then suspended in 0,32 M sucrose (pH 7.4).

2) Cell viability determination.

Exclusion of trypan blue was used to assess cell viability, which was typically 80-85%.

3) Phosphatidylserine vesicles were prepared in 25 mM TRIS-HCl (pH 7.4) as previously described (8).

4) Treatment of cells.

0.64 µMoles of phosphatidylserine vesicles were added to 1 mg cell suspension and incubated in an isosmotic medium consisting of 50 mM TRIS-HCl pH 7.4 and 0.3 mM sucrose, for different times at 37°C. After the incubation, the suspensions were layered on 0.32 M sucrose and centrifuged at 2500 g for 30 min.
The cells with bound phospholipids sedimented to the bottom of the centrifuge tube, whereas the unbound vesicles remained afloat above the sucrose solution. The pellets were resuspended in a small volume of 0.32 M sucrose (pH 7.4).

5) Crude membranes preparation.

In some experiments membranes of phospholipid-enriched cells were obtained by exposing the cells to a hypothonic shock and centrifuging at 4500 g for 30 min. The pellet resuspended in a small volume of 0.32 M sucrose (pH 7.4) represented the crude membranes.

6) Extraction and analysis of phospholipids.

The phospholipids were extracted and purified from the crude membranes prepared from normal and treated cells by the method of Folch et al (9). The separation of phospholipids was carried out on plates coated with Silica Gel H (Merck) containing Mg-acetate according to Rouser (10). The spots were evidentiated with J_2, and scraped off for the quantitation of phosphorus by the method of Bartlett (11).

7) Assays.

ATPase activities were measured by the release of inorganic phosphate from ATP. P_i was assayed according to Fiske and Subbarow (12).
The incubation medium contained 50 mM TRIS-HCl (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 100-200 µg protein

in a final volume of 1 ml. The reaction was initiated by the addition of ATP. Enzyme incubation lasted for 20 min at 37°C in Dubnoff bath and the reaction was stopped by the addition of 12% (v/v) cold trichloroacetic acid.
Mg^{2+} dependent, ouabain-insensitive, ATPase activity was determined by adding 0.2 mM ouabain to the incubation medium. The Na^+/K^+ ATPase activity was calculated as the difference between total ATPase and Mg^{2+}-ATPase activity.
Lactate-dehydrogenase activity was assayed spectrophotometrically as reported in Whittaker and Barker (13), by estimating the transfer of H^+ from $NADH_2$ to pyruvate in a medium (3 ml) consisting of 50 mM TRIS-HCl pH 7.4, 93 µM $NADH_2$, 360 µM Na-pyruvate and about 100 µg of cell suspension. The sample was read against water at 340 nm at 20°C for 5 min. 3% Triton X-100 was added at the end to verify the activity of the occluded enzyme. A change of extintion of 1 corresponded to 0.48 µMoles of $NADH_2$ oxidized. The specific activity was expressed in units of lactate-dehydrogenase/mg protein, being a unit the amount of enzyme that oxidized 1 µMole $NADH_2$/min. Protein content was measured according to Lowry (14).

Results

The total phosphorus content was measured in BHK 21 cells incubated for different times at 37°C in an isosmotic medium with and without 0.64 µMoles of phosphatidylserine/mg protein. This concentration of phosphatidylserine was chosen on the basis of our previous experiments on the interaction between phosphatidylserine liposomes and synaptosomes (8). The cells were then whashed on sucrose gradient to separate the unreacted phospholipid vesicles.
The average content of phosphorus in BHK 21 cells was 30.11 ± 0.64 µg phosphorus/mg protein, that is 0.971 µMoles phosphorus/mg protein.
A time-dependent uptake of phosphatidylserine by the cells occurred. The maximal incoporation was reached after 10 min of incubation and it did not increase at longer incubation times (Figure 1A). The phosphatidylserine-enriched cells were structurally integral, as it was shown by their values of lactate-dehydrogenase activity similar to those of normal preparations: control and phosphatidylserine-enriched cells showed 39 ± 1.5 Units and 34 ± 2 Units without detergent and 250 ± 82 and 222 ± 78 Units after addition of 3% Triton, respectively. Phosphatidylserine was not internalized but remained at the membrane level. The cells in fact were broken by hypothonic treatment after incubation with phosphatidylserine and the pellets were collected after centrifugation. In this crude membrane fraction phosphatidylserine was incorporated in

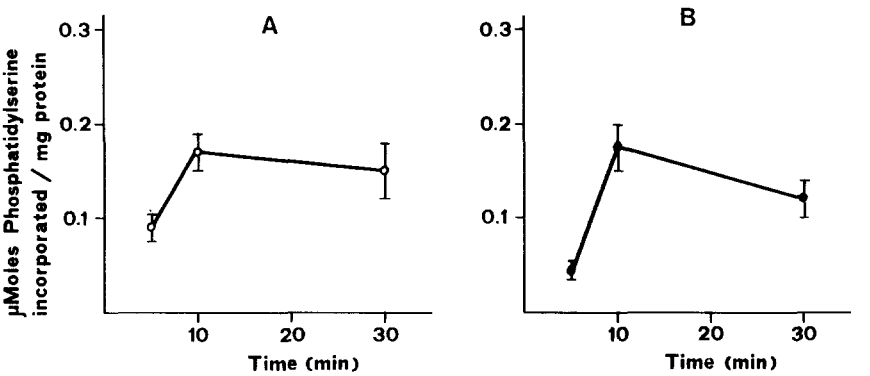

Figure 1 : Time-course of incorporation of added phosholipid by BHK 21 cells.

