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Changes of the compositional asymmetry of phospholipids associated to the increment in the membrane surface potential

Jorge Cerbón and Víctor Calderón

Department of Biochemistry, Centro de Investigación y de Estudios Avanzados del IPN, México, D.F. (Mexico)

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The contribution of phosphatidylinositol (PI) and phosphatidylserine (PS) to the outer negative membrane surface potential was studied in normal, PS-rich and PI-rich yeast cells. Under carefully defined conditions; PS and PE were quantified by using the non-penetrating chemical probe trinitrobenzenesulfonic acid (TNBS) and the PI by degradation with a specific phospholipase C. An asymmetric distribution of phospholipids in the plasma membrane with more PS (80–90%), PI (70–85%) and PE (70–85%) in the inner leaflet was found. When compared to normal cells there were 3-times more PI and 2-times more PS in the outer leaflet of the PI-rich and PS-rich cells. These values are consistent with the two-times increased surface potential in these cells. Interestingly, the contribution of PI was around twice the contribution of PS to the surface potential in the cells studied. When compared to normal cells there was a two-times increased accessibility of PS to TNBS in the PI-rich cells and the accessibility of PI to phospholipase C was also increased two-times in the PS-rich cells, while the proportion of derivatized PE was similar in all cells. Taking into account that the amount of PI is similar in normal cells and PS-rich cells and the amount of PS is similar in PI-rich cells and normal cells, a charge driven transbilayer transport of acidic phospholipids can be proposed.

Introduction

Recently, we found that yeast cells rich in anionic phospholipids, phosphatidylinositol-rich cells (PI-rich) and phosphatidylserine rich cells (PS-rich) show a negative surface potential 2-times higher than normal cells. This increased surface potential activates the high affinity proton-linked transport systems and inhibits the transport of anions from high external concentrations. The reduction of the surface potential by counter ions reverses completely the above mentioned alterations [1]. When the anionic phospholipid rich cells were maintained in the absence of carbon and nitrogen sources and in the presence of cycloheximide, the excess of anionic phospholipids was reduced as well as the surface potential [2]. Since all processes of biosynthesis, transformation and degradation of phospholipids occur at the cytoplasmic side, the above mentioned results indicate that the anionic phospholipids travel through the membrane to contribute to the changes in the external surface charge. In the present study the distribution of the anionic PS and PI that may contribute to the negative membrane surface potential was determined in normal cells, PS-rich and PI-rich cells to know:

(1) The compositional asymmetry of phospholipids in yeast.

(2) If the increment in negative surface potential observed in the PI-rich and PS-rich cells is due to an increase in the amount of anionic phospholipids in the outer membrane leaflet.

(3) Which is the contribution of each phospholipid to the membrane surface potential. The impermeant reagent 2,4,6-trinitrobenzene sulfonate (TNBS) was used [3] for the amino phospholipids and for PI a phospholipase c specific for phosphatidylinositol purified from *Bacilius cereus* was utilized [4].

The results indicate that anionic phospholipids contribute to the membrane surface potential, with a more important participation of PI as compared to PS. The data also suggest that the surface potential could modulate the translocation of the anionic phospholipids in

Correspondence: J. Cerbón, Department of Biochemistry, CINVESTAV-IPN, Apartado Postal 14-740, 07000 México, D.F., México.

the membrane. PS, PE and PI are asymmetrically distributed with a higher concentration in the inner leaflet.

Materials and Methods

Organisms

All the strains used were derived from *Saccharomyces carlsbergensis* ATCC-9080. PI-rich cells were obtained by growing the cells in a medium containing 10 mM arsenate, as previously described [2], and PS-rich cells by adding hydroxylamine (10 mM final concentration) to growing cells (absorbance $A = 0.25$). The cultures were further continued 18 h. Hydroxylamine at 10 mM caused the maximum increase in PS content of cells without affecting viability [5]. All cells were grown at 37 °C in nutrient broth containing 1% (w/v) maltose and 0.5% (w/v) yeast extract.

