Biochimica et Biophysica Acta, 1150 (1993) 45–50

BBAMEM 76023

Evidence in favor of the existence of a kinetic barrier for proton transfer from a surface of bilayer phospholipid membrane to bulk water

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(Received 6 November 1992)

Key words: Proton transfer; Bilayer lipid membrane; Nigericin; Monensin; Energy coupling; Boundary potential difference

When the hydrogen-ion flux is induced by nigericin across the planar bilayer lipid membrane (BLM) with bulk pH values being equal at the opposite sides of the BLM, formation of a difference in boundary potentials ($\Delta\phi_b$) on the membrane is observed by the method of inner membrane field compensation. pH gradients are titrated routinely by the addition of sodium acetate at one side of the membrane. The increase in buffer concentration (citrate, phosphate, Mes) leads to a decrease in $\Delta\phi_b$. $\Delta\phi_b$ forms in the presence of phosphatidylserine in the membrane-forming solution only. It is concluded that the steady-state difference of the hydrogen ion binding to the opposite surfaces of the membrane (HIBD) is created under the conditions of equal pH values near surfaces of the BLM. The model of the processes implies that nigericin transfers proton predominantly from interface to interface while acetate transfers the proton from bulk phase to bulk phase. In the other series of experiments the monensin-mediated formation of the HIBD leads to the formation of an potassium-ion gradient in the presence of nigericin. Thus, a possibility of performing a work due to the formation of HIBD is demonstrated. Owing to these properties the hydrogen-ion binding difference can be interpreted in a first approximation as a difference of surface hydrogen-ion concentration at the opposite sides of the membrane, arising due to the existence of a kinetic barrier for the proton transfer at the membrane interfaces. These findings can be significant for the mechanism of energy transduction in membrane phosphorylation in mitochondria and chloroplasts.

Introduction

The processes of proton transfer in membranes attract much attention, since hydrogen ions are involved directly in the processes of energy transformation in membrane phosphorylation in mitochondria and chloroplasts [1]. In this connection the question of the existence of a barrier to the proton transfer from the surface of the membrane to the adjacent water phase is considered to be important. In a series of theoretical and experimental studies the possibility of lateral proton conduction along the phospholipid/water interface was postulated and demonstrated [2–6]. This phenomenon favors the existence of the barrier to proton at the interface and supports the idea of the involvement of lateral proton pathway in the coupling of electron transfer and phosphorylation in mitochondria and chloroplasts (for reviews see Refs. 7,8). In a recent paper of Heberle and Dencher the existance of the kinetic barrier for proton transfer at the interface was demonstrated for a case of the comparatively simple proton pump *Bacteriorhodopsin* [9]. However, the reliability of these results was questioned [10,11]. Besides in experiments of Gutman and co-workers [12,13] the presence of the barrier contradicts with the experimental data. In the present work, we used the model system described by us previously [14] where the nigericin-induced hydrogen ion flux through a planar bilayer phospholipid membrane (BLM) depended substantially on the buffer concentration in the solutions. Our study supports the idea of the existence of a kinetic barrier for proton transfer from the membrane to the bulk water.

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Abbreviations: BLM, bilayer lipid membrane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $\Delta\phi_b$, the difference of boundary potentials across BLM; TTFB, tetrachlorotrifluoromethylbenzimidazole; HIBD, the hydrogen-ion binding difference at the opposite sides of the membrane.

Materials and Methods

BLM is formed on a Teflon partition 0.4 mm in diameter, by a conventional method [15]. Typically, a membrane-forming solution contains 20 mg phosphatidylcholine from soy beans (asolectin, Sigma) and 10 mg cholesterol (Serva) in 1 ml of n-decane unless otherwise stated. The thinning of the BLM is observed both visually and by measuring its capacity. The experiments were carried out at room temperature (21–23°C). Nigericin and monensin are from Calbiochem, buffers citrate, Mes, Tris are from Serva. D_2O is from Merck. Phosphatidylserine is from Sigma, Diphytanoylphosphatidylcholine from Avanti Polar Lipids. Other chemicals are from Reachim (Russia). The hydrogen ion electroneutral flux (J_H) induced by nigericin in the presence of potassium ion gradient is measured by the method described earlier [16]. Briefly, the pH gradient in the unstirred layers (calculated from the membrane potential in the presence of a protonophore TTFB) is reduced to zero by means of the formation of the gradient of acetic acid across the BLM. Under these conditions J_H is equal to the acetate flux. In our recent experiments, we compared the values of pH gradients determined from potentials in the presence of a protonophore and directly by pH microelectrode. A quantitative agreement was observed [17]. Acetate concentrations are converted into J_H according to Ref. 16. The solutions were stirred by magnetic bars, the rate of the stirring were constant throughout all experiments.

