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ORIGINAL PAPER

Microinjection in combination with microfluorimetry to study proton diffusion along phospholipid membranes

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Abstract Proton diffusion along the surface of a planar bilayer lipid membrane was measured by means of acid/base injection with a micropipette and recording of the kinetics of fluorescence changes of fluorescein-labelled lipid on the surface. The dimensionality of the process was assayed by fitting the kinetic curves with two-dimensional (2D) or three-dimensional (3D) diffusion equations. In agreement with Serowy et al. (Biophys J 84:1031–1037, 2003), lateral proton diffusion proceeded via bulk phase by means of buffer molecules as proton carriers ($D = 600$ μm^2/s) under the conditions of 1 mM buffer in the solution. Introduction of proton binding sites on the membrane surface led to the appearance of a considerable contribution of two-dimensional proton diffusion on the membrane surface with $D = 1{,}100$ μm^2/s. The system described can be used to study the dependence of the proton diffusion rate on the phospholipid and protein composition of the membrane.

Keywords Proton transfer · Membrane surface · Unstirred layer · Fixed and mobile buffers

Abbreviations

BLM	Bilayer lipid membrane
DPhPC	Diphytanoylphosphatidylcholine
2D	Surface diffusion
3D	Bulk diffusion
Fl-SO_4	Fluorescein-5-(and-6)-sulfonic acid
Fl-PLL	Fluorescein-poly-L-lysine

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Introduction

Proton-transfer processes play an important role in biology because proton pumps are key enzymes in cell energetics (Mitchell 1966). The process of oxidative phosphorylation includes also the proton transfer between proton pumps, which promotes the study of the kinetic mechanisms of proton diffusion along the membrane surface (Mulkidjanian et al. 2006). Many theoretical and experimental investigations have been aimed at elucidation of relative contributions of bulk (3D) diffusion and enzyme/membrane surface (2D) diffusion (Tocanne and Teissie 1990; Gutman and Nachliel 1990; Georgievskii et al. 2002a, b; Cherepanov et al. 2004; Serowy et al. 2003, and references therein). The proton transfer pathway along the membrane is critically dependent on: (a) the distance between the source and the sink; (b) the composition of the solution; and (c) the composition of the membrane.

In the present paper, we used the injection of acid/base solution from a micropipette as a proton source in contrast to our previous approach using UV flash-induced release of protons from a caged-proton compound (Serowy et al. 2003; Geissler et al. 2005). This technique produced larger responses enabling us to work at higher buffer capacity of the medium (typically 1 mM). Besides, the size of the source area was considerably smaller which simplified the theoretical analysis of the data. The experimental approach apparently resembled that of Teissie et al. (1985), Prats et al. (1986) and Gabriel et al. (1994). However, there were several critical differences such as working with lipid

bilayers, not monolayers, and smaller distances of the proton diffusion measured. These differences led to considerably different results although both approaches showed the involvement of surface 2D diffusion in the process of proton transfer along the lipid-water interface.

Experimental

Horizontal planar bilayers were made from diphytanoyl-phosphatidyl-choline (DPhPC, Avanti Polar Lipids, Alabaster, AL) dissolved at 20 mg/ml in *n*-decane by a method of Mueller et al. (1963). The membrane-forming solution contained also 5% (mol of DPhPC) of *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycerol-3-phosphoethanolamine (fluorescein-DHPE, Molecular Probes). Formation of the membrane was performed by spreading this solution across a circular hole 0.2–0.3 mm in diameter in a diaphragm separating two aqueous phases of a Teflon chamber. The scheme of the experimental setup is presented in Fig. 1. The septum was pretreated with 2% DPhPC in *n*-hexane. The experimental chamber consisted of a lower compartment (2 ml) with the 50-μm distance from the transparent bottom film to the septum holding the membrane and the 0.5-ml upper compartment (Brutyan et al. 1995). The formation of black BLMs was observed visually both in transparent and fluorescent modes. The experiments were performed in the solution containing 100 mM NaCl and 1 mM HEPES, buffered at pH 6.5 or 8.5.

The chamber was placed on the top of the objective of an inverse microscope Olympus IX-70 (with a 20×). Fluorescence was excited by a 150-W Xenon lamp attached to a monochromator set at 488 nm. For fluorescence detection from the horizontally mounted BLM, a photodiode connected to a current amplifier (EPC9, HEKA electronics) was used. The observation spot had normally a square form (10 × 10 μm) via a system of diaphragms (Till Photonics).

