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Interfacial pH modulation of membrane protein function in vivo. Effect of anionic phospholipids

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In yeast cells, the magnitude of the membrane surface potential (φ) is determined to a large extent by the relative amount of anionic phospholipids (Cerbón and Calderón (1990) Biochim. Biophys. Acta 1028, 261–267). When a significant surface potential exists, the pH at the membrane surface (interfacial pH) will be different to that in the bulk suspending medium. We now report that: (1) In cells with higher φ (phosphatidylinositol-rich cells (PI-rich) and phosphatidylserine-rich cells (PS-rich) a 10-times lower proton concentration in the bulk was enough to achieve the maximum transport activity of H^+-linked transport systems when compared to normal cells. (2) When the φ was reduced by increasing the concentration of cations in the medium, more protons were required to achieve maximum transport, that is, the pH activity curves shifted downwards to a more acidic pH. (3) The magnitude of the downward pH shift was around 2.5-times higher for the more charged membranes. (4) Around 10-times more KCl than $MgCl_2$ was necessary to give an equivalent pH shift, in agreement with their capacity to reduce the φ of artificial bilayers. The interfacial pH calculated from the values of φ indicates that it was 0.4 pH units lower in the anionic phospholipid rich cells as compared to normal cells. The results indicate that membrane surface potential may explain the complex relationship between pH, ionic strength and membrane protein function. Maximum transport activities were found for glutamate at interfacial pH of 4.2–4.8 and were inhibited at interfacial pH = 3.2–3.4, suggesting that surface groups of the carrier proteins with pK values in the region 3.8–4.2 (aspartyl and glutamyl) are involved in binding and/or release of charged substrates.

Introduction

Membrane lipid composition has been modified in different ways. The modifications achieved have been extensive enough to alter a number of cellular functions, including carrier-mediated transport, the properties of certain membrane-bound enzymes, binding of insulin to their receptor, phagocytosis, endocytosis, etc. [1,2]. What remains to be described is the physical basis for the biochemical requirement membrane proteins have for certain membrane lipids. Recently, we found that yeast cells rich in phosphatidylinositol or in phosphatidylserine showed a negative surface potential (φ) 2-times higher than in normal cells. The anionic phospholipid-rich cells exhibited an increase in activity (lower the K_m) of H^+-linked transport systems (from low external concentration) and reduced the transport of anions (increase the K_m) from high external concentrations. The reduction of φ by counter ions or by decreasing the concentration of anionic phospholipids, reverses completely the above-mentioned transport alterations [3,4]. These effects were interpreted as due to an increased concentration of protons in the vicinity of the carrier proteins as counterions to the anionic phospholipids. Therefore, an increase in anionic phospholipids in the yeast, should originate changes in the interfacial proton concentration that could alter the dissociation of charged amino acids in the carrier protein and modify the binding or release of charged substrates. In order to test the above-mentioned possibilities we studied in normal cells (N-cs) and anionic-phospholipids rich cells (APLR-cs), the role of the proton gradient concentration upon the activity of the carriers of glutamate (anionic), arginine (cationic) and glycine (zwitterionic) from low external concentrations. Once the pH activity curves were obtained the effect of varying the interfacial proton concentration while maintaining the bulk pH (pH_b) constant upon the activity of the carrier proteins was investigated. The results showed that the interfacial proton concentration and not the bulk proton concentration regulates the activity of the carriers. It was also found that the

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rate of diffusion of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which is a weak acid that enters the yeast cells protonated [5], was 2-times higher in the APLR-cs when compared to N-cs. This increase can only be explained by a higher proton-concentration at the membrane of the APLR-cs (i.e., a lower interfacial pH). The results suggested that the dissociation of aspartic and/or glutamic residues ($pK \sim 3.8$–4.2) of the carrier, located at or very near the lipid aqueous interface, was altered by the changes in the interfacial pH (IpH) originated by the different concentration of anionic phospholipids at the yeast cell membrane.

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 [3] and PS-rich cells by adding hydroxylamine (10 mM final concentration) to growing cells (absorbance $A = 0.25$). The cultures were further continued 10–18 h. As previously reported, hydroxylamine at 10 mM caused the maximum increment in PS content of cells without affecting viability [6]. All cells were grown at 37°C in nutrient broth containing 1.5% (w/v) yeast extract and 1% (w/v) maltose.