A = increase of phosphorus recovery in whole cells treated with 0.64 µMoles phosphatidylserine vesicles/mg protein.

B = increase of phosphorus recovery in crude membranes prepared from 0.64 µMoles phospholipid-treated cells.

Experimental conditions as described in the Methods. The results are the mean of 6 experiments ± S.E.M.

a time-dependent manner similar to that observed in intact cells (Figure 1B).

The thin-layer chromatography of phospholipids, extracted from the crude membranes of cells treated for 10 min with phosphatidylserine, demonstrated that phosphatidylserine was not degraded during incorporation in the cell membranes (Table 1). In the phosphatidylserine-treated cells the amount of phosphatidylserine in the membranes increased from 8% to 21%, whereas that of other phospholipids was not modified.

The treatment of cells with phosphatidylserine vesicles resulted in an altered Na^+/K^+ ATPase activity. Table 2 shows an increase of 85% of Na^+/K^+ ATPase activity measured in intact cells, while Mg^{2+} ATPase activity was unchanged. The activity did not change in the crude membrane preparation (data not shown).

Discussion

Our results show that the exposure of BHK 21 cells to phosphatidylserine vesicles caused a significant incorporation of the phospholipid in the cells. The increase of total phosphorus content in the membranes prepared from phosphatidylserine-treated cells and the specific increase of phosphatidylserine extracted from the membranes, suggest that phosphatidylserine vesicles fused, without further

TABLE 1 : Phospholipid composition of crude membranes prepared from normal and phosphatidylserine-enriched cells.

	Control	Phosphatidylserine-enriched cells	
Phosphatidylserine + phosphatidylinositol	8.02 ± 0.98	21.94 ± 6.18	$p < 0.001$
diphosphatidylglycerol	6.18 ± 2.27	6.45 ± 2.81	
phosphatidylcholine	53.29 ± 1.53	52.82 ± 0.37	
phosphatidylethanolamine	26.45 ± 3.48	31.16 ± 1.38	p = n.s.

Experimental conditions as described under Methods.

The percent of phospholipids was calculated by subtracting the amount of phosphorus recovered as phosphatidylserine+phosphatidylinositol from the total phosphorus recovered from normal and phosphatidylserine-enriched preparations.

The results are mean of 6 experiments ± S.E.M. For statistical analysis the t Student's test was used.

transformation, with the plasma membrane. Fusion is accepted as the most likely mechanism of interaction between cells and negatively-charged phospholipid vesicles (15,16). On the other hand we cannot exclude a tight adsorption of vesicles on the cellular surface.

As already observed in synaptosomes (8), the addition of phosphatidylserine to BHK 21 cells resulted in an increase of Na^+/K^+ ATPase activity in intact cells, but not in the broken ones. The values of lactate-dehydrogenase activity confirmed the integrity of the phosphatidylserine-enriched cells. The

TABLE 2 : Modification of Mg^{2+}ATPase and Na^+/K^+ ATPase activity of BHK 21 cells treated with 0.64 μMoles phosphatidylserine/mg protein.

Enzymatic activity	Control	Phosphatidylserine-treated cells
Mg^{2+}ATPase	3.03 ± 0.21	3.72 ± 0.32
Na^+/K^+ATPase	0.61 ± 0.06	0.967 ± 0.08 ¨

Experimental conditions as described under Methods.

The results are mean of 6 experiments ± S.E.M.

¨ $p < 0.005$

For statistical analysis the t Student's test was used.

disappearance of the increase of Na^+/K^+ ATPase activity after hypothonic shock of the phosphatidylserine-enriched cells roules out a direct effect of this phospholipid on the enzyme and suggests that the modification of the sodium pump was correlated to the cellular integrity. As it is admitted that in cell and membrane models fusion is accompanied by an increase in membrane permeability (15,16), phosphatidylserine could create some domains of altered permeability on BHK plasma membrane through which ions or substrates could pass more easily. The increase of Na^+/K^+ ATPase activity but not of Mg^{2+} ATPase, that is present in BHK 21 cells as an ectoenzyme (17), could be an indirect evidence for an effect on permeability. Na^+/K^+ ATPase is involved in the control of cell volume and maintains within physiological limits the concentration of Na^+ and K^+ ions that are important for protein and DNA synthesis (18) and for the transport of non-electrolytes (sugars and aminoacids) (19). Thus alterations of the activity of this enzyme following the phosphatidylserine vesicles interaction could drammatically affect the cellular physiology.

In conclusion these results confirm that phosphatidylserine vesicles have intrinsic biological properties. The possible use of phosphatidylserine vesicles as drug carriers is therefore complicated by the fact that they are able to modify some biochemical parameters of the cells, thus interfering with the pharmacological actions of eventually entrapped compounds.

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