The injection of acid/base and other substances [fluorescein-5-(and-6)-sulfonic acid (Fl-SO_4, molecular probes), fluorescein-PLL (Sigma, M.W. 29,800; Fluorescein 0.008 mole/mole lysine)] close to the membrane was carried out by means of glass pipettes with typical tip diameter of several μm [prepared by patch pipette puller (Narishige PP83)] filled with appropriate solution. The pipette was connected via a tube to a 200-μl pipette. It was important to equilibrate the pressure in the patch pipette and in the solution, which was followed by the motions of microparticles normally present in the solution and in the membrane. The sampling resulted in the fast deposition of a pipette solution producing a sphere of about 5–10 μm in diameter as judged by the visual control of fluorescence (for the case of Fl-SO_4).

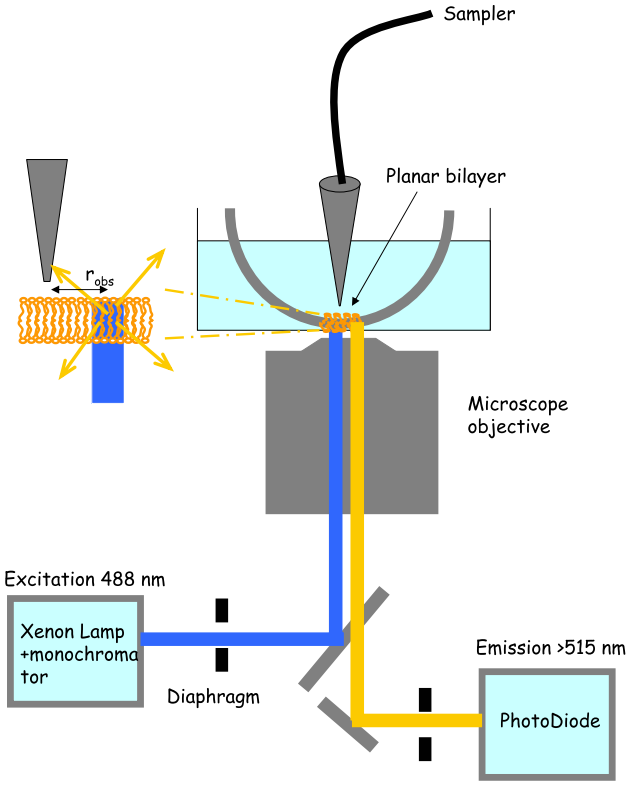

Fig. 1 Scheme of the experimental setup. Horizontal planar bilayers were formed in a chamber placed in a fluorescent microscope. The shift in local pH was carried out by microinjection using micropipette connected to a sampler. The fluorescence from the observation area was collected by a system of diaphragms

Results and discussion

The procedure of the injection of acid or base in a region close to the membrane was developed by using a water-soluble fluorescein analogue, fluorescein-5-(and-6)- sulfonic acid (Fl-SO_4). Figure 2 shows the kinetics of fluorescence changes at different distances from the pipette, r_{obs}, namely 90 and 170 μm for panels A and B, respectively, which were elicited by the pressure pulse in the pipette with Fl-SO_4 solution at $t = 0$. In these experiments we used a pure DPhPC membrane without fluorescein-DHPE and measured the diffusion of a marker (Fl-SO_4) from the pipette tip to the observation spot located at different distances r_{obs}. The increase in the distance resulted in deceleration of the kinetic curves which can be nevertheless well fitted by the equation for three-dimensional (3D) diffusion (Eq. 1, grey lines in Fig. 2) with similar diffusion coefficients D being equal on average to 300 ± 20 $\mu m^2/s$.

$$F(t) = \frac{A}{(Dt)^{3/2}} \exp(-r_{obs}^2/4Dt) \quad (1)$$

Figure 3 shows the results of the experiment when fluorescein-labeled polylysine was added in the pipette.

