Activity of Transmembrane Proteins Induces Magnification of Shape Fluctuations of Lipid Membranes

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(Received 19 November 1998)

We report the first experimental evidence of the effect of the activity of transmembrane proteins on shape fluctuations of a lipid membrane. We incorporate a light-driven proton pump, the bacteriorhodopsin (BR), inside the phospholipid bilayer of fluctuating giant vesicles. Using the micropipet technique, we measure the excess surface area due to the fluctuations of the vesicles. The excess surface area is larger when the BR pumps protons than when it is not activated. [S0031-9007(99)09182-6]

PACS numbers: 87.16.–b, 82.65.Dp

Biological membranes are not simple lipid bilayers but consist of a complex mixture of different kinds of lipids and proteins interacting with the cell cytoskeleton and the extracellular matrix. A rich machinery associated with the activity of molecular motors, ion pumps, or channels resides within these membranes. As a first step to describe the physical properties of biological membranes, simplified model systems have been studied [1]. For instance, pure lipid membranes fluctuating around their mean equilibrium shape because of Brownian motion have been investigated both experimentally and theoretically in planar geometry as black lipid films or in spherical geometry as vesicles [2]. Studies on charged membranes [3] have shown that charge surface and electrostatic interactions have an effect on lipid bilayer bending elasticity. Multicomponent membranes with different kinds of lipids or inclusions of proteins exhibit phase separations and/or morphological changes [4]. Recently, interest has grown for polymerized membranes, mimicking the role of the cell cytoskeleton. Interactions of fluctuating membranes with polymers [5], microtubules [6], or actin shells [7] interestingly couple curvature, tension, and bending elasticity of the membrane with the rheological properties of the polymers. In all these examples, the membranes have been investigated at thermodynamic equilibrium: the only noise source for such (passive) membranes is thermal noise, which satisfies the fluctuation-dissipation theorem. At the opposite, biological membranes are clearly nonequilibrium membranes. For this reason, including a nonequilibrium behavior in physical models of complex membranes is a necessary step towards a complete description of biological membranes.

In this Letter, we study one important example of nonequilibrium behavior of biological membranes, namely, the case of active proteins embedded inside the lipid bilayer. Ion pumps, voltage, or ligand dependent channels can dissipate energy provided by concentration gradients, ATP (adenosine triphosphate) hydrolysis, or an external light source. When activated, a pump translocates ions from one side of the membrane to the other, and a channel lets ions and water flow through the membrane pore. This ion translocation or ion flow exerts a localized force on the membrane. The noise associated with these local forces is nonthermal and, as a result, does not satisfy the fluctuation-dissipation theorem. Thus, a nonthermal noise source adds to thermal noise, making biological membranes nonequilibrium (active) membranes.

From the point of view of nonequilibrium statistical physics, understanding the behavior of active membranes is challenging. Recent theoretical results [8,9] have shown that the shape fluctuations of active membranes should differ both qualitatively and quantitatively from that of passive membranes. The presence of active proteins embedded in the membrane is expected to induce a magnification of the shape fluctuations due to a modification of the fluctuation spectrum. Experiments on red blood cells [10] have demonstrated the importance of ATP as an energy source in the "flickering" phenomenon of red blood cells, questioning the classical analysis of Brochard and Lennon [11]. The role of the viscosity of the medium surrounding the membrane has also been studied on red blood cells [12]. Viscosity should play no role on the fluctuations of a passive membrane according to the fluctuation-dissipation theorem, whereas it should have an influence in the case of an active membrane, which is indeed the case for the flickering of red blood cells. Red blood cells have extensively been studied as model lipid membranes [13] but still constitute a complex model especially because of the presence of the cytoskeleton. In search for a simpler model, we have studied the shape fluctuations of giant phospholipid vesicles with an active protein, the bacteriorhodopsin (BR), incorporated inside the lipid bilayer.

The bacteriorhodopsin [14] is a 27 kDa protein, with a light-driven proton pump activity. The BR is purified from the "purple" membrane of the bacteria *Halobacterium Salinarium* using a classical procedure [15]. The BR structure and function, as well as its photocycle are well known and characterized [16]. Briefly, a photon is absorbed maximally at 566 nm and induces a change of

 0031-9007/99/82(21)/4356(4)$15.00 © 1999 The American Physical Society

conformation of the protein leading to the translocation of a proton in about 5 ms from one side of the membrane to the other. In the dark, or when illuminated at a wavelength out of the absorption band, the BR has no pumping activity. When illuminated in the absorption band, the BR exhibits a pumping activity. For instance, in the case of BR reconstituted in vesicles in the size range 0.2–0.5 μm in diameter, protons are pumped inwards indicating an asymmetric orientation of the protein [17]. This activity can be inhibited by a sufficiently large electrochemical potential gradient. Adding KCl to the solution suppresses this inhibiting gradient via passive diffusion of HCl through the membrane [18].

