Kinetic properties of cation/H$^+$-exchange: calcimycin (A23187)-mediated Ca^{2+}/2H$^+$-exchange on the bilayer lipid membrane

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The calcimycin (A23187)-mediated electrically silent flux of hydrogen ions coupled with a counter transport of calcium or magnesium ions was measured by the method of local pH changes recording in the unstirred layers near the planar bilayer lipid membrane (BLM). It was shown that: (1) the pH dependence of calcimycin-mediated Ca^{2+}/2H$^+$ exchange had a maximum at pH 7; (2) the apparent Michaelis constant for the alkali earth cations were higher at acidic pH than the corresponding values at alkaline pH; (3) the apparent Michaelis constant for calcium was similar to that for magnesium ions in agreement with calcimycine cation binding constants; (4) the ratio of calcium and magnesium fluxes was independent of pH in the pH range from 5 to 8. (5) the flux was proportional to the calcimycin concentration at pH > 6.3 and proportional to the square of the carrier concentration at pH < 5; (6) the addition of calcium ion chelator EDTA increased the flux significantly. These data were discussed in terms of the model of cation/H$^+$-exchange and it was concluded that the dissociation of the cation-carrier complex at the membrane/water interface played an important role in the process of calcimycine operation. The comparison of the kinetic properties of calcimycin with the previously described kinetics of nigericin (Antonenko and Yaguzhinsky (1988) Biol. Membr. (Russian) 5, 718–728) revealed much similarity. On the other hand, a significant difference was found between the mechanism of the nigericin K/Na selectivity and calcimycin Ca/Mg selectivity.

Introduction

It was shown recently that membranes of different kinds contained enzymatic systems catalyzing nonelectrogenic cation/H$^+$-exchange; namely the Na$^+$/H$^+$-antiporter [1], the organic cation/H$^+$-exchanger of rat renal brush-border membranes [2], Na$^+$/H$^+$-, K$^+$/H$^+$- and Ca^{2+}/2H$^+$-exchangers of the inner membrane of

mitochondria [3–6], Na$^+$/H$^+$-antiporter of bacterial membrane [7] and some others. The study of the mechanism of these complex systems imply the necessity of the study of comparatively simple cation/H$^+$-exchange catalyzed by a substance such as nigericin and calcimycin (A23187 or calcium ionophore). The present work deals with the kinetic properties of calcimycin-mediated Ca^{2+}/2H$^+$-exchange. This ionophorous antibiotic is widely used in physiological experiments for the study of Ca-dependent processes [8–10]. The thermodynamics and the kinetics of the cation binding with calcimycine in the solution [11–14] and at the interface [15–16] were studied thoroughly. The calcimycin-mediated calcium ion transport was studied in planar BLM [17–21], liposomes [22–27], thick liquid membrane [28] and natural membranes [29–31]. It was proposed that the calcium dissociation step was rate-determining in the case of a thick liquid membrane [28]. The question of the rate-limiting step for the transport through lipid bilayers remains open. It was shown that cholesterol reduced the calcium flux [26] provided that the translocation across the membrane is the rate-determining step. On the other

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hand, recent works of Riddell and co-workers showed that the nigericin- and monensin-mediated transport of cations was determined by the rate of interaction of cations with the carrier [32,33]. It is unclear also whether the mechanism of Ca/Mg selectivity of calcimycin is based on the difference in cation dissociation rates [28] or on the difference in translocation rate across the membrane [21]. Under different conditions the dependence of the rate of cation transport on calcimycin concentration was observed to be linear, quadratic or with an intermediate slope [17–25]. So the data available do not present the distinct kinetic picture of calcimycin-mediated cation transport and the mechanism of its operation.

In the present work, our method to measure electrically silent fluxes of hydrogen ions based on pH-shift recording in the unstirred layers near BLM [20,21] was applied. The method proved useful for the study of the kinetics of nigericin-mediated K^+/H^+-exchange [34] as well as for the measurements of cation selectivity of the polyether antibiotics [20,21]. The comparison of the kinetic properties of calcimycin with the previously described kinetics of nigericin [34] revealed much similarity. The data obtained are discussed in terms of two competitive models: (1) The rate-determining step of the transport is the translocation of the cation-ionophore complex across the membranes. (2) The rate-determining step of the transport is a reaction of cation binding with the ionophore at the membrane/water interface.