Surface potential

The surface potential was estimated by the binding of 9-aminoacridine (9-AA) as previously described [6]. The dye binds to negatively charged surfaces and quenches its fluorescence [7], therefore the amount of dye bound to the cells, due to surface charge effects, can be calculated from the decrease of fluorescence in the supernatant and the recovery of fluorescence observed after the addition of 100 mM $MgCl_2$ to the cells suspensions. The addition of $MgCl_2$ released the cells-bound 9-AA due to a reduction of the negative surface potential. The binding of the dye was measured at 23 °C. Routinely the buffer contained 2 μM 9-AA. The cells were washed and resuspended in 20 mM Tris–citrate buffer (pH 6.0) containing 9-AA. immediately after exposure, the suspensions were centrifuged and the fluorescence of the supernatants measured in an Aminco-Bowman spectrofluorometer, using the wavelength pairs 400 nm and 454 nm, for excitation and emission, respectively. The dye concentration was calculated from a calibration curve.

Localization of phosphatidylserine and phosphatidylethanolamine by reaction with trinitrobenzenesulfonate (TNBS)

Aminophospholipids on the external surface of the membrane react much more rapidly with TNBS than internal aminophospholipids [3]. After growing the cells in the presence of [^{32}P]P_i, under the conditions mentioned above, the cells were harvested by centrifugation, water washed and treated with TNBS. The cells were washed twice with one volume of 0.1 M KCL containing 50 mM potassium phosphate at pH 8.2 and resuspended in the same buffer containing 5 mM TNBS and maintained at 4 °C in darkness. Samples were taken at times indicated. Lipids were extracted with chloroform/methanol (2:1, v/v) and the phases separated by brief centrifugation. The aqueous layer was removed and the chloroform extract, after the addition of carrier lipid, was taken to dryness. The lipids were disolved in 0.3 ml chloroform/methanol (2:1, v/v) and aliquots used for analysis. At 4 °C after 30 min all the externally exposed aminophospholipids were converted in TNBS-derivates and at 25 °C both external and internal aminophospholipids were labelled (see Results). Trinitrophenylated PS and PE were prepared from yeast-lipid extracts and utilized as standards and carriers.

Analytical procedure

Phospholipids were separated by two-dimensional thin-layer chromatography (developing solvents: first dimension chloroform/methanol/water (65:25:4, by vol.); second dimension chloroform/methanol/acetic acid/water (85:20:8:4, by vol.)). Spots on thin-layer plates were visualized by iodine vapour and scrapped off. Radioactivity of individual lipids was measured using aquasol in a Packard Tricarb scintillation counter. All cpm values were corrected for quenching effects by the use of an external standard and the channel ratio method.

Localization of phosphatidylinositol

To discriminate between PI outside and inside the cells, the hydrolysis obtained by phospholipase treatment of intact cells was compared to those of sonicated cells and yeast-lipid extracts, which served as control preparations with a similar phospholipid composition but a random availability of phospholipids. Phospholipase c specific for PI was purified from *Bacillus cereus* ATRF-247, as described previously [43]. A unit of enzyme was defined as the amount of enzyme causing the hydrolysis of 1 mmol of substrate per min. Secondary effects on the molecular organization of the membrane due to PI-hydrolysis were expected to be minimal since the concentration of PI is relatively small and the enzyme does not show activity upon the other lipid components (see Results). In control experiments alterations on the permeability of small molecules were not found i.e., ions and amino acids (data not shown). The reaction mixture containing yeast cells (10 mg dry wt/ml) and 2.0 U/ml of enzyme in 20 mM Tris-borate buffer (pH 7.0) were incubated at 36 °C. At different periods of time, aliquots were withdrawn, cells were separated in a microfuge (2 min, $10000 \times g$) and lipids extracted from the pellet and separated by thin-layer chromatography. The ^{32}P of phosphatidyl[^{32}P]inositol was estimated and compared to the [^{32}P]PI from an identical cell suspension incubated in the same buffer but without enzyme. The PI accessible to phospholipase C in the intact yeast represents the PI of the outer membrane leaflet and the PI total composition was determined with ultrasonically treated yeasts in the presence of phospholipase C. The activity of the puri-

fied phospholipase C (1 U/ml) upon micelles of pure PI in 0.8% deoxycholate was 93% and 69% in a mixture of yeast phospholipids. Yeasts were sonicated four times for 1 min each, at 30-s intervals and 60 watts.

Chemicals

[^{32}P]P_i as orthophosphoric acid was purchased from New England Research products. The other reagents used: hydroxylamine, 9-aminoacridine, solvents, salts, etc. were purchased from Sigma, J.T. Baker and E. Merck.