We used the electroformation method described in [19] to grow giant (radius >20 μm) unilamellar phospholipid [Egg Phosphatidylcholine (EPC), Avanti Polar Lipids] vesicles, modified according to [20] in order to incorporate BR in the membrane. The vesicles were grown in sucrose 50 mM and then transferred in glucose 50 mM to magnify the contrast in phase contrast microscopy. In some experiments, we also added KCl (2 mM) to both the internal and external media to get rid of a potential electrochemical gradient, and/or glycerol (16% and 25%) to increase the viscosity. Using fluorescent (FITC, fluorescein isothiocyanate) labeling of the BR [21], we observed that the intensity of fluorescence differs from one vesicle to the other, even if these vesicles are grown from the same initial dried film with a given *a priori* ratio of lipid per BR (1000 to 60). Consequently, we used the intensity of fluorescence I_F as a relative indicator of the total amount of BR incorporated inside the vesicles.

We used the micropipet technique [22] to measure the excess surface area due to the shape fluctuations of the vesicles around their mean spherical shape. The experimental setup is directly derived from that of Evans and Needham [23], including a temperature control at 15–16 °C to prevent evaporation of the vesicle solution, and an inverted microscope (Axiovert 135, Zeiss), with phase contrast or differential interference contrast optics. The pressure was measured by a pressure transducer (Validyne, SEI-3D, France) and the position of the membrane inside the pipet was followed by videomicroscopy. The vesicles were imaged and made, respectively, passive or active by shining, respectively, red (high-pass filter 650 nm) or green-yellow light (high-pass filter 500 nm) through the transillumination pathway. This provided us with a very simple system to monitor selectively the effects of the wavelength change. Fluorescence images were obtained with an argon laser (488 nm) through the epi-illumination pathway and were then quantified by computer image analysis.

In the micropipet technique, a pressure difference is applied between the inside and the outside of a micropipet creating tension in the membrane and pulling out the excess area due to the shape fluctuations inside the micropipet.

In the low-tension regime [22], for a passive membrane, the slope of the logarithm of the tension σ versus the areal strain $\alpha = \Delta S/S$ is a measure of the ratio of the bending modulus of the membrane κ over thermal energy k_BT,

$$\alpha \frac{8\pi\kappa}{k_BT} = \ln\frac{\sigma}{\sigma_i}, \tag{1}$$

where k_B is the Boltzmann constant and σ_i is the initial tension corresponding to $\alpha = 0$. This results directly from the expression of the fluctuation spectrum given by $\langle u^2(q)\rangle = k_BT/(\kappa q^4 + \sigma q^2)$ [24], where $u(q)$ is the displacement of the membrane over its mean position for a given wavelength q. Using Eq. (1) when the membrane is passive yields the value of κ. Qualitatively, because of the expected magnification of the fluctuations due to the activity of the proteins, for the same tension, we should be able to pull out more excess area when the membrane is active than when it is passive. The slope of the logarithm of the tension versus the areal strain should thus be smaller than in the passive case. For an active membrane, following [8,9], we could expect, in principle, a deviation from the logarithmic dependence. However, the micropipet technique is hardly sensitive to corrections to the logarithmic law in the accessible areal strain range. Consequently, in the following, we use Eq. (1) and the concept of effective temperature to measure the nonthermal noise strength resulting from the activity. The detailed analysis of the micropipet experiments, including a full derivation of the dependence of the tension with the areal strain for active membranes, has been performed and will be reported in another paper [25].

We first carried out micropipet experiments on vesicles containing an increasing amount of BR to investigate the influence of BR incorporation on the bending modulus of the membrane. The vesicles were illuminated with red light so that the BR was inactive. The bending modulus κ is plotted versus the fluorescence intensity on Fig. 1. For pure EPC vesicles, we reproduce the standard value of $\kappa = 10 \pm 1 k_BT$ [26]. The bending

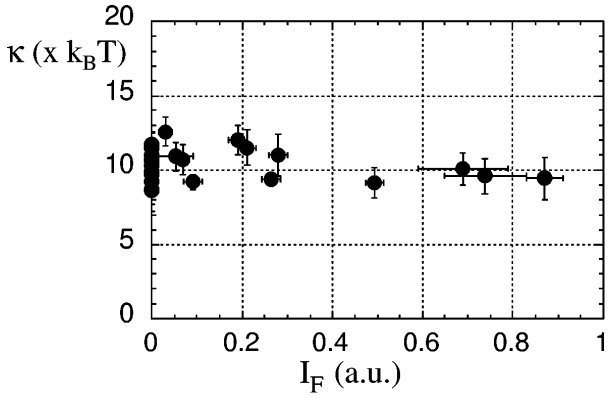

FIG. 1. Plot of the passive bending modulus κ versus the fluorescence intensity I_F (I_F is proportional to the BR concentration).

modulus does not show any measurable dependence on the BR concentration in the vesicles. This absence of bending modulus renormalization is a favorable situation for checking unambiguously the effect of activity; it is in good agreement with the absence of transition temperature shift in EPC membranes reported in [27].