Materials and Methods

The methods and materials were mainly the same as described in our previous paper [34] concerning the nigericin-mediated K^+/H^+-exchange. Calcium ionophore A23187 was from Calbiochem-Behring (La Jolla, CA), EDTA and EGTA were from Serva (Heidelberg, F.R.G.), $CaCl_2$ and $MgCl_2$ were from Reachim (Moscow, U.S.S.R.). In short, J_H was calibrated against the potential by adding increasing concentrations of sodium acetate at different pH values as described earlier [13]. The buffer mixture Tris, Mes and β-alanine slightly affected the J_H values in the pH range 4.5–8.5. The J_H value corresponding to 10 mV potential varied from $4.7 \cdot 10^{-11}$ to $5.5 \cdot 10^{-11}$ mol H^+/cm^2 per s (the average was $5 \cdot 10^{-11}$) under these conditions.

Calcium ionophore A23187 was dissolved in ethanol solution, sampled in a number of vessels and evaporated. The membrane-forming solution was added to dry vessel before the beginning of the experiment.

Results

Fig. 1 provides an example of the measurements of calcimycin-mediated J_H flux. It displays the kinetics of
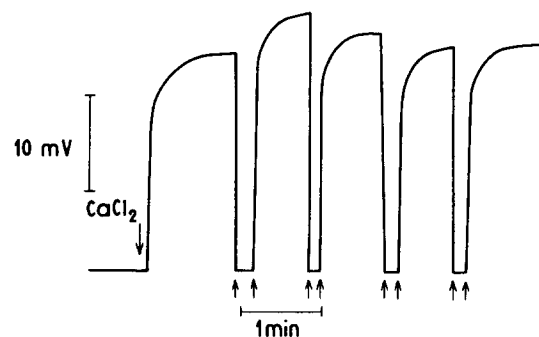

Fig. 1. The generation of the BLM potential after the formation of the difference of calcium ion concentration (10 mM at one side of the membrane and 1 mM at the other) in the presence of calcimycine (0.5 mM in the membrane-forming solution) and a TTFB protonophore. The potential has plus sign on the side of BLM where the concentration of $CaCl_2$ is lower. The solution was: 1 mM Tris, 1 mM Mes, 1 mM β-alanine, 100 mM choline chloride, 0.01 mM TTFB (pH 7.5). At the moments marked by arrows the potential recording was switched off or on after formation and disruption of BLM.

the formation of the electrical potential on BLM induced by the difference in calcium ion concentrations at the opposite sides of the membrane in the presence of
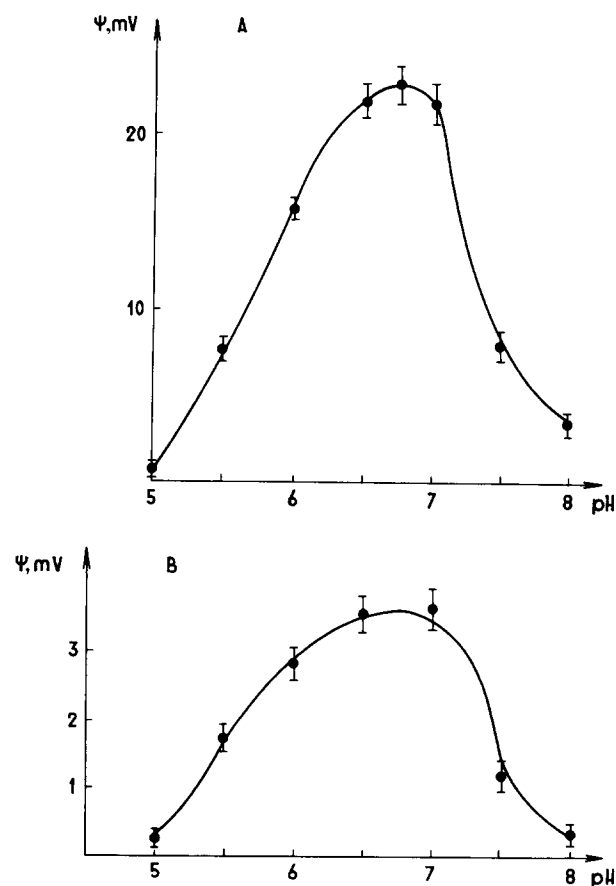