Results and Discussion

The compositional asymmetry of a phospholipid in the membrane i.e., the unequal distribution between the inner and outer halves of the bilayer can be viewed as a kind of concentration gradient across the membrane [3]. Therefore, if this is the case, it would be expected that the amount of anionic phospholipids in the PS-rich and PI-rich cells would increase in the outer membrane leaflet.

Treatment of yeast cells with trinitrobenzensulfonate

Normal cells were utilized to established the reaction conditions leading to a limited final level of modification, which would represent minimal reagent penetration and membrane disturbance. These conditions were 4°C, 5.0 mM TNBS, pH 8.2 in darkness. The same conditions were then used to compare the accessibility of aminophospholipids in the PS-rich and PI-rich cells. It can be seen in Fig. 1 that the amino-group modification by TNBS in yeast cells reaches a plateau (approx. 20% of total aminophospholipids) in 30–40 min, which remains unchanged for up to 90 min.

Yeasts were incubated at 4°C with TNBS, and 50 min later they were sonicated. Then the TNBS non-

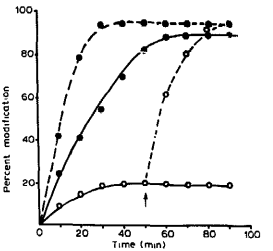

Fig. 1. Treatment of yeast cells with trinitrobenzensulfonate. Percent modification of aminophospholipids determined by measuring the [^{32}P]P_i-labelled derivatized lipids are shown. Open circles 4°C, solid circles 25°C. Continuous lines, intact cells; discontinuous lines, broken cells by sonication. Conditions: pH 8.2; darkness; 5 mM trinitrobenzensulfonate. Arrow indicates the time at which sonication was performed upon the cells exposed at 4°C.

permeability of the yeasts disappeared and 90 to 95% of the total aminophospholipids were bound to TNBS.

At 25°C (penetrating conditions), the same proportion (90%) of aminophospholipids were derivatized. Sonication of the cells at zero time of incubation with TNBS at 25°C, does not increase significatively the degree of modification. Also, the use of higher concentrations of TNBS up to 25 mM gave the same degree of derivatized aminogroups (not shown). Thus, with 5 mM TNBS all the accessible amino-groups of the phospholipids can, in principle, be modified and the fraction accessible at 4°C can be taken as external. Under these conditions, the concentration of cytoplasmic TNBS was in the micromolar range (data not shown). Table I shows the phospholipid composition and the results of external aminophospholipids derivatization

TABLE I

Phospholipid composition and trinitrobenzensulfonate (TNBS) derivatized aminophospholipids in yeast cells under non-penetrating conditions

The phospholipid composition was expressed in cpm($\times 10^{-3}$) and by percentage distribution of ^{32}P-labelled lipid in brackets. (a) The cells were cultivated in the Presence of [^{32}P]P_i; hydroxylamine was added when the absorbance reached 0.3 at 600 nm and the incubation continued for 16 hs more. Final concentration of hydroxylamine was 10 mM. (b) The cells were cultivated in presence of [^{32}P]P_i in a medium containing 10 mM sodium-arsenate for 48 h. The data represent the average of three separate experiments by duplicate. S.D. was never higher than 10%. TNBS concentration was 5.0 mM, pH 8.2, darkness. 4°C. Since the maximum derivatization of aminophospholipids occurred within 30–40 min (Fig. 1), the cells were incubated 50 min to ensure optimal outer leaflet labelling.

Cells	Phospholipid composition					
	PS	(TNBS)-PS	PE	(TNBS)-PE	PI	PC
Normal	52.8	3.8	173	17.97	87.49	337.39
	(7.8)	(0.56)	(25.7)	(2.6)	(13.0)	(50.1)
PS-rich	131.95	7.8	78.9	14.16	79.53	316.86
(a)	(21.0)	(1.23)	(12.6)	(2.24)	(12.6)	(50.2)
PI-rich	63.6	8.7	122.2	18.3	147.55	348.7
(b)	(8.9)	(1.22)	(17.2)	(2.58)	(20.8)	(49.18)

