Proton-induced endocytosis is dependent on cell membrane fluidity, lipid-phase order and the membrane resting potential

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ABSTRACT

Recently it has been shown that decreasing the extracellular pH of cells stimulates the formation of inward membrane invaginations and vesicles, accompanied by an enhanced uptake of macromolecules. This type of endocytosis was coined as proton-induced uptake (PIU). Though the initial induction of inward membrane curvature was rationalized in terms of proton-based increase of charge asymmetry across the membrane, the dependence of the phenomenon on plasma membrane characteristics is still unknown. The present study shows that depolarization of the membrane resting potential elevates PIU by 25%, while hyperpolarization attenuates it by 25%. Comparison of uptake in suspended and adherent cells implicates that the resting-potential affects PIU through remodeling the actin-cytoskeleton. The pH at the external interface of the cell membrane rather than the pH gradient across it determines the extent of PIU. PIU increases linearly upon temperature increase in the range of 4–36 °C, in correlation with the membrane fluidity. The plasma membrane fluidity and the lipid phase order are modulated by enriching the cell's membrane with cholesterol, tergitol, dimethylsulfoxide, 6-ketocholestanol and phloretin and by cholesterol depletion. These treatments are shown to alter the extent of PIU and are better correlated with membrane fluidity than with the lipid phase order. We suggest that the lipid phase order and fluidity influence PIU by regulating the lipid order gradient across the perimeter of the lipid-condensed microdomains (rafts) and alter the characteristic tension line that separates the higher ordered lipid-domains from the lesser ordered ones.

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1. Introduction

Endocytosis usually proceeds via three main steps; inward bending of the plasma membrane, followed by the formation of a connected bud and finally bud scission to form a free vesicle. These steps are commonly governed by curve forming proteins (e.g. BAR and epsin), coat proteins (e.g. caveolin and clathrin) and scission proteins (e.g. dynamin GTPase) [1]. However, endocytic vesicles are also formed when these proteins are inhibited or absent altogether, suggesting that other less defined mechanisms exist. Several clathrin/caveolin independent endocytic pathways have been implicated, such as flotillin coated vesicles, GPI-AP enriched compartments (GEEC) and others pathways which are characterized by their dependence on small G proteins, i.e. RhoA, CDC42 or ARF6 [2,3].

The possible involvement of the physico-chemical properties of the phospholipid polar heads, such as their charge, has been suggested to affect membrane curvature in model lipid bilayers [4,5]. The curvature of the lipid bilayer membrane is primarily expressed through the spontaneous curvature of each monolayer in the coupled bilayer. The decrease in the surface charge density of only one layer is expected to enforce the membrane to adopt a new curvature [6,7]. The electrostatic repulsion between membrane phospholipid polar-heads modulates the area each polar-head occupies, while not affecting the packing order of the lipid hydrocarbon tails [8]. Hence, the local tension produced by a surface area asymmetry between the two membrane monolayers, can be rebalanced by the bilayer adapting its spontaneous curvature, since the equilibrium curvature of a membrane is the product of minimizing the membrane elasto-static and electro-static energies [4,5,9,10]. It has been theoretically argued that for a critical value of membrane surface charged density, the membrane will spontaneously bud in the absence of any applied external force [11,12].

Recently we have shown, using fluorescent and electron microscopies, flow cytometry and spectrometry, that receptor-independent endocytic-like events can be triggered by exposing the cells to external pH < 6 which leads to a highly enhanced proton-induced-uptake (PIU) of macromolecules, in the absence of specific receptor ligands or fusion peptide [13,14]. Fluorescent optical sections clearly demonstrated the homogeneous distribution of the dextran cargo in the cytosol due to an efficient endosomal escape, while the fast recycling of membrane vesicles was supported by TEM experiments [13]. Moreover, we have validated flow cytometry as the quantitative method for the uptake of the dextran by employing enzymatic and quenching methods for

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removing residual adsorbed dextran from the cell content analyses. The insensitivity of PIU to inhibiting agents of the known endocytic pathways and to ATP depletion, suggests that PIU proceeds via a new route [13]. Lowering the cell's pH induced partial disruption of the actin cytoskeleton in adherent cells and PIU was shown to depend on cytoskeleton reorganization [15]. Limited disruption of actin membrane scaffolds allows for enhanced PIU, but severe depolymerization of actin filaments impedes it [15]. The suggested mechanism for the initiation of PIU was attributed to the ability of the high proton concentrations at the external surface of the plasma membrane to neutralize part of the negative charge of the phospholipid polar-heads, thus reducing their electrostatic repulsion and consequently the area occupied per molecule. The surface asymmetry that develops across the plasma membrane in terms of surface area and charge density creates an electro-mechanic disparity, reflected by enhanced membrane tension [16], can be balanced by the membrane adapting a new conformation to its spontaneous curvature. This effect was demonstrated in studies where a pH jump triggered spontaneous vesicle formation from planar dispersions of phospholipid acid (PtdOH) [17] and the exposure of composite phospholipid vesicles to localized pulses of low pH was shown to produce the formation of inward tubular membrane invaginations [18–20]. We theorize that the membrane tension will thermodynamically favor the development of membrane invaginations in places where negative curvature values pre-exist, such as in tension lines at the border membrane microdamains of high lipid packing order [8].

In the current study we show that membrane lipid parameters such as phase order and fluidity affect the extent of PIU. We suggest that this effect is mediated by the tension energy associated with the borderline of ordered lipid microdomains.

