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Spatial distribution and activity of Na $^{+}$/K $^{+}$-ATPase in lipid bilayer membranes with phase boundaries

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ABSTRACT

We have reconstituted functional Na $^{+}$/K $^{+}$-ATPase (NKA) into giant unilamellar vesicles (GUVs) of well-defined binary and ternary lipid composition including cholesterol. The activity of the membrane system can be turned on and off by ATP. The hydrolytic activity of NKA is found to depend on membrane phase, and the water relaxation in the membrane on the presence of NKA. By collapsing and fixating the GUVs onto a solid support and using high-resolution atomic-force microscopy (AFM) imaging we determine the protein orientation and spatial distribution at the single-molecule level and find that NKA is preferentially located at l_o/l_d interfaces in two-phase GUVs and homogeneously distributed in single-phase GUVs. When turned active, the membrane is found to unbind from the support suggesting that the protein function leads to softening of the membrane.

1. Introduction

For more than three decades it has been hypothesized that the meso-scale lateral organization of biological membranes is an important factor for membrane-protein interactions [1]. The lipid rafts hypothesis proposes that specialized lipid nanodomains, termed rafts, that are rich in saturated lipid chains and cholesterol form platforms for protein sorting and function [2,3,4,5,6,7]. Interestingly, from a purely membrane biophysics perspective, lipid membranes with and without proteins do show lipid-mediated lateral heterogeneity [8,9,10,11,12]. We have earlier imaged small domains of size ≈100 nm deep inside the liquid ordered (l_o)–liquid disordered (l_d) coexistence region [13], which are spontaneously generated due to low line tension ≈1 pN [12]. The domains are considered to be pseudo-critical fluctuations and are detected in the one-component saturated and mono-saturated lipid monolayers and bilayers near the main phase transition and in the ternary lipid mixture over a wide range of compositions [8,9,10,11,12,13]. These domains are suggested to provide a mechanism for lipid aggregation and stabilization of lipid–protein complexes, away from phase coexistence [14], and have major influence on bilayer mechanics and permeability [15,16]. In the present work we analyze the lateral structure of well-defined model membranes at mesoscopic and nanoscopic length scales using giant unilamellar vesicles (GUVs) composed of two or three lipid components, i.e., DOPC-chol and DOPC–DPPC-chol, containing Na $^{+}$/K $^{+}$-ATPase (NKA), a trans-membrane (TM) protein suggested to be associated with specialized domains rafts [17,18]. NKA hydrolyzes adenosine triphosphate (ATP) and uses the free energy of hydrolysis for maintaining the TM Na $^{+}$ and K $^{+}$ ions-gradients across cell membranes. The kinetics of ions transport is described by Albers–Post reaction cycle or E_1–E_2 model, where E_1 and E_2 are the two conformations of the NKA in the lipid-bilayer with high Na $^{+}$ and high K $^{+}$ affinities respectively [19,20]. The two main sub-units of the NKA are an α-subunit (containing the catalytic residues, ion occlusion, transport pathways, and inhibitor binding sites) with 10 TM segments and a single TM glycosylated β-subunit (important for protein folding, trafficking to the plasma membrane, stabilization, K-occlusion, and cell-adhesion) as shown in Fig. 1a. The bilayer can adjust in the vicinity of NKA by stretching/compressing and bending of lipids in order to match the TM hydrophobic thickness as shown in Fig. 1a. A third small, regulatory protein called FXYD is also associated. The cross sectional area of NKA varies from cytoplasmic (cyt) to extracellular (ext) side and with the conformation and is approx 12.6 nm^2 in E_1-conformation near the ext