Fig. 2. pH dependence of the BLM potential in the presence of 1.3 mM calcimycin in the membrane-forming solution. The composition of the medium was as in the caption to Fig. 1. (A) The gradient of calcium ions was 10/0 mM. (B) The gradient of magnesium ions was 10/0 mM.
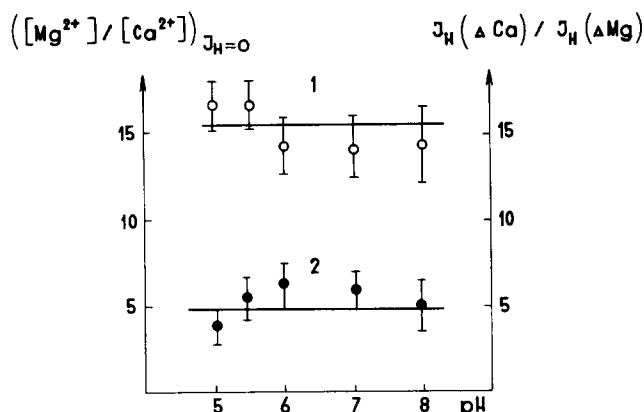
Fig. 3. Curve 1, pH dependence of the parameter of Ca/Mg selectivity of calcimycin measured according to Ref. 21. Curve 2, pH dependence of the ratio of hydrogen ion fluxes induced by difference in the $CaCl_2$ and $MgCl_2$ concentrations induced by calcimycin. The conditions are the same as in the caption to Fig. 2.

Fig. 5. Effect of magnesium ion concentration on the BLM potential at pH 5 (curve 1) and pH 8 (curve 2) under the conditions as in the caption to Fig. 1. The magnesium concentration at the other BLM side consisted 10%. The calcimycin concentration was 2.6 mM in the membrane-forming solution.

TTFB protonophore. After measuring the steady-state values of potentials the BLM was destroyed and a new one formed. A small diameter of the hole prevented the solutions from mixing. This procedure was repeated many times. The average value of the potential was used.

Fig. 2A shows the pH dependence of J_H, measured under the conditions of 10 mM $CaCl_2$ at one side of BLM and 1 mM at the other. It has a maximum at pH about 7.0. Similar variation was observed when the calcium ion gradient was 0.15 : 0 mM (data not shown). Fig. 2B shows the same dependence as Fig. 2A but magnesium ions were used instead of calcium. The ratio of calcium and magnesium ion fluxes was independent of pH (Fig. 3, curve 2) and equal to approx. 5. It is seen from the Fig. 3, curve 1 that the parameter of Ca/Mg selectivity ($S_{Ca/Mg}$) proposed in Ref. 21 is pH independent also.

Noteworthy, we found similar pH dependence with a maximum at pH 7 for the potassium ionophore nigericin in our previous work [34]. This coincidence could mean that this pH dependence is the property of the method, but not of the ionophore. However, the pH
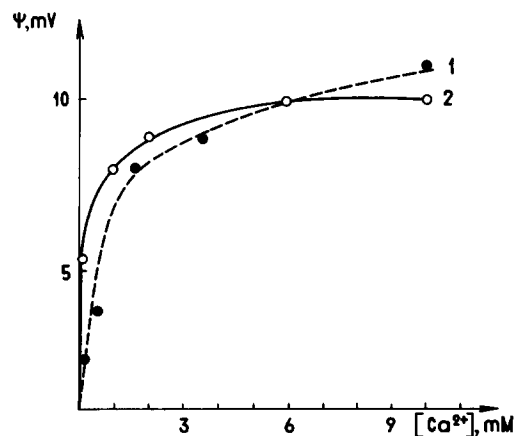