The difference of boundary potentials $\Delta\phi_b$ is measured by the method of inner membrane field compensation (IFC method) by recording of a capacitive current of double-frequency according to Ref. 18. The electric scheme includes a generator (G3-112, Russia, 395 Hz cycle is used) in series with CD offset (B5-43, Russia), a filter of double frequency (Band-pass filter type 1617, Brüel and Kjær, Denmark), a cell for the BLM with two reference electrodes, an amplifier (Keithley 301 (USA), the feedback resistance is 1 MΩ), a home-made active filter of first frequency, a selective amplifier set at 790 Hz (Selective nanovoltmeter type 237, Unipan, Poland) and an oscilloscope. This method can not be applied to the measurements of $\Delta\phi_b$ in the

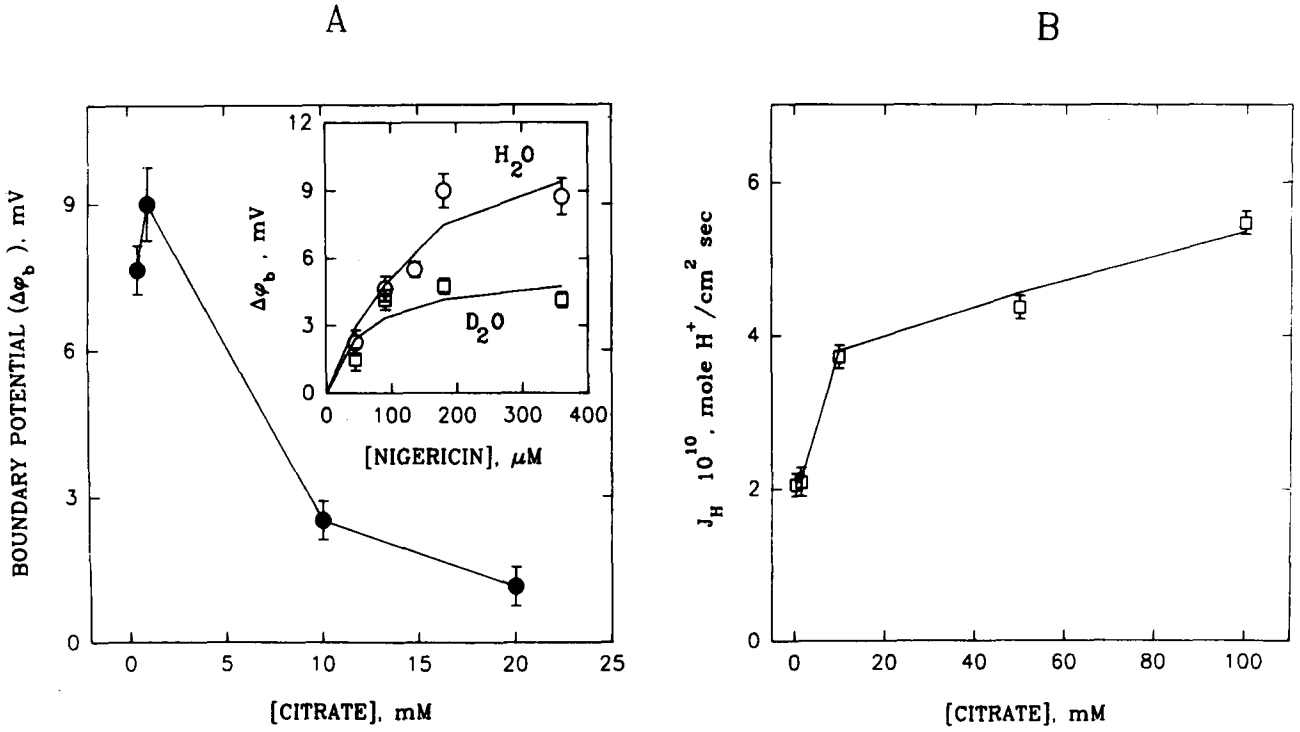

Fig. 1. (A) The effect of citrate buffer concentration on the boundary potential difference of BLM ($\Delta\phi_b$) induced by nigericin-mediated K^+/H^+ exchange. $\Delta\phi_b$ was measured by the inner membrane field compensation method [18]. The potassium ion gradient was 95 mM on one side (*cis*) and 5 mM on the other. The choline chloride concentration varied in a way that the total concentration of citrate and choline chloride was 20 mM (pH 6.5). The nigericin concentration in the membrane-forming solution was 180 μM. Inset: the effect of nigericin concentration in the membrane-forming solution on the $\Delta\phi_b$. The solution was 1 mM citrate, 95 mM KCl on *cis* side and 5 mM KCl on the *trans* side, 20 mM choline chloride (pH 6.5). Curve 1, solution in H_2O, curve 2, D_2O. Lines were drawn by eye. (B) The effect of citrate buffer on the nigericin-induced electroneutral hydrogen-ion flux through the BLM. The flux was measured under the conditions of zero pH gradient on the membrane by adding acetate on the cis side of the BLM according to Ref. 16. The KCl gradient was 95 mM to 5 mM. The choline chloride concentration varied in a way that the total concentration of citrate and choline chloride was 100 mM (pH 6.5). The nigericin concentration in the membrane forming solution was 180 μM.

presence of an electrogenic ionophore which increases the conductivity of BLM [18]. Since neither nigericin nor monensin increases the conductivity of BLM under our experimental conditions, we consider them to induce electroneutral fluxes only.

Results and Discussion