For each vesicle, we have performed the micropipet experiment twice: first on the active membrane (passive), then on the passive (active) membrane, by adjusting the wavelength of the illumination. We further checked that the results do not depend on the measuring sequence. A typical plot of the logarithm of the tension versus the areal strain is given in Fig. 2a: the effective temperature T_{eff} defined above for an active membrane is about twice larger than the real temperature T of the corresponding passive membrane. The relative change of absolute temperature $\Delta T/T = (T_{\text{eff}} - T)/T$ when the membrane is activated is plotted in Fig. 2b for increasing contents of BR. For pure lipid vesicles containing no BR, no change is observed within the error bars of the method. For the lowest content of BR reached in the experiments, the effective temperature increases by about 100% and does not further depend on the concentration of BR in the vesicle, even for high protein content. At first sight, this result may look surprising. In fact, according to theory [8,9], one could expect a linear dependence of the membrane excess fluctuation in the low BR density limit. This regime is squeezed below the lowest BR concentration in our experiments. The coupling between the membrane curvature gradients and the protein fluxes may be responsible for this unexpected behavior [25,28].

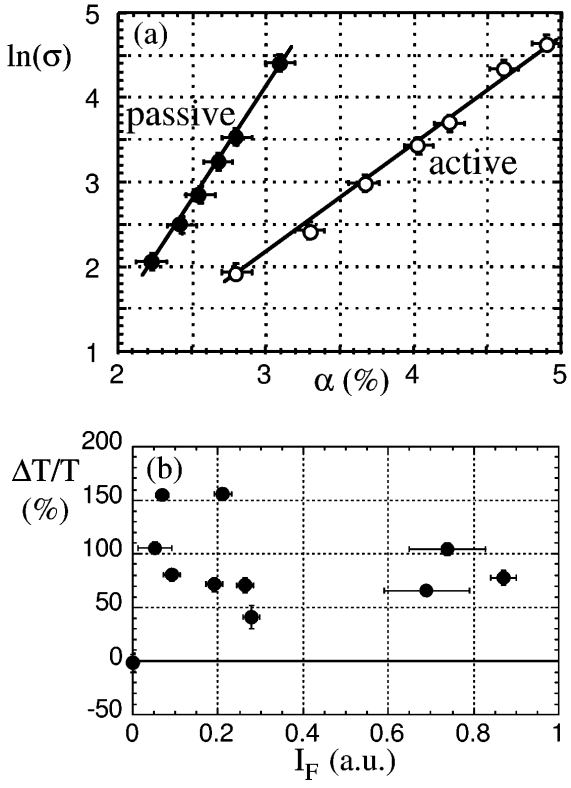

FIG. 2. Plot of the logarithm of the tension σ versus the areal strain α for one vesicle under passive and active conditions. (b) Plot of the relative change of the absolute temperature $\Delta T/T = (T_{\text{eff}} - T)/T$ versus the fluorescence intensity I_F (I_F is proportional to the BR concentration).

We also investigated the effect of the addition of salt (KCl 2 mM) to both the internal and external solutions. We did not observe any significant modulation of the effect of the activity (Table I), showing that the BR activity is not inhibited by any electrochemical gradient. On the other hand, increasing the viscosity by a factor of 2 by adding glycerol up to 25% inhibits significantly the effect (Table I). Taken together, our results prove that the activity induces an enhancement of the amplitude of the fluctuations measured by a strong increase of the effective temperature of the membrane.

In this Letter, we report the first, to our knowledge, experimental evidence of an effect of the activity of proteins on the shape fluctuations of a lipid membrane. Obviously, the lipid molecules are not responsible for this effect since no change in κ/k_BT could be detected in the control experiments with pure lipid vesicles when switching the light from yellow-green to red. We have estimated the direct temperature increase of the membrane as a result of the yellow-green photon absorption by BR, to be significantly smaller than 0.1 K. This is definitively too small to account for the strong effect observed when the BR is activated [29]. An additional argument proving the nonequilibrium origin of the observed effect concerns the role of the medium viscosity. We indeed observed a significant decrease of the effect of the BR activation when the viscosity is increased: no change at all would be detectable if the origin was purely thermodynamical.

We are gratefully indebted to J.-L. Rigaud for communicating to us his vast experience with BR, especially for the obtention and purification of BR. K. Ritchie, M. Prévost, and F. Pincet gave us technical assistance with the micropipet technique. We thank F. Jülicher for carefully reading the manuscript. We also thank E. Evans, R. Bruinsma, S. Ramaswamy, A. Parsegian, H. Strey, and P. L. Hansen for illuminating discussions.

TABLE I. Effect of the presence of 2 mM KCl and of increasing the viscosity on the relative change in absolute temperature $\Delta T/T$. The addition of glycerol yields viscosities $\eta = (1.5-2) \times \eta_w$, where η_w is the viscosity of water. The results correspond to the average ($\pm$ standard error) of at least ten independant experiments at different BR concentrations.

	$\Delta T/T$ (no BR)	$\Delta T/T$ (BR, no KCl)	$\Delta T/T$ (BR, KCl)
$\eta/\eta_w = 1$	0.018 ± 0.025	0.736 ± 0.101	0.583 ± 0.078
$\eta/\eta_w = 1.5$	...	...	0.365 ± 0.045
$\eta/\eta_w = 2$	...	...	0.200 ± 0.040

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