Fig. 4. Effect of calcium ion concentration on the BLM potential at pH 5 (curve 1) and pH 8 (curve 2) under the conditions as in the caption to Fig. 1. The calcium concentration at the other BLM side consisted 10%. The calcimycin concentration was 0.8 mM in the membrane-forming solution.
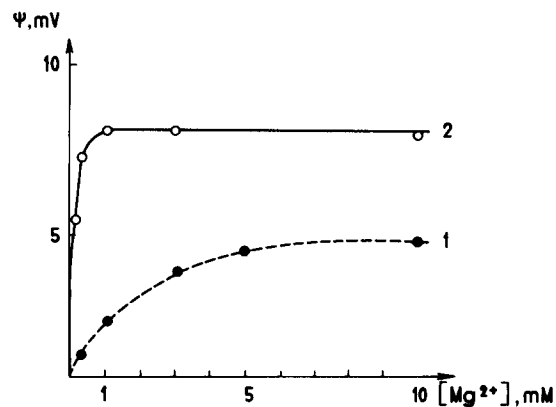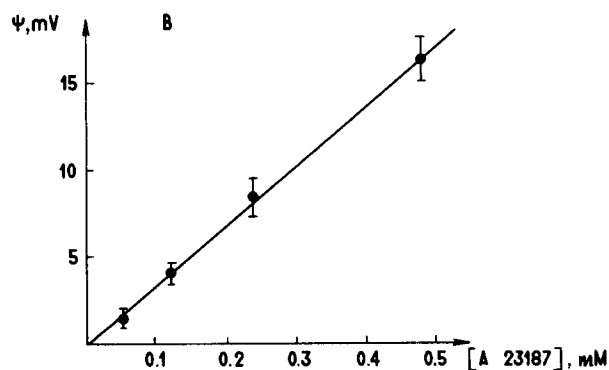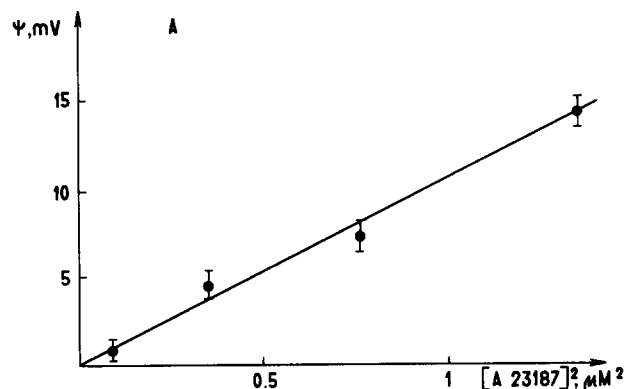
Fig. 6. Effect of calcimycin concentration on the BLM potential. (A) pH = 5, calcium ion gradient was 10 mM-1 mM, ionophore was added in the water solution. (B) pH = 6.3, the same gradient of calcium ions and the ionophore was in the membrane-forming solution. Other conditions as in the caption to Fig. 1.
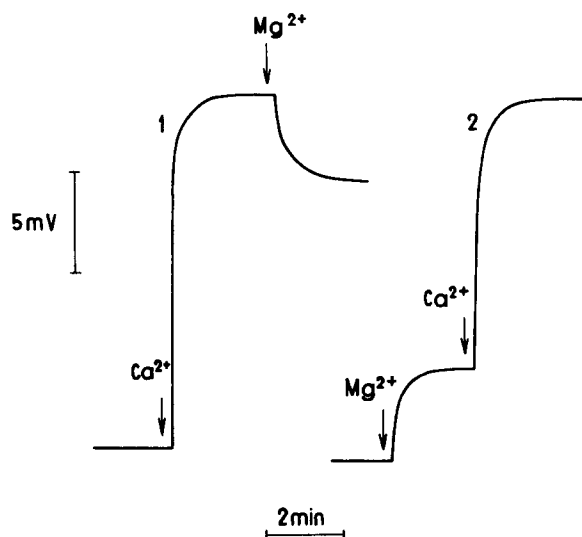
Fig. 7. The inhibitory effect of magnesium ions on the Ca^{2+}-induced (10 mM/1 mM) BLM potential. Mg^{2+}, the addition of 10 mM/1 mM of magnesium ions. The calcimycin concentration in the membrane-forming solution was 1.2 mM, pH 6.0. Other conditions as in the caption to Fig. 1.

dependence of sodium ionophore monensin measured by the same method had a maximum at pH 5.5 (data not shown) in agreement with Ref. 7. Therefore this possibility is excluded.