Fig. 1A shows the effect of citrate buffer concentration on the boundary potential difference on BLM ($\Delta\phi_b$) under the conditions of the operation of a K^+/H^+ exchanger nigericin in the presence of a potassium-ion gradient on the membrane. The nigericin-induced values of $\Delta\phi_b$ are calculated by subtracting the $\Delta\phi_b$ values found without nigericin from the $\Delta\phi_b$ values found with nigericin. $\Delta\phi_b$ decreases with the increase in the citrate concentration (Fig. 1A). The plus sign is on the *cis* side of the BLM (where the KCl concentration is higher). It should be pointed out that the measurements of $\Delta\phi_b$ are carried out at zero pH gradient on the membrane which is normally formed under the conditions of K^+/H^+ exchange process [16]. pH gradients were zeroed routinely by the addition of sodium acetate on the *cis* side of the BLM [16]. The value of $\Delta\phi_b$ practically does not change after addition of sodium acetate on the *cis* side of BLM (data not shown). The decrease in $\Delta\phi_b$ with the increase of citrate concentration (Fig. 1A) is not due to the increase of ionic strength of the solution, since the increase in the citrate concentration is accompanied by the decrease in choline chloride concentration. Qualitatively similar results are obtained with other buffer solutions, phosphate and Mes, namely 3.0 mV and 6.0 mV at 1 mM and 1.0 mV and 1.0 mV at 20 mM phospate and Mes, respectively. The decrease in $\Delta\phi_b$ upon the increase in citrate concentration is observed also under the conditions of monensin-mediated Na^+/H^+ exchange in the presence of NaCl gradient (3.5 mV at 1 mM citrate and 1.7 mV at 10 mM citrate). The inset of Fig. 1A shows the dependence of $\Delta\phi_b$ on the nigericin concentration in solutions of H_2O and D_2O. The maximum isotope effect is observed at high nigericin concentration. The effect of D_2O along with the effect buffer indicates that hydrogen ions are involved in the process. All experiments described are carried out on membranes formed from asolectin, the natural mixture of phospholipids purified from soy beans. Experiments with the BLM formed from phosphatidylcholine (to be more precise, synthetic diphytanoylphosphatidylcholine) show that $\Delta\phi_b$ is not elicited (under the conditions of Fig. 1A, the experimental error is 0.5 mV). However, $\Delta\phi_b$ is generated on membranes formed from 1:1 mixtures of diphytanoylphosphatidylcholine and phosphatidylserine ($\Delta\phi_b = 6.5$ mV at 1 mM citrate).