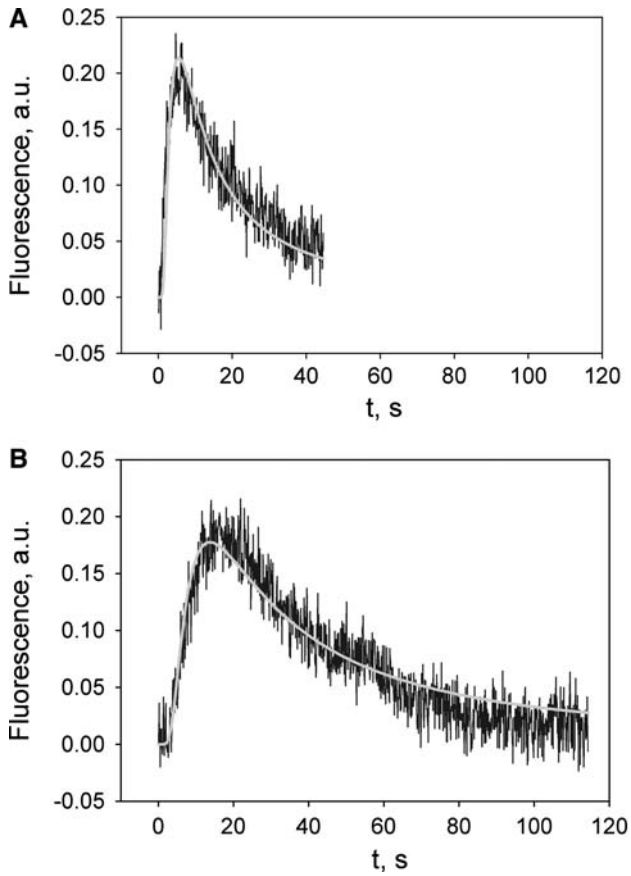

Fig. 2 Kinetics fluorescence changes corresponding to sulfated fluorescein (Fl-SO_4) diffusion from the pipette to the selected region of the planar membrane. The measurements were carried out at two distances, $r_{obs} = 90$ μm (**a**), and 170 μm (**b**). *Grey curves* are best fits of the experimental curves by Eq. 1 with $D = 260$ μm^2/s (**a**), and 340 μm^2/s (**b**). Concentration of Fl-SO_4 in the pipette was 0.03 mg/ml. The solution was 100 mM NaCl, 1 mM HEPES, pH 8.5

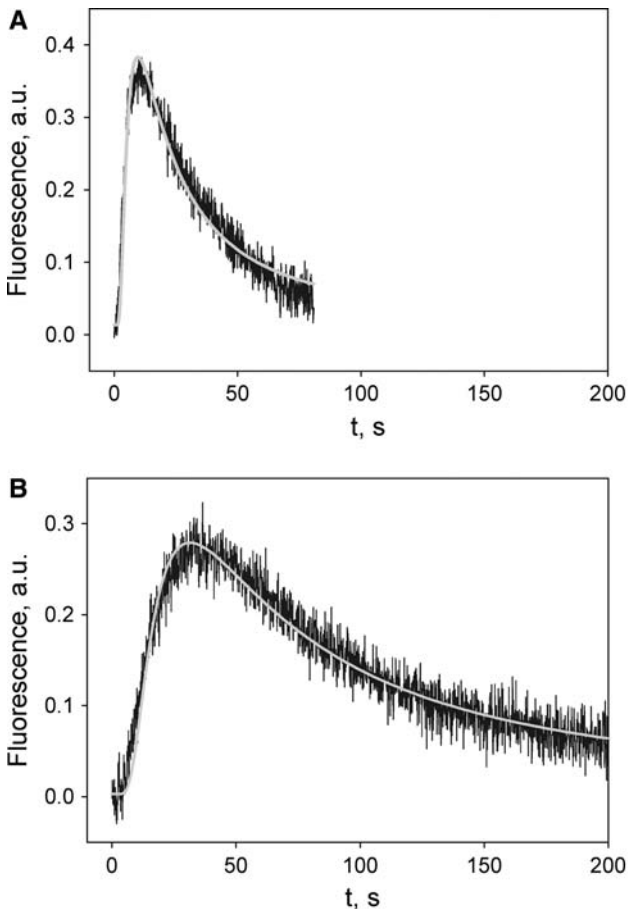

Fig. 3 Kinetics fluorescence changes corresponding to sulfated fluorescein (Fl-SO_4) diffusion (**a**) and FITS-PLL diffusion (**b**) from the pipette to the selected region of the planar membrane. The measurements were carried out at $r_{obs} = 125$ μm. *Grey curves* are best fits of the experimental curves by Eq. 1 with $D = 290$ μm^2/s (**a**), and 85 μm^2/s (**b**). Concentration of Fl-SO_4 and FITS-PLL in the pipette was 0.03 mg/ml (**a**) and 1 mg/ml (**b**). The solution was 100 mM NaCl, 1 mM HEPES, pH 8.5

The comparison of the fluorescence kinetics of these two markers obtained at approximately equal distances showed that the increase in the molecular weight (polylysine had M.W. of 30,000) led to substantial deceleration of the diffusion kinetics. This deceleration can be quantitatively estimated by the decrease in the diffusion coefficient to 110 ± 20 μm^2/s.