Analysis of phospholipids and membrane surface potential measurements

Phospholipids were extracted from yeast cultures uniformely labelled with $[^{32}P]P_i$, by chloroform/methanol (2:1) and separated by thin-layer chromatography, as previously described [3]. From the lipid composition the ratio anionic/zwitterionic phospholipids was calculated (A/Z). Surface potential was determined by the binding of 9-aminoacridine (9-AA) and their release by monovalent (KCl) and divalent cations (methyl viologen $(MV)Cl_2$) as in Ref. 4. When suspended in a low cation-containing medium, yeast cells quench the fluorescence of 9-AA. Relief of this quenching is achieved by adding cations to the suspending medium, with the order of effectiveness being $C^{3+} > C^{2+} > C^{+}$, showing that the fluorescence acts as an indicator of the membrane surface potential. Surface charge densities were calculated as in Ref. 4, using concentrations of KCl (C') and $(MV)Cl_2$ (C'') that gave a F/F_{max} value = 0.5. F is the fluorescence yield of a particular experiment and F_{max} is the maximum fluorescence observed in the presence of 100 mM $MgCl_2$. Eqn. 4 of Ref. 7 was utilized.

$$\sigma = -[3.444 \cdot 10^{6}(C' - 4C'C'')/C'']^{1/2}$$

Transport assays

The cells were harvested, washed twice with water and resuspended for 20 min in 1% (w/v) glucose at 23°C. Thereafter, the cells were collected, water washed and used at once as follows: The uptake of substrates was assayed at 23°C. The cells were resuspended (1.0 mg dry wt./ml) in prewarmed 20 mM Tris solution containing 200 μg/ml cycloheximide, adjusted at the desired pH with HCl or citric acid when indicated. Cells were incubated for 5 min before the substrate and when indicated cations were added. The uptake of the labelled compounds was determined by separation of the cells from the suspension medium by centrifugation of the cell suspension through dibutyl phthalate [4] in a Beckman E microfuge ($12000 \times g$, acceleration time 6 s). Initial rates (30 s) were obtained. Radioactivity of the cell pellet was determined by scintillation counting in a Packard TriCarb scintillation counter. Aquasol was utilized as a scintillation liquid. All cpm values were corrected with the use of an external standard and the channel ratio method. The rates of uptake appear linear for at least 1.0 min.

Determination of intracellular pH

Cells in the stationary phase were harvested, water washed twice and resuspended in 50 mM Tris-citrate buffer (pH 4.0) containing 0. 2–1.0 mM $[^{14}C]$propionate (0.1 μCi/ml). The uptake of propionate by the yeast reached a maximum in about 4–5 min. The intracellular pH was computed on the basis of a pK_a value of 4.87 for propionate [8]. Also, the pH was determined potentiometrically after rupture of the cells by freezing and thawing. Both methods gave similar results.

Determination of carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) uptake

Since CCCP is a weak acid (pK value about 6.1) and enters yeast cells by diffusior [5], in the absence of a proton gradient concentration, it is possible to suggest a difference of uptake between the normal cells and the cells rich in anionic phospholipids if there were significant differences in the interfacial pH. Water washed cells were resuspended in 10 mM Tris pH 6.5 (1.6 mg dry wt./ml) final concentration. Aliquots of 5.0 ml were taken and 30 nmol CCCP/ml were added. At different times cells were separated by centrifugation (30 s) in a microfuge and the absorbance of the supernatant determined: at pH 6.5 CCCP shows a maximum of absorbance at 375 nm. The remaining CCCP concentration of the supernatants was calculated making use of a calibration curve. There is a linear relationship between CCCP concentration and absorbance at 375 nm in the range 5–50 nmol/ml.

Chemicals

[^{32}P]P_i as orthophosphoric acid, L-[2,3-^{3}H] glutamic acid, L-[2,3-^{3}H]arginine, [2-^{3}H]glycine and [^{14}C]propionate were purchased from New England Research Products. The other reagents used: 9-aminoacridine, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), tetracaine, solvents, salts, etc. were purchased from Sigma, J.T. Baker and E. Merck, A.C, dibutyl phthalate was purchased from Eastman Kodak Co.

Results and Discussion

Phospholipid composition and surface potential (φ)

As previously reported [3,4], the ratio of anionic phospholipids to zwitterionic phospholipids (A/Z), as well as the surface potential (9-AA-binding), increased in the PS-rich and in the PI-rich cells (anionic-phospholipid rich cells (APLR-cs)) relative to normal cells (N-cs). The A/Z ratio was 0.23–0.25 in N-cs and 0.49–0.51 in the APLR-cs. The 9-AA binding was 25.0–27.5 in N-cs and 61–64 in the APLR-cs. 9-AA binding was expressed in nmol/g dry wt of cells.