with TNBS (4 °C). It can be seen that the amount of PS (cpm) in the PS-rich cells was around 2-times higher than in normal cells and in PI-rich cells. The proportion of TNBS-PS was 2.0-times higher in the PS-rich cells and 1.7-times higher in the PI-rich cells when compared to normal cells. On the other hand, the proportion of derivatized PE was similar in all cases (2–2.6%) in spite that the amount of PE in the normal cells (cpm) was 2-times and 1.5-times higher than in the PS-rich cells and PI-rich cells, respectively. The decrease in PE concentration observed in the PS-rich cells correlates perfectly well with the increment in PS. PE decreases from 28% in the normal cells to 15% in the PS-rich cells, while PS increases from 8% in the normal cells to 22% in the PS-rich cells. These results show that the PS-decarboxylase pathway is the main route in the PE biosynthesis. The concentration of PC remained constant (50 ± 3%), showing that their biosynthesis takes place exclusively by the CDP-choline-$\alpha\beta$-diglyceride pathway and not by the succesive methylation of PE. It is known that in yeast cells, growing in a rich medium, the methylation reactions are repressed [8]. The amount of PC, therefore, can be utilized as an internal reference to detect changes in the proportion of the other phospholipids.

Treatment of yeast cells with phospholipase C specific for PI

The cell wall of yeast cells allowed the passage of certain proteins, i.e., endoglucanase and exoglucanase are among the many proteins that are found in the culture media of growing yeast. A general overview on the properties of the yeast cell wall is that it shows higher permeability for macromolecules than bacterial walls [9]. Then it was decided to test the activity of phospholipase C specific for PI upon intact yeast cells. It can be seen in Fig. 2 that a plateau level of hydrolysis (around 10–20%) was attained in 20–30 min and remained without change for up to one hour. An aliquot of the same suspension incubated without phospholipase C serves as the control. In yeast broken by sonication, PI hydrolysis reaches a maximum value of 68%. The same degree of hydrolysis was found in yeast-lipid extracts. Therefore, the accessible PI in the intact cells can be taken as external. PS, PE and pC were found not to be hydroiyzed by the phospholipase in both intact and broken cells. It can be seen (Table II) that the accessibility of PI to the phospholipase was higher in the anionic phospholipids-rich cells when compared to normal cells. The distribution of phospholipids over the outer leaflet of biomembranes can, in principle, be deduced from their availability to the action of agents as TNBS and phospholipases on intact cells. The results indicate that there is an asymmetric distribution of PS, PE and PI with less of one fifth present in the outer membrane leaflet. The conditions

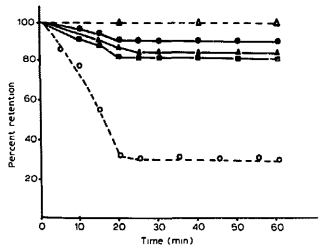

Fig. 2. Treatment of yeast cells with phospholipase C specific for PI. Percent retention of phospholipids was determined by measuring the [^{32}P]P_i-labelled phospholipids. Conditions: pH 7.0, 36 °C, 20 mM Tris-borate buffer, 2.0 UI of enzyme/ml, 10 mg dry wt. of cells/ml. (●) PI, normal cells; (■) PI, PI-rich cells; (▲) PI, PS-rich cells; (○——○) PI, broken cells by sonication; and (△——△) PC, PS and PE, intact and broken cells by sonication. The cells were sonicated at zero time of incubation.

can be considered valid, since, the cells were not significantly penetrated by TNBS and the phospholipase C was highly specific for PI [4].

The results indicate that PC should be more abundant in the membrane outer leaflet to compensate the asymmetric distribution of PS, PI and PE (more in the inner leaflet). Other attempts with a phospholipase C (EC 3.1.4.3) specific for PC, suggest that this seems to be the case. The early phase of enzyme treatment showed a large hydrolysis of PC as compared to PE, but it was not possible to know the real concentration of PC because with time a large degradation of phospholipids occurred and an increase of the permeability of small molecules was presented (data not shown),

TABLE II

Levels of phospholipid hydrolysis with purified phospholipase C (PI-specific) from Bacillus cereus

Results are expressed as percent of phospholipid hydrolyzed in yeast cells. Incubation conditions as in Fig. 2. Incubation time 45 min to ensure optimal PI hydrolysis of the outer membrane leaflet. The results are an average of at least three separate experiments by duplicate. The data of other phospholipids are shown as control of the specificity of the enzyme. Control incubations omitting phospholipase were carried out to show the absence of endogenous phospholipase activity under the conditions tested. There was no hydrolysis of PS and PE.