The diffusion coefficients were in good agreement with the literature data. The diffusion coefficient of biotin-fluorescein was estimated to be 340 μm^2/s (Kamholz et al. 2001). The diffusion coefficient of a polypeptide with molecular weight of 30,000 should be about 100 μm^2/s according to (Dubin et al. 1967). It can be concluded that the process of injection of the marker in the region close to the membrane did not induce the convective flows in the system and the mass transfer here was determined by the diffusion only.

In order to measure the proton diffusion along the membrane surface, fluorescein-labeled lipid (Fl-PE) was added to the membrane. The fluorescence of fluorescein is strongly pH-dependent being higher at alkaline pH. Two types of experiments were carried out: (a) acid injection at pH 8.5 (Fig. 4a), and (b) base injection at pH 6.5 (Fig. 4b). The comparison of these data with those of Figs. 2 and 3 shows that the proton diffusion proceeded faster compared to Fl-SO_4 or Fl-PLL and that the kinetic curves can be quite well fitted by Eq. 1 for 3D diffusion with $D = 600$ μm^2/s (grey curves).

Figure 5 shows the dependence of the proton diffusion curves on the distance r_{obs} between the injection and observation areas. Experimental data were averaged over 5–7 repeats of each experiment. The kinetic curves were fitted both by the Eq. 1 (blue curves) and Eq.2 (2D diffusion, red curves).

$$F(t) = \frac{A}{(Dt)} \exp(-r_{obs}^2/4Dt) \quad (2)$$

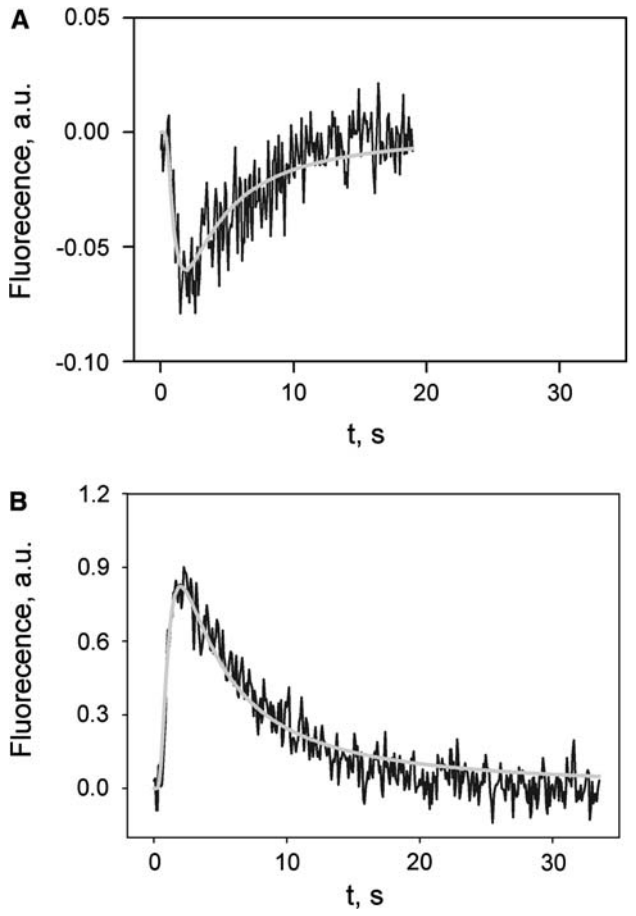

Fig. 4 Kinetics of protonation/deprotonation of Fl-PE on the surface of the membrane as a result of diffusion of protons from the pipette to the selected region of the planar membrane. The measurements were carried out at different pH of the bathing solution [8.5 in (**a**) and 6.5 in (**b**)]. The pipette contained a solution of acidic solution of 0.2 M HEPES and was at $r_{obs} = 80$ μm away from the recording area (**a**); and alkaline solution of 0.2 M TRIS and was at $r_{obs} = 90$ μm away from the recording area (**b**). *Grey curves* are best 3D fits (Eq. 1) of the experimental curves with $D = 560$ μm^2/s (**a**), and 650 μm^2/s (**b**). The membrane-bathing solution was 100 mM NaCl, 1 mM HEPES