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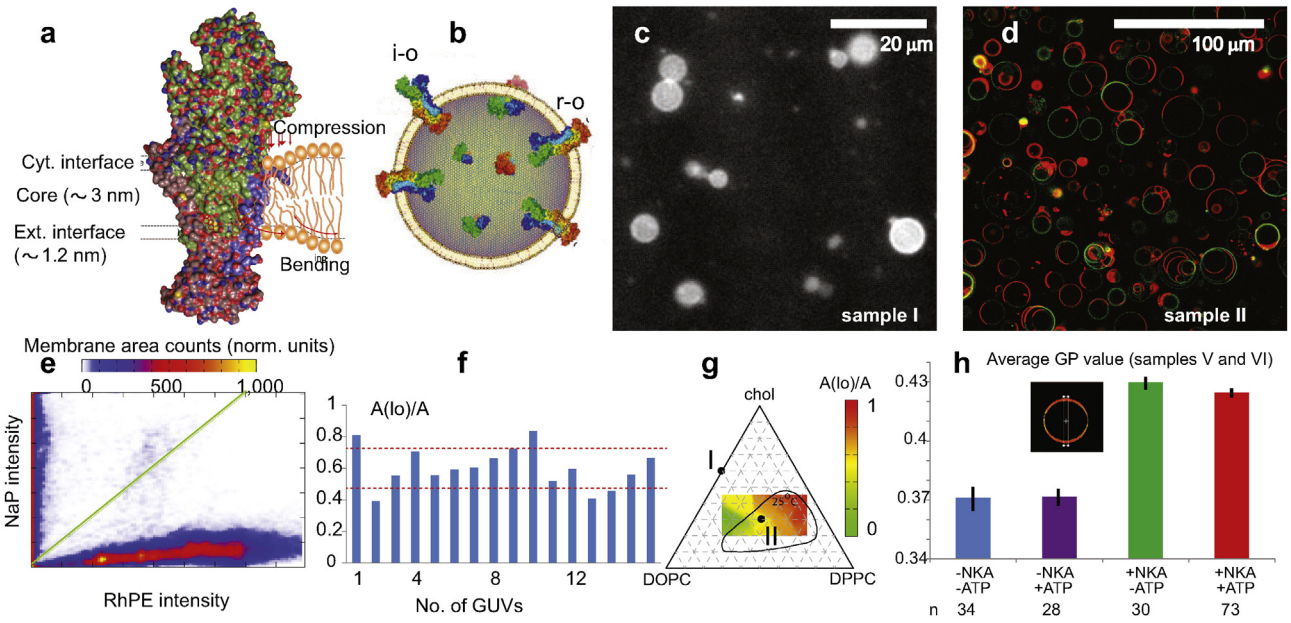

Fig. 1. NKA graphical illustration, GUVs of two- and three-component lipid mixtures, area fractions of separated phases, orientation of NKA in vesicles, and GP measurements. (a) A cartoon showing the membrane deformation adjacent to the protein and the NKA subunits: α-subunit (green), β-subunit (raspberry) and FXYD-subunit (blue). Cytoplasmic (cyt) to extracellular (ext) sides are shown (reproduced from [21]). (b) A graphical illustration of the orientations of NKA in a proteoliposome (taken from [19]). Rightside-out (r-o) is the native membrane configuration and inside-out (i-o) is opposite of the r-o orientation. A third orientation non-oriented (n-o) is not shown. (c) Epi-fluorescence image of GUVs (sample I) and (d) confocal-fluorescence image of GUVs (sample II) containing NKA settled at the bottom in an observation chamber. In ternary GUVs, the two fluorescence dyes NaP (green) and RhPE (red) are known to preferentially partition into the l_o and l_d phases, respectively. (e) A typical 2D-histogram of the fluorescence intensities of NaP and RhPE on an arbitrary vesicle surface. The green line is the threshold used to distinguish between l_o and l_d membrane domains. (f) The area-fraction of the l_o phase $A(l_o)/A$ is plotted for the 16 random GUVs (for sample II) analyzed. The standard deviation ($\pm$10.2%) is indicated by the gap between the two red-dashed lines. (g) Phase-diagram of the ternary lipid mixture displaying liquid–liquid coexistence region at 25°C, adapted from [35]. The black dots represent samples I and II. The color map display the $A(l_o)/A$ measured in GUVs prepared by dissolving lipids in organic solvent (taken from [36]). (h) The average GP value measured before and after adding ATP (to a final concentration of 2 mM) in samples V and VI, with error bars displaying standard error on the mean (SEM). The different measurements are shown by different colors; blue for GUVs, violet for GUVs with ATP added, green for GUVs containing NKA but, no ATP added and red for GUVs containing NKA with ATP added. In the inset, a selected GUV in the l_o membrane phase (sample V) displaying the Laurdan GP image at the equatorial region is shown. The white box is where the fluorescence counts for the GP are measured.