Two possibilities of nigericin-induced steady-state $\Delta\phi_b$ generation should be discussed. (1) A case of slow proton transfer between carboxylic groups of nigericin and acidic-basic groups of phospholipids (fast proton exchange between nigericin and bulk water). In this case the value of $\Delta\phi_b$ is determined by the difference of steady-state concentrations of charged forms of nigericin (mainly anionic) at different surfaces of the membrane under the conditions of nigericin turnover. (2) A case of fast proton transfer between carboxylic groups of nigericin and acidic-basic groups of phospholipids on the surfaces of BLM. This is possible if the transfer of protons from nigericin to bulk water is hindered by some kind of a kinetic barrier. In this case the occupation of the membrane surface by hydrogen ions and/or the opposite surface by hydroxyl ions should occur under the conditions of nigericin-induced transmembrane hydrogen ion flux. To decide which case is realized in our experiments we should compare the results of $\Delta\phi_b$ measurements on membranes formed from different phospholipids. The surface of the membrane formed from phosphatidylcholine is covered by weak bases only, phosphate groups, the proton binding ability of which is less than that of carboxylic groups of nigericin molecules [19]. So the access of protons released from nigericin to the surface of the BLM is highly restricted under these conditions. That is why there is no $\Delta\phi_b$ formation on these membranes in the experiment. The pK value of the carboxylic group of phosphatidylserine is similar to the pK of nigericin and the presence of this lipid in the membrane correlates with the phenomenon of $\Delta\phi_b$ formation. Since the membrane composition (the other conditions being equal) should not substantially alter the rate of nigericin turnover in the membrane one can say conclusively that the $\Delta\phi_b$ formation is due to fast lateral proton transfer between carboxylic groups of nigericin and acidic-basic groups of phospholipids on the surface of BLM, in particular the carboxylic groups of phosphatidylserine. The decrease in $\Delta\phi_b$ with the increase in concentration of citrate (Fig. 1A), as well as of phosphate or Mes concomitant with the increase in hydrogen ion flux across the membrane (Fig. 1B) can be accounted for by the process of catalysis by these compounds of proton transfer from acidic-basic groups of phospholipids on the membrane surface to water near the membrane.

We have carried out an independent set of experiments where in the absence of nigericin we have measured $\Delta\phi_b$ on the BLM formed from asolectin upon the change of pH at one side of the membrane. It is shown that the shift of pH from 6.5 to 5.1 induces the $\Delta\phi_b$ formation of 9 mV and -7.5 mV at pH shift from 6.5 to 8.3. Thus these results show that the membrane has enough acidic-basic groups to elicit the difference of boundary potentials of the observed values. We have not observed $\Delta\phi_b$ formation on membranes formed

from diphytanoylphosphatidylcholine in the above range of pH shifts. Small values of $\Delta\phi_b$ (−1.5 mV) were observed upon pH shift from 6.5 to 9.0.

Fig. 1B shows the effect of citrate buffer concentration on the hydrogen ion flux across the membrane induced by nigericin in the presence of KCl concentration gradient under the conditions of zero pH gradient on BLM. It is seen that the flux increases with the increase in citrate concentration in accordance with our previous results [14]. The increase in the flux correlates with the decrease in the difference of boundary potentials which enables us to conclude that the increase in the flux is a result of the decrease in the difference of hydrogen ions binding at the opposite BLM surface (HIBD). This correlation shows that the HIBD determines the transmembrane hydrogen-ion flux induced by nigericin under our experimental conditions. It is worth noting that fluxes measured on the membrane formed from diphytanoylphosphatidylcholine are substantially lower than the fluxes on the BLM formed from asolectin under the same conditions (data not shown). It can be proposed that in the former case the surface of BLM is not covered with hydrogen ions and the area of the interaction of citrate molecules with bound protons is reduced.