Phospholipid	Cells		
	normal	PS-rich	PI-rich
PI	9.14 ± 0.5	19.0 ± 1.3	15.88 ± 0.73
PC	1.4 ± 0.15	2.6 ± 0.2	2.0 ± 0.2
PS	0.0	0.0	0.0
PE	0.0	0.0	0.0

TABLE III

Phospholipid asymmetry of the yeast plasma membrane

(a) Measured with phospholipase C specific for PI, purified from *B. cereus*. (b) Measured by labelling with TNBS. Results were obtained with radioactivity labelled yeast cells. Values were calculated from data of Tables I and II taking 63% of the total phospholipid as belonging to the plasma membrane.

Phospholipid/Cells	% of individual phospholipids	
	outer leaflet	inner leaflet
Phosphatidylinositiol (a)		
Normal	14.5	85.5
PS-rich	30.15	69.85
PI-rich	25.2	74.8
Phosphatidylserine (b)		
Normal	10.3	89.7
PS-rich	8.7	91.3
PI-rich	19.0	81.0
Phosphatidylethanolamine (b)		
Normal	14.6	85.3
PS-rich	23.8	76.2
PI-rich	20.6	79.3

and indicated important alterations on the structural organization of the membrane.

To our knowledge, the topology of phospholipids in yeast cells has not been reported. More adequate percent values can be obtained taking into account that not all the phospholipids are in the plasma membrane. An average value of 63% (55–71%) of the total phospholipids, as belonging to the plasma membrane, was obtained from preliminary studies (data not shown). These values are in agreement with data from the literature [10–14]. Since no significant difference has been found between total cellular phospholipid composition and the composition of the plasma membrane [8,10], the outside-inside distribution of PI, PS and PE were calculated taking a value of 63% of the total phospholipids as plasma membrane lipids (Table III). From the amount of PI and PS present in each cell type and their accessibility, around 2-times more PI than PS was found present in the outer membrane leaflet of the three types of cells studied. The unequal distribution of PI, PS and PE can be viewed as a kind of phospholipid concentration gradient across the membrane. However, this gradient alone can not explain the higher accessibility of PS to TNBS observed in the PI-rich cells (2-times), when compared to normal cells (Table I), since the concentration of PS was similar in both cell types (10% and 8.9%, respectively). Also, there was a higher accessibility of PI to phospholipase C in the PS-rich cells (2-times), when compared to normal cells (Table II), in spite that the PI concentration was similar in both cell types (12.6 and 13%, respectively). An exagerated increment of surface charge at the inner membrane leaflet, during phospholipid biosynthesis in the anionic phospholipids-rich cells could be responsible for the increased trans location of anionic phospholipids to the outer leaflet. Work is being conducted to further substantiate this possibility. The results showed that there is a direct correlation between the increase of anionic phospholipids in the outer membrane leaflet and the increase in the negative surface potential. The 9-AA binding (nmol/g dry wt. of cells) was $27.1 + 1.3$ (normal cells), $56.2 + 2.7$ (PS-rich cells) and 64.1 ± 3.9 (PI-rich cells).

In order to further substantiate the correlation between surface potential and phospholipid composition, the surface potential was measured subsequent to the hydrolysis of PI with phospholipasase C. The 9-AA binding decreased from 27.1 ± 1.3 nmol/g dry wt of cells to 18.7 ± 1.7 after exposure to phospholipase C specific for PI. Also the surface potential increased the 9-AA binding to 38.0 ± 2.6 nmol/g dry wt. of normal cells after derivatization of the external aminophospholipids with TNBS.

The previous finding showing that the membrane surface potential of the PI-rich cells decreases when the PI-content was reduced [2], argues also in favor of a concentration gradient of phospholipids across the membrane. It has been found that the compositional asymmetry of PE in membranes of *Bacillus megaterium* is independent of metabolic energy [3]. However, the maintenance of lipid asymmetry in red blood cells requires an ATP-dependent aminophospholipid translocase [15] that specifically transports PS and PE from the outer to the inner leaflet.

The decrease in surface potential of the PI-rich cells mentioned above takes place when the cells were maintained for 20–24 h in the absence of carbon and nitrogen sources and in the presence of cycloheximide. Therefore, it is possible that the asymmetric composition in yeast may not depend directly upon metabolic energy. Otherwise with time, an equal distribution of anionic phospholipids will take place and the surface potential should increase because the higher concentration of anionic phospholipids in the inner membrane leaflet.

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