interphase [21]. From the crystal structures of NKA in the E_2-P and E_1~P.ADP conformations the cyt and ext protrusions from the membrane faces are approximately 4 nm and 8 nm [22,23]. Fig. 1b shows the possible orientations of the reconstituted NKA in the proteoliposomes: rightside-out (r-o) which is the native membrane configuration, inside-out (i-o) is the opposite of r-o orientation, and a third orientation non-oriented (n-o) has both sides exposed to the medium (not shown) [19,24,22,23]. NKA accounts for about 25% of standard metabolic rate [25] in animal cells (level reaches 70% in brain). NKA concentration in tissues varies significantly with around a 160,000-fold difference between the lowest (erythrocytes) and the highest (brain cortex) value. NKA has major contributions in brain, immune system, kidney, heart, skeletal muscles, vascular smooth muscles, erythrocytes etc. as described in [20].

The paper is organized as follows, we reconstitute NKA into GUVs of precisely controlled single-phase (l_o) and two-phase (l_o/l_d) membrane fluid phases. The measurements of protein activity and density in GUVs are discussed, followed by the investigation of the extent of water relaxation in the membrane in the active and non-active state of NKA. The spatial distribution and orientation of NKA is investigated at the single-molecule level in free-standing membranes by collapsing GUVs and rapidly immobilizing the collapsed membrane on a solid support. Unbinding of vesicle patches from the solid support is observed as a consequence of NKA activity. Finally, we discuss the implications of our work and possible adaptations of the method for other lipid–protein systems.

2. Materials and methods

1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol and 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Corden-Pharma. The fluorescence probes, N-Lissamine rhodamine B 1, 2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (RhPE), and naphthopyrene (NaP) were purchased from Molecular Probes and Sigma, respectively. Chloroform was of HPLC grade quality purchased from Rathburn (Micro-lab, Aarhus, Denmark). 8.65 mM stock solutions in chloroform of each lipid are prepared separately. Glucose and sucrose were from Sigma, NaCl (sodium chloride, purity >99.5%) was from Fluka, whereas L-histidine (purity >99%) and $MgCl_2$ (magnesium dichloride, purity >99%) were from Sigma-Aldrich. Ultra-pure MilliQ water (18.3 MOhm cm) was used in all steps involving water. The osmolarity of solutions was checked using an osmometer (Osmomat 030, Gonotec GmbH, Berlin, Germany). Four types of buffers are prepared, buffer-A (200 mM sucrose, 30 mM NaCl, 30 mM histidine, 2 mM $MgCl_2$ at pH 7) and buffer-B (200 mM glucose, 30 mM NaCl, 30 mM histidine, 2 mM $MgCl_2$, buffer-C (200 mM sucrose, 30 mM NaCl, 30 mM histidine at pH 7) and buffer-D (200 mM glucose, 30 mM NaCl, 30 mM histidine) at pH 7. Na2ATP was purchased from SIGMA and a stock solution of 1 M was prepared. The pH of the Na2ATP was adjusted to 7.4 by adding 0.5 M TRIS.

2.1. Preparation of functional proteoliposomes

NKA purified from shark rectal glands was reconstituted into small unilamellar vesicles (SUVs) [24]. Essentially, NKA and the lipids, (a) DOPC and (b) DOPC-chol (60%–40%), were co-solubilized in 130 mM NaCl, 4 mM $MgCl_2$, and 30 mM histidine, pH 7.0 at a lipid/protein weight ratio of 10 using the nonionic detergent $C_{12}E_8$ (ethylene glycol dodecyl monoether) at 4 mg/mg protein. After equilibration the