The variations of J_H with the concentration of Ca^{2+} and Mg^{2+} are illustrated in Figs. 4 and 5, respectively. The nonlinear regression analysis of these data calcu-
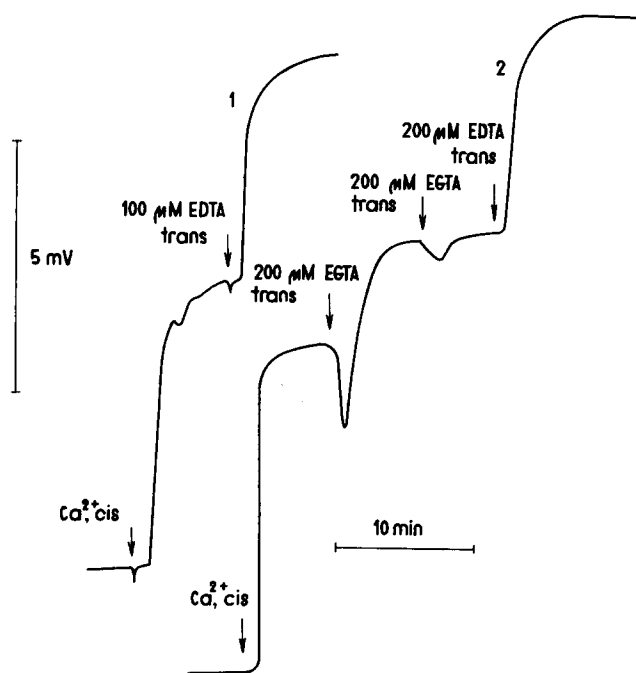

Fig. 8. The effect of EDTA and EGTA (trans side) on the Ca^{2+}-induced (0.15 mM on the cis side) BLM potential in the presence of 1 mM calcimycine in the membrane-forming solution. The conditions were as in the caption to Fig. 1.
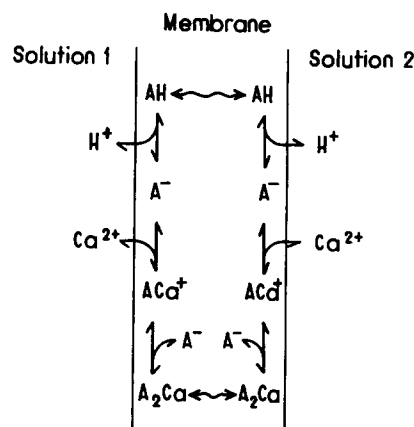

Fig. 9. The model of cation/proton antiport across the bilayer lipid membrane catalyzed by calcimycin. A^+, ACa^+, A_2Ca and AH, four forms of calcimycin. Straight lines, chemical reactions; waved lines, translocation across the membrane.

lated from the Michaelis-Menten equation gave the following K_m values: for Ca^{2+} 1.3 ± 0.2 mM (pH 5), 0.08 ± 0.01 mM (pH 8) and for Mg^{2+} 1.5 ± 0.2 mM (pH 5), 0.05 ± 0.01 mM (pH 8).

The effect of calcimycin concentration on the J_H flux is presented in Fig. 6. At high pH (6.3 and higher) this variation was linear, while at low pH (5 and lower) the J_H was proportional to the square of carrier concentration.

The addition of magnesium ions to the same side of the membrane where the calcium ions had been added resulted in the decrease of the flux (Fig. 7). This experiment showed that the rate of calcimycin-mediated calcium transfer is higher than the magnesium transfer. The reverse order of additions (Fig. 7, curve 2) showed that the J_H flux induced by magnesium increased after the addition of calcium.

Fig. 8 shows that the addition of calcium chelator EDTA at the BLM side devoid of calcium increased the rate of calcium-induced $Ca^{2+}/2H^+$-exchange. The addition of EGTA affected the cation exchange similarly though to a lower extent.