Proton-linked transport systems as surface pH indicators

When the dependence of glutamate uptake (initial rate) on external pH was analyzed, it was found (Fig. 1) that the whole pH activity curve was shifted towards lower pH values in N-cs when compared to APLR-cs. The optimum pH values found were pH 5.0 in Ncs against pH 6.0 in the APLR-cs. Having found that the mechanism of cotransport is one in which protons bind before the anion [4], the results suggested that up to 10-times less protons in the suspension medium were required in the APLR-cs for achieving the maximum initial rate of uptake. An increase in cations should

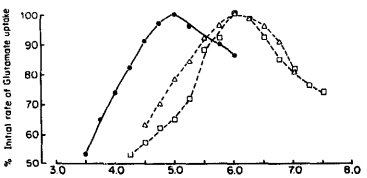

Fig. 1. Dependence of glutamate uptake on external pH. Cells were kept for 20 min at 23°C in 1% glucose, water washed twice and resuspended in 20 mM Tris-citrate buffer. After 5.0 min labelled glutamate (20 μM) was added. Data are expressed as percent of the maximum initial rate of uptake (30 s) and each value is the average of at least six independent determinations. S.D. was never higher than 7%. ●——●, Normal cells; △——△, phosphatidylserine-rich cells; □——□, phosphatidylinositol-rich cells. The 100% values expressed in nmol min^{-1} g^{-1} were: 27 in normal cells and 40 in the anionic-phospholipid rich cells.

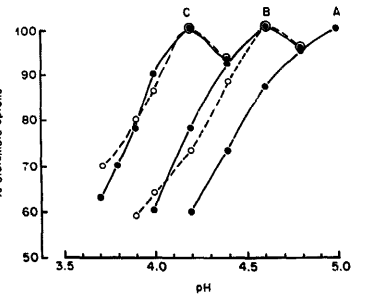

Fig. 2. Effect of reducing the surface potential on the dependence of glutamate uptake on external pH in normal cells. Experimental conditions as described in Fig. 1. The surface potential was reduced by adding either KCl closed circles, or $MgCl_2$ open circles. (A) 20 mM Tris-citrate (control); (B) 20 mM Tris-citrate plus 25 mM KCl or 2.5 mM $MgCl_2$; (C) 20 mM Tris plus 50 mM KCl or 5.0 mM $MgCl_2$. Cations were added 10 s before the addition of substrate. The 100% values expressed in nmol min^{-1} g^{-1} were: curve (A) 28; curve (B) 25.5 (closed circles), 23.8 (open circles); curve (C) 22.2 (closed circles), 20.5 (open circles).

lower the magnitude of the surface potential, reduce the concentration of protons at the outer membrane/solution interface and shift the pH activity curves to more acidic values. To determine whether this explanation was adequate the effect of KCl (from 5 to 50 mM) and $MgCl_2$ (from 1 to 5 mM) was investigated. It can be seen (Fig. 2, N-cs) that the pH–activity curves of transport were shifted towards more acidic values. The optimum pH shifted from pH 5.0 in 20 mM Tris towards pH 4.62 in the presence of 25 mM KCl and towards pH 4.2 with 50 mM KCl. In other words, it was necessary to increase the proton concentration in order to obtain the optimum initial rate of transport when the surface potential was reduced. Similar pH shifts were obtained with 2.5 mM $MgCl_2$ and 5.0 mM $MgCl_2$ instead of 25 mM and 50 mM KCl, respectively. It is known that divalent cations are at least one order of magnitude more efficient than monovalent cations in reducing the surface potential of artificial lipid bilayers [9]. With intermediate concentrations of both KCl and $MgCl_2$, shifts in the pH-activity curves were also obtained but were omitted in the figure to make it clear. The results obtained when reducing the surface potential in APLR-cs can be observed in Fig. 3, 10 mM KCl was enough to shift the pH–activity curve of transport towards more acidic values. The optimum pH shifted from pH 6.0 in 20 mM Tris towards pH 5.0 in the presence of 10 mM KCl or 1 mM $MgCl_2$. As anticipated theoretically, the shift downwards with increas-

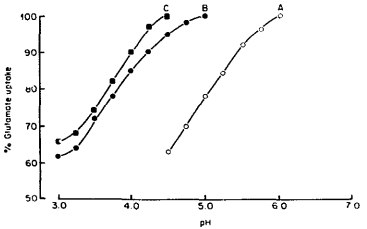

Fig. 3. Downward shifts of the pH-activity curves of glutamate uptake when reducing the surface potential of the anionic phospholipid rich cells. Experimental conditions as described in Figs. 1 and 2. Curve (A) control, 20 mM Tris. Curve (B) addition of 1.0 mM $MgCl_2$. Curve (C) addition of 0.5 mM tetracaine. The 100% values expressed in nmol min^{-1} g^{-1} were: curve (A) 40; curve (B) 24; curve (C) 20.