It seems important to show the possibility of performing work by the difference in the hydrogen ions binding at the opposite surfaces of the BLM, since these protons can carry the additional energy. It was shown previously by us [20] that in the presence of sodium ion gradient on BLM the potassium ion gradient was formed upon the addition of both monensin and nigericin. We estimated the value of the potassium ion gradient by measuring the open circuit potential in the presence of valinomycin. The coupling of Na^+/H^+- and H^+/K^+-exchangers was a result of the formation of pH gradient in the unstirred layers by monensin [20]. In the present work we have repeated the same kind of experiments with lithium acetate (monensin and nigericin both have poor affinity to lithium ions) being added on the *cis* side of the membrane for zeroing the pH gradient on the membrane. The concentrations of lithium acetate are chosen in independent experiments so that the BLM potentials in the presence of a protonophore and monensin are zero. Under these conditions the only energy source for the formation of the potassium ion gradient is the difference of hydrogen ions bound at the opposite surfaces of BLM.

Fig. 2, curve 1 shows an example of a trace of BLM potential formation in the presence of valinomycin under the conditions described above. It is seen that after the complete titration of pH gradient with lithium acetate the bulk sodium-ion gradient continues to transform into potassium-ion gradient in the unstirred layers. Control experiments show that potentials do not form in the presence of only one ionophore (nigericin

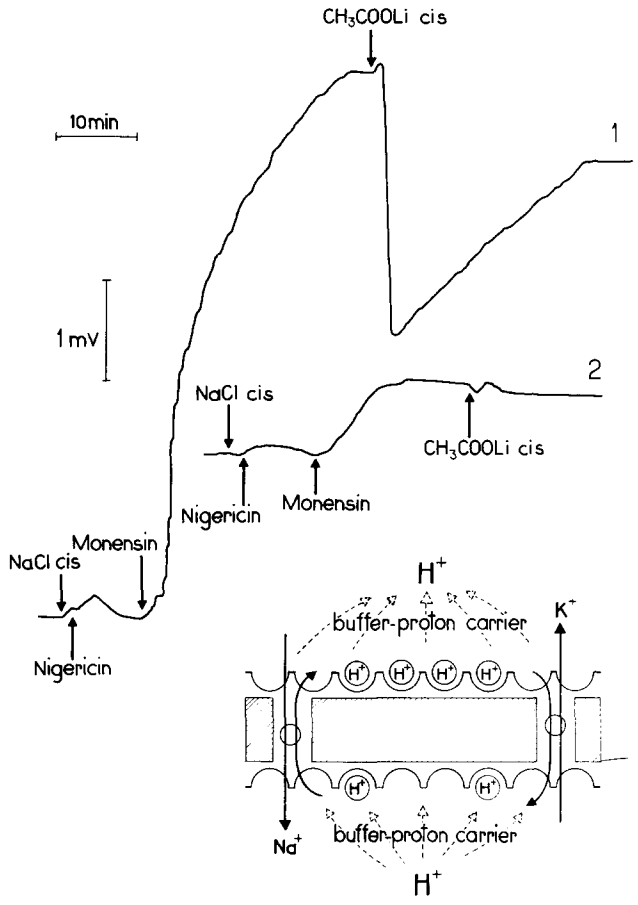

Fig. 2. The example of the generation of the BLM potential in the presence of valinomycin, monensin and nigericin upon the formation of NaCl gradient on the membrane (95 mM *cis* and 5 mM *trans*) in the absence of pH gradient on BLM. Curve 1, the solution was 1 mM citrate, 0.5 mM KCl, 50 mM choline chloride (pH 6.5). Curve 2, 50 mM citrate, 0.5 mM KCl (pH 6.5). Ionophores valinomycin (added before the start of the recording), nigericin and monensin were added in bathing solutions in concentrations 1 μM, 1 μM and 0.1 μM, respectively. Inset: the scheme of the process of coupling of monensin-mediated Na^+/H^+-exchange and nigericin-mediated K^+/H^+-exchange via the gradient of surface proton concentration (H^+ in circles).