The sums of square deviations from the mean were 5.6, 8.1, and 3.3 times lower for 3D fit compared to 2D fit for panels A, B, and C, respectively. The averaged diffusion coefficient was about 600 μm^2/s. This value of D corresponds to the diffusion coefficient of HEPES used in these experiments as a buffer. These data show that the proton diffusion represented mostly 3D diffusion via the buffer molecules under these conditions in agreement with Serowy et al. 2003.

Proton diffusion along the membrane should strongly depend on the presence of proton-binding sites on the surface. Serowy et al. (2003) used A23187 to increase the number of the binding sites in their experiments. Actually,

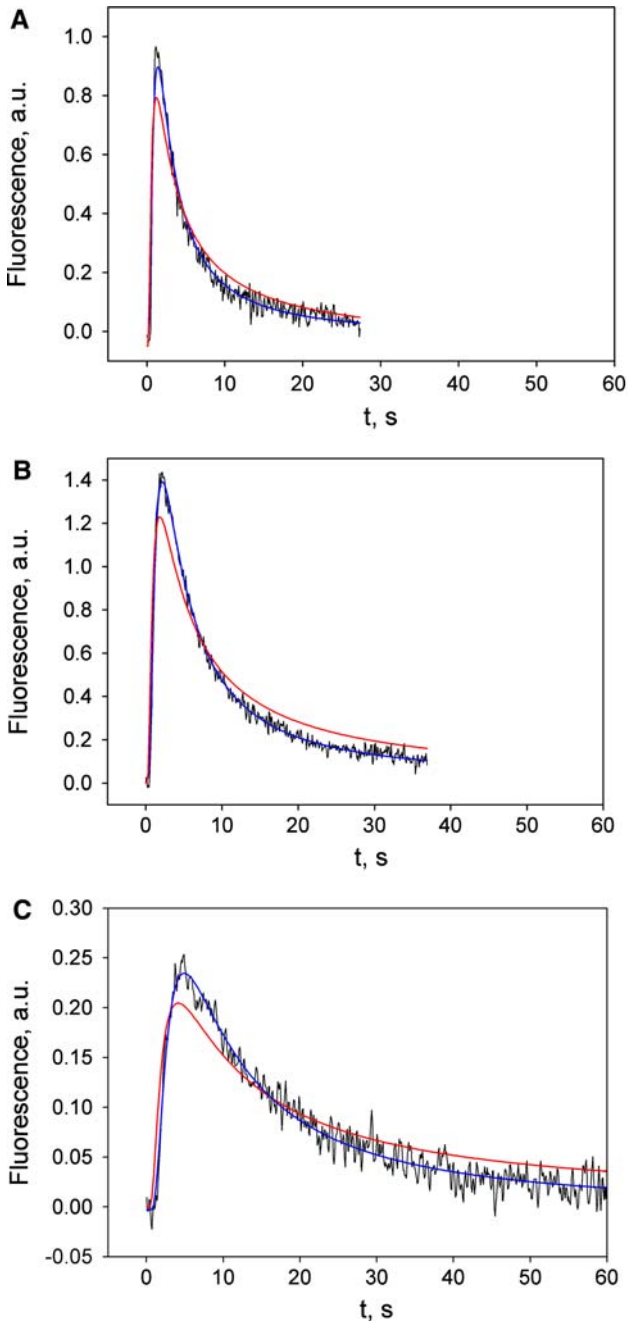

Fig. 5 Kinetics fluorescence changes corresponding to the diffusion of protons from the pipette to the selected region of the planar membrane. The measurements were carried out at different distances: $r_{obs} = 70$ μm (**a**), 90 μm (**b**), and 130 μm (**c**). Experimental curves were collected for the same conditions (5–9 measurements) and averaged. *Blue curves* are best 3D fits (Eq. 1) of the experimental curves with $D = 580$ μm^2/s (**a**), 630 μm^2/s (**b**) and 560 μm^2/s (**c**). *Red curves* are best 2D fits (Eq. 2) of the experimental curves with $D_s = 990$ μm^2/s (**a**), 1,100 μm^2/s (**b**) and 1,000 μm^2/s (**c**). The pipette contained a solution of 0.2 M TRIS. The membrane-bathing solution was 100 mM NaCl, 1 mM HEPES, pH 6.5