ing electrolyte concentration, was greater for the more charged membranes [10]. The values of KCl (C') and MV Cl_2 (C'') used for the surface charge density calculations expressed in mM were: 6.8 (C') and 0.42 (C'') for N-cs, 15 (C') and 0.74 (C'') for the PS-rich cells, 16 (C') and 0.68 (C'') for the PI-rich cells. The corresponding σ values were 0.0169 (C/m^2) in N-cs against 0.029–0.032 (C/m^2) in the APLR-cs.

pH at membrane surfaces

φ and IpH were calculated as in Ref. 11. Using the surface charge values mentioned above, the surface potential (φ) was calculated by their relation in the Gouy-Chapman Theory for an electrolite consisting of a 1:1 salt [7]. Since a significant φ exists, the pH at the membrane surface (IpH) was calculated from the expression [11]

$$IpH = pH_b + (F\varphi / 2.3RT)$$

where R, T and F are the gas constant, temperature in K and the Faraday, respectively. When the membrane was bathed in 20 mM Tris (pH 5.0), conditions where the maximum transport activity was found, a $\varphi = -45.18$ mV and an interfacial pH = 4.25 were obtained in Ncs. The increased level of protons at the surface may simply act as counterions to the lipid fixed negative charges ($pK_a = 1.5$ [12]). On the other hand, protonation will occur in the surface groups of the carrier proteins if their pK values are in the region of 3.8–4.2 (aspartyl and glutamyl groups) and above. In Ncs, when 50 mM KCl was added to the cell suspension, φ decreased to -26.29 mV and at a $pH_b = 5.0$, IpH increased to 4.46 (0.21 pH units). Due to this reduction in H^+ concentration at the interface, glutamate uptake was reduced. To restore the maximum transport activity it was necessary to decrease the pH_b from 5.0 to 4.4 (Fig. 2). In the APLR-cs in 20 mM Tris $pH_b = 6.0$, where maximum transport activity was observed, $\varphi = -67.4$ mV and IpH = 4.88. When 10 mM KCl was added φ decreased to -58.72 mV, IpH increased to 5.026 (i.e., H^+ concentration decreases $3.76 \cdot 10^{-6}$ M at the interface) and protons have to be added to restore transport activity. It was necessary to decrease pH_b from 6.0 to 5.0. The excess of protons added to the medium (2.66-times more than needed theoretically) to obtain the maximum activity, can be explained if the 1.96-times increment in the K_m of glutamate uptake found previously [4] is taken into account. The K_m of glutamate changes from $8 \cdot 10^{-5}$ M towards $1.57 \cdot 10^{-4}$ M when 1.0 mM $MgCl_2$ was added to the suspending medium (20 mM Tris). In all cases 40–45% of inhibition was observed at IpH = 3.2–3.4 or below. The results clearly showed that the proton concentration at the membrane interface, and not the proton concentration in the bulk allow the transport system to act at its maximum capacity.

The dependence of glutamate transport activity on interfacial pH was further analyzed by using the cationic local anesthetic tetracaine. This compound reduced the negative surface charge, and therefore the surface potential more efficiently, due to its capacity to become anchored to the bilayer [13]. It was found that 0.5 mM tetracaine shifted the activity curve of transport up to 1.5 pH units downwards (Fig. 3). Similar results were obtained with the proton linked transport systems of arginine and glycine, that is, the pH activity curves were shifted upwards in the APLR-cs. They did not show, however, a single well defined pH optimum as glutamate (Fig. 4). The proton-linked transport system of glutamate behaves as a very efficient surface pH indicator (biosensor); therefore, data as those shown in Fig. 2 can be used to determine differences in membrane surface potential (for a detailed discussion see Fromherz [14].

Diffusion of CCCP

To further substantiate the presence of a higher proton concentration at the membrane solution interface, relative to the bulk, the uptake of CCCP, a weak acid with a pK_a of about 6.1 [5], was studied at an external pH of 6.5 when there is no ΔpH gradient in its favor. The internal pH was more acidic in the APLR-cs than in N-cs (pH 6.3 in Ncs vs. pH 6.1 in APLR-cs). It can be seen (Fig. 5) that the rate of CCCP uptake was 2-times higher in the APLR-cs as compared to N-cs. The apparent equilibrium concentration was obtained at around 20 min in the APLR-cs vs. 40 min in the N-cs. This higher rate of diffusion can only be explained by the presence of an increased concentration of protonated CCCP at the membrane solution interface of the APLR-cs. At $pH_b = 6.5$ in 10