2. Materials and methods

2.1. Solutions and reagents

Modified Karnovsky's fixative solution ($2\times$ strength), 6% paraformaldehyde, 1% glutaraldehyde and 0.2% Triton X-100 in 0.2 M cacodylate buffer; MES buffered saline (MBS), 0.1 mg/ml CaCl, 0.1 mg/ml MgCl–$6H_2O$, KCl 0.4 mg/ml, NaCl 8 mg/ml, 2.06 mg/ml MES (2-(N-morpholino)ethanesulfonic acid, hemisodium salt) and 2 mg/ml Glucose–H_2O, titrated with HCl to pH 5.2.

70 kD Dextran-FITC, Phalloidin-TRITC, Tergitol-40 (nonylphenol ethoxylate), DMSO (dimethylsulfoxide), 6KC (6-keto 3-hydroxy cholesten), MβC (methyl-β-cyclodextrin), Phloretin, Laurdan (6-dodecanoyl-2 dimethylaminonaphthalene), Cholesterol-MβC (40 mg per 1 g MβC) and TMA-DPH (1-(4-Trimethylammoniophenyl)-6-diphenyl-1,3,5-hexatriene) were purchased from Sigma-Aldrich, Rehovot, Israel.

BCECF-AM (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester) were purchased from Invitrogen, CA, USA).

2.2. Tissue culture

PBS (phosphate buffered saline), PBS (Ca^{+2} and Mg^{+2} free), DMEM (Dulbecco's Modified Eagle Medium, 4.5 mg/ml glucose), RPMI 1640 (Roswell Park Memorial Institute) culture media, HBSS (Henk's balanced salt solution), FCS (fetal calf serum), trypsin solution (0.25% with 0.05% EDTA), PSN (penicillin 10,000 unit/ml, streptomycin 10 mg/ml, nistatin 1250 unit/ml) L-glutamine solution (200 mM), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 M) and Tryphan-blue (0.4%), were purchased from Biological Industries, Beit Ha'emek, Israel). Human keratenocytes (HaCaT [21]) were cultured in DMEM, supplemented with 2 mM L-glutamine, 10% FCS and 0.2% PSN solution and were grown at 37° C, in a humid atmosphere of 5% CO_2 in air. Cells were harvested before reaching ~80% confluence by employing trypsin solution for 5 min at RT. The harvested cells were centrifuged for 2 min at 400 g. The supernatant was aspirated and the cell pellet was re-suspended in fresh growth media.

Whenever required, iso-osmolarity (290 mOs) of buffer solutions verified by osmometer (Wescor Vapro 5520, Logan, UT, USA).

2.3. Determination of Intracellular pH

The pH-dependent spectral shifts exhibited by BCECF allow calibration of the pH response in terms of the ratio of fluorescence intensity at 535 nm when using two different excitation wavelengths $\lambda_1 = 490$ nm and $\lambda_2 = 440$ nm [22]. BCECF-AM is a non-fluorescent, cell permeable derivative that is transformed by intracellular esterase into the charged, non-permeable and fluorescent BCECF molecule.

Calibration curve for BCECF excitation ratio, presented in Table 1a, shows the relative shift in the ratio of BCECF fluorescence in solution as a function of measured pH (Mettler-Toledo SevenEasy pH electrode, Columbus, OH, USA). Cell loading with 10 μM BCECF-AM was performed by incubating the cells for 30 min in HBSS at 37 °C. Following the incubation period, the cells were centrifuged at 400 g for 1 min, the solution aspirated, and the cells re-suspended in PBS. For analysis of intracellular pH, the cells were transferred to black 96 well microplates and their emission ratio at 535 nm was determined when excited at 430 nm and 490 nm, respectively (Molecular Devices M5^e, Sunnyvale, CA, USA). Table 1b shows that within 3 min from introducing 20 mM acetic acid into the wells (lowering of the external solution pH to 5), the cells possessed a stable cytoplasmic pH of 5.

2.4. Uptake studies

For the studies of adherent cultures, cells were seeded on surface treated 24 well plates and incubated in growth medium at 37 °C humid atmosphere with 5% CO_2. Experiments were performed when cells reached approximate confluence (~5×10^5 cell/well). In studies of cells in suspensions, harvesting the cells was performed before they were subjected to the treatment protocol. Cell cultures in wells were washed twice with PBS before being subjected to the experimental procedure. During the experiments, each well or vial contained 250 μl of the designated test solution. Termination of the acidic exposure was accomplished by adding 1 ml of cold DMEM into each well or vial, thus recovering the physiological pH of 7.4. Experiments were planned and conducted in a manner that enables all wells in a single plate to enter the washing step at the same time. The wells were washed twice with cold PBS and the cells were harvested by 10 min incubation with a mixture of 0.25 ml trypsin solution and 0.25 ml PBS (deprived of Ca^{2+} and Mg^{2+}) at room temperature. The harvested cells from each well were transferred to a 5 ml vial containing 1 ml cold DMEM with 10% FCS. The cells were washed twice by centrifugation and the cell pellets were re-suspended in 0.5 ml cold PBS. The fraction of cells with compromised membrane integrity was determined during FACS analysis by their disability to exclude trypan-blue from their cytoplasm (5% ± 3%, n = 300 samples).

For uptake studies involving cells enriched or depleted of cholesterol, the cells were incubated in the presence of 0.1 mM cholesterol or 10 mM MβC, respectively, for 45 min at 37 °C, followed by centrifugation (2 min at 400 g) and re-suspension in 0.3 ml of either PBS or the MBS. For uptake studies with amphipathic molecules, the cells were

Table 1a
Calibration of BCECF ratio to pH.

pH of BCEF sol.	BCECF Fl ratio	Shift in Fl ratio
7.4	7.352 ± 0.173	
6.5	3.997 ± 0.021	0.544
6	2.830 ± 0.022	0.385
5.4	2.343 ± 0.008	0.319
5.05	2.208 ± 0.01	0.300