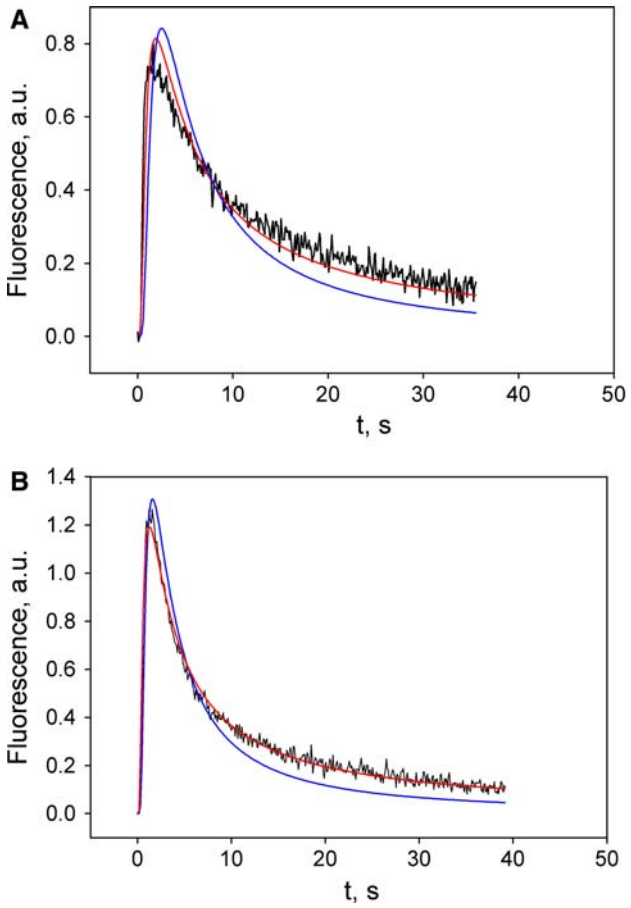

Fig. 6 Kinetics fluorescence changes corresponding to the diffusion of protons from the pipette to the selected region of the planar membrane in the presence of 10 μM A23187 (**a**) and both A23187 and 1 mM $CaCl_2$ (**b**). The measurements were carried out at $r_{obs} = 70$ μm. Experimental curves were collected for the same conditions (5–7 measurements) and averaged. *Blue curves* are best 3D fits (Eq. 1) of the experimental curves with $D = 330$ μm^2/s (**a**), and 510 μm^2/s (**b**). *Red curves* are best 2D fits (Eq. 2) of the experimental curves with $D_s = 630$ μm^2/s (**a**), and 940 μm^2/s (**b**). The pipette contained a solution of 0.2 M TRIS. The membrane-bathing solution was 100 mM NaCl, 1 mM HEPES, pH 6.5

the addition of A23187 to the experimental system changed dramatically the character of the kinetic curves (Fig. 6a). This lipophylic compound carries one carboxylic group, which can effectively bind protons and calcium ions (Pfeiffer and Lardy 1976). Kinetic curves were fitted substantially better by Eq. 2 compared to Eq. 1, i.e. the sum of square deviations from the mean was 5.1 times higher for Eq. 1 compared to that for Eq. 2. This suggests that protons diffused preferentially via membrane surface with $D = 700$ μm^2/s. The addition of calcium ions shifted the experimental curves further toward shorter times (Fig. 6b). The diffusion coefficient averaged for the measurements at different distances was estimated to be 1,100 μm^2/s (using Eq. 2). Again the sum of square deviations from the mean was much higher (9.9 times) for Eq. 1 compared to that for Eq. 2. The increase in the diffusion coefficient can be accounted for by the competition for the binding sites between protons and calcium ions. Importantly, the process of diffusion was two-dimensional even in the presence of $CaCl_2$. This shows that the proton pathway along the membrane can prevail over the transfer to the bulk solution even under the conditions of the decrease in the proton affinity of the membrane surface suggesting an existence of the barrier for proton transfer from the surface to the bulk in accordance to (Antonenko et al. 1993; Heberle et al. 1994; Cherepanov et al. 2004).

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