or monensin). The value of the potential diminishes as expected with the increase in the citrate concentration which reduces the HIBD (Fig. 2, curve 2). Mes buffer acts in a similar way (data not shown). We measured the difference of boundary potentials in the absence of valinomycin in parallel experiments: $\Delta\phi_b = 7$ mV at 1 mM citrate and $\Delta\phi_b = 1.5$ mV at 50 mM citrate. The correlation between $\Delta\phi_b$ and potassium-ion gradient formation shows that the energy source for the formation of the potassium-ion gradient in our system is the difference in hydrogen-ion binding at opposite surfaces of BLM elicited by Na^+/H^+ exchange.

Control experiments with membranes formed from diphytanoylphosphatidylcholine, which lack the ability to form the HIBD (see above), show that there is no

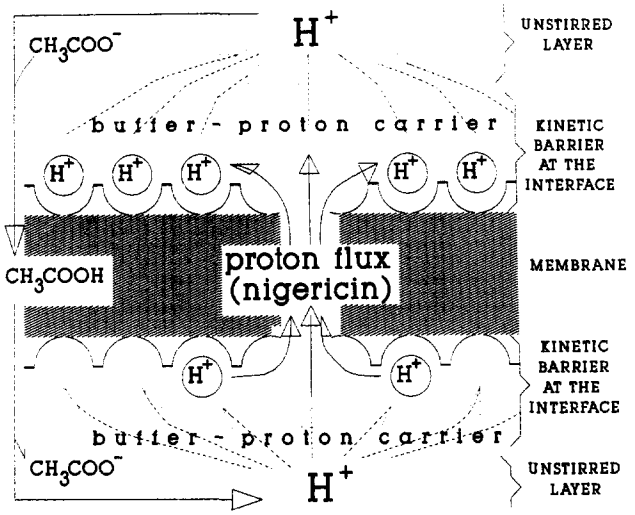

Fig. 3. Scheme of the process of the formation of the difference of hydrogen-ion binding to the opposite surfaces of the membrane (H^+ in circles) induced by the nigericin-mediated transmembrane hydrogen ion flux. Free and buffer bound protons in the solution are transferred to the opposite side of the membrane by acetate in a way that the pH difference is maintained constant. Buffer molecules play a dual role in the system: first, protons source and sink in the solution and second, carriers in the process of the proton transfer from the surface of the membrane to acetate in the bulk solution.

process of coupling of monensin and nigericin operation under the conditions of Fig. 1A (data not shown).

Fig. 3 shows the scheme of the process of the formation of the difference in the hydrogen-ion binding at the opposite surfaces of the membrane (H^+ in circles) under the conditions of equal bulk pH values in both solutions bathing the membrane (H^+ without circles) which is induced by the nigericin (monensin)-mediated transmembrane hydrogen-ion flux. Acetate transfers protons from one bulk phase to the other (to be more precise from one unstirred layer to the opposite unstirred layer) across the membrane without affecting the bound protons while nigericin transfers protons from one interface to the other. Buffer molecules (citrate, Mes, phosphate) play a dual role in the system. In the unstirred layers near the membrane they reduce the steady-state pH shifts [20]. This action of buffers differs from their routine effect in a single-phase system, since it involves the diffusion through the unstirred layers [21,22]. In the present work, in agreement with the results of Refs. 12 and 13, it was shown that buffer molecules can play a role of catalyzers, carriers of protons by facilitating the acid-base equilibration between the interface and bulk water. The catalytic effect of buffer molecules was clearly demonstrated by the experiments published in our previous paper [14] where after the titration of a nigericin-induced pH gradient with acetate the addition of an extra amount of buffer led again to the appearance of the pH gradient. Thus, instead of decreasing pH gradients, the addition of buffer induced the 'paradoxical' phenomenon of increasing the pH gradient on BLM. The catalytic effect of buffer molecules confirms the existence of the kinetic barrier for protons at the interface which is concluded from the phenomenon of the formation of the hydrogen-ion binding difference at the opposite sides of the membrane. The phenomenon of the formation of the HIBD can be interpreted as the formation of the gradient of the surface proton concentration at the opposite sides of the membrane. This term (surface proton concentration) implies that the protons bound to the membrane can diffuse freely along the membrane. This situation was described in works of Teissié and co-workers [4] and Morgan and co-workers [5]. The real situation should be complicated by the different binding abilities of acidic-basic groups at the surface of the membrane characterized by their different pK values.