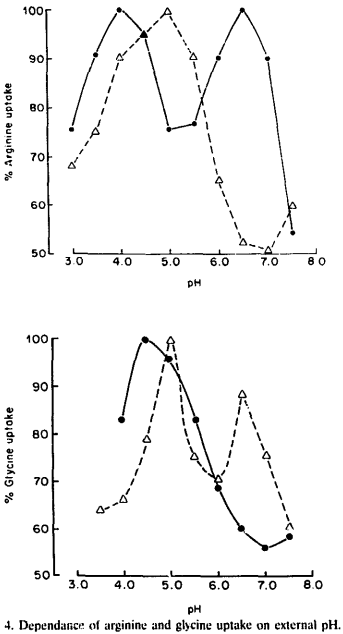

Fig. 4. Dependance of arginine and glycine uptake on external pH. Experimental conditions as in Fig. 1. ●——●, Normal cells; △——△, anionic phospholipid-rich cells. The 100% values expressed in nmol min^{-1} g^{-1} were: for arginine 6 in normal cells and 14 in PLR-cs and for glycine 83.6 in normal cells and 142 in APLR-cs.

mM Tris, the $\varphi = -59.9$ mV and the IpH = 5.51 in Ncs and the $\varphi = -83.22$ mV and IpH 5.12 in APLR-cs.

In a few cases, the influence of surface charge and surface potential on transport activity has been analyzed in situ, i.e., with membrane proteins in their natural surroundings [10,15–17]. Experiments have been reported were the surface charge was artificially changed within the natural membrane, for instance by adding charged surfactants to mitochondria [18] or by using reconstituted systems [19,20].

When measuring φ, complications may arise due to the use of probes whose binding properties and localization may induce changes directly or indirectly upon the membrane surface potential [10]. In principle, direct methods are superior than the use of probes, because they are non-perturbing. In the present case, the activity of the proton-linked transport systems studied were capable of clearly detecting the differences in surface charge and surface potential between N-cs and APLR-cs (between 0.0169 C/m^2 and 0.0288–0.0328 C/m^2). Regulation of the membrane surface potential and transport by anionic phospholipids has also been observed in *Escherichia coli* [21], and changes in the *A/Z* phospholipid ratio during growth in response to increases in the external pH were found in *Bacillus stearothermophylus* [22]. These results indicate that anionic phospholipids play an important physiological role in regulating the interfacial pH. The apparent competitive inhibition of H^+-linked transport systems by cations [4,23] can be explained by the reduction of the proton concentration at the cell membrane interface altering the dissociation of ionizable groups of the carrier proteins involved in substrate recognition (binding and release).

From all the carriers studied that are regulated by the membrane surface potential [4], the complete DNA sequence of the CAN 1 locus of the yeast *Saccharomyces cerevisiae* is the only known. The CAN 1 gene encodes an amino acid permease specific for arginine, but transports the basic amino acids lysine, hystidine and ornithine, with much lower affinities [24]. At present it is not known how the CAN 1 gene product is inserted into the membrane. However, when the charge difference of the flanking regions of the most N-terminal membrane spanning segment of the arginine permease was analyzed according to Hartmann et al. [25], a N-exoplasmic/C-cytoplasmic orientation was found (data not shown). The arginine permease has an extremely hydrophilic 93 amino acid long N-terminal

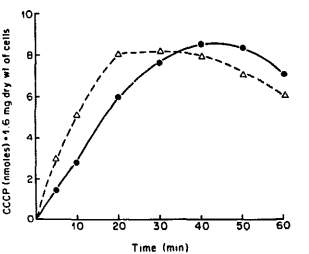

Fig. 5. Comparative CCCP uptake. Cells (1.6 mg dry wt./ml) were suspended in 10 mM Tris buffer (pH 6.5). Uptake was started by the addition of 30 nmol of CCCP/ml. At indicated times the cells were separated by centrifugation (30 s) in a microfuge and the absorbance of the supernatant determined at 375 nm. The amount of CCCP was determined by making use of a calibration curve. ●——●, Normal cells; △——△, anionic phospholipid-rich cells.

domain with a total charge of −9. A cluster of negatively charged amino acids (aspartic and glutamic acid residues) is located near the most N-terminal signal anchor sequence and could be at or very near the lipid/aqueous interface and sense the changes in the interfacial pH. By studying the changes in the compositional asymmetry of phospholipids associated to the increase in membrane surface potential in yeasts, a 1.7-times higher concentration of anionic phospholipids was found in the exoplasmic side of the plasma membrane of the PS-rich and the PI-rich cells when compared to Ncs [26].

Acknowledgements

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