The most closely-related system described in the literature is a system for the measurement of lateral proton conductance, as described in a review by Tocanne and Teissié [4]. In our system, a lateral proton transfer from nigericin to proton-acceptor groups at the membrane surface is essential as well. It is interesting to note that contrary to our findings the lateral proton conductance was shown to be independent of the phospholipid headgroup structure [4]. This apparent contradiction can be accounted for by the significant differences between these two systems. In particular, our system includes the transmembrane proton flux along with the lateral proton transfer at the surface. Furthermore, due to the lateral mobility of nigericin molecules it is not necessary to assume the participation of the lateral proton conductance along the phospholipid headgroups in the process of formation of the difference of hydrogen-ion binding to the opposite surfaces of the membrane.

The model presented in Fig. 3 implies the different mechanisms of the proton transfer for nigericin (monensin) on one side and acetate and a protonophore TTFB on the other. In particular, acetate and TTFB transfer protons from bulk water to bulk water and nigericin transfer protons from interface to interface. The causes of this difference are unclear now. One of the reasons can be the different hydrophobicity of nigericin and TTFB (and acetate). Though the most probable scheme of the TTFB turnover is from interface to interface [24], it may interact predominantly (in contrast to nigericin) with bulk protons rather than with bound protons. The ability of TTFB protonophore to be sensitive to bulk pH (not to surface protons) enables us to apply the method of the BLM potential measurements in the presence of TTFB to the measurements of hydrogen ion flux across the membrane mediated by the stage of proton release (association) from the surface of the membrane. It is a question of

further study to test the sensitivity of other protonophores to surface protons.

In the recent experiments of Gutman and co-workers [12,13] it was shown, in agreement with our findings, that BLM formed from phosphatidylserine can retain protons at the surface much longer than membranes formed from phosphatidylcholine. They used a flash-photolysis setup to monitor transient capacitance-current changes after pH jumps induced by laser flashes. These authors concluded the absence of the kinetic barrier for protons at the micelle interface from the experiments with proton emitters of different hydrophobicity [13]. The experimental technique used by Gutman and co-workers (kinetic measurements) differs significantly from ours (steady-state experiments). It is essential that Gutman and co-workers studied only one surface process occurring at the lipid/water interface, whereas we examined two surface processes taking place at opposite sides of BLM. The reasons for the obvious contradiction with our main conclusion are still unclear. One possibility is that different proton emitters used by us (nigericin, monensin) and by Gutman and co-workers (2-naphthol and 2-naphthol-3,6-disulfonate) can inject protons to different regions of the interface, predominantly to the membrane surface (nigericin) and to the bulk phases (the naphthols). The other possibility is that the existence of the barrier is not a general case but requires some specific conditions. So we can conclude that, at least in our experimental system, there is a kinetic barrier for protons at the interface.

Summarizing the discussion we can conclude that under our experimental conditions:

(1) The steady-state surface hydrogen-ion concentration can be not in equilibrium with the bulk hydrogen-ion concentration due to the presence of a kinetic barrier for the proton transfer from the surface of the membrane to the bulk phase. Buffer molecules facilitate the proton transfer at the interface thereby decreasing the barrier for the proton transfer. The transmembrane gradient of the surface proton concentration can be observed under the conditions of zero transmembrane gradient of bulk phase proton concentration.

(2) It is shown also that the surface-proton concentration gradient can play the role of coupling factor for the process of conversion of sodium gradient consumed by monensin into potassium gradient produced by nigericin and, therefore, can store the energy. It can be pointed out that the properties of the surface protons described in the present work allow them to participate directly in the processes of energy transduction like membrane phosphorylation in mitochondria and chloroplasts.

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