Discussion

Fig. 9 shows the scheme of calcimycin-mediated $Ca^{2+}/2H^+$-exchange. The scheme is based on the model of Pressman and co-workers [35]. There are four forms of the carrier at membrane surfaces: A^-, AH, ACa^+ and A_2Ca. AH and A_2Ca forms can translocate across the membrane. The important problem of this kind of model is what kinetic step limits the whole process: the translocation across the membrane or the reactions of cation binding at the membrane surfaces. If one assumes that the binding process reaches the equilibrium and it is the translocation of AH and/or A_2Ca across the membrane which limits the process than the most

part of our experimental data can be accounted for as follows: (1) At acidic pH the process is determined by the translocation of the A_2Ca form, the concentration of which diminishes with decreasing pH. In the alkaline pH range the rate-limiting step turns to the translocation of the AH form, the concentration of which decreases with increasing pH. These considerations explain a bell-like shape of pH dependence of J_H flux as well as the linear and quadratic variation of J_H flux with the carrier concentration at different pH ranges. (2) The decrease in the apparent value of the Michaelis constant with the pH increase can be accounted for by the competition between the cation and hydrogen ion for the single binding site. (3) The similarity of the Michaelis constants for Ca^{2+} and Mg^{2+} corresponds to the similarity of their binding constants in one-phase [13,29] and two-phase [18] systems. (4) The Ca^{2+}-induced J_H flux reduction upon the Mg^{2+} addition (Fig. 7) can be explained if one assumes that the A_2Mg translocation rate constant is lower than the A_2Ca constant provided that the cation binding constants are similar.

However, this model contradicts the data presented in Fig. 3, curve 2 illustrating the pH independence of the ratio of J_H fluxes induced by Ca^{2+} and Mg^{2+}. According to the model, in the alkaline pH range the process is limited by the translocation of the AH form and the $J_H(Ca)/J_H(Mg)$ ratio should reach unity at this pH. This was really observed for the ratio of K^+ and Na^+ fluxes induced by nigericin [34]. On the other hand, the assumption that the translocation of the A_2Ca (A_2Mg) form is a rate-determining step in the whole range of pH leads to new difficult questions: (1) Why does the concentration of this form pass though maximum at pH 7? (2) Why is the flux proportional to the concentration? And finally, in terms of this model it is hard to explain the increase of $Ca^{2+}/2H^+$-exchange rate upon the addition of EDTA (Fig. 8). It may be proposed that EDTA reduced the Ca^{2+} concentration in the unstirred layer near BLM which had increased as a result of the Ca^{2+} flux across the membrane. EDTA chelated Ca^{2+} and suppressed the inhibitory effect of calcium ions. However, Fig. 8, curve 2 shows that the effect of EDTA remained in the presence of saturating concentrations of EGTA under the conditions when all calcium ions had to be chelated. This experiment shows that the explanation is unsatisfactory.

If one rejects the main assumption that the carrier translocation across the membrane is a rate-determining step and proposes that the cation binding reactions at the membrane/water interface limit the process than the experimental data can be accounted for as follows: (1) The above mentioned effect of EDTA means that it is the dissociation of A_2Ca (or ACa^+) complex which limits the whole process. (2) The difference between the EDTA and EGTA action can be accounted for by their

specific interactions with A_2Ca and ACa^+ forms according to the reactions:

$$A_2Ca_M + Che_S^{2-} \rightleftharpoons 2A_M^- + CheCa_S$$

$$ACa_M^+ + Che_S^{2-} \rightleftharpoons A_M^- + CheCa_S$$
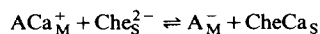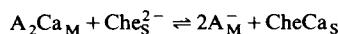
where M and S subscripts refer to the membrane and the solution accordingly. In terms of this model the dissociation of the A_2Ca form limits at acidic pH (quadratic variation with the carrier concentration) and the dissociation of ACa^+ limits at alkaline pH (linear variation with the carrier concentration). (3) The dissociation of the calcium-calcimycin complex is complicated by the interaction with hydrogen ions [11] and its rate increases with the hydrogen ion concentration [11]. This may account for the decrease in J_H flux with pH shift from 7 to 8 (Fig. 2). The lowering of J_H flux from pH 7 to pH 5 is caused by the decrease in the surface concentration of calcium-calcimycin complexes due to transformation of the carrier into the AH form. (4) The virtual pH independence of $J_H(Ca)/J_H(Mg)$ does not contradict the model since the AH form does not limit the process. (5) In terms of the model the reduction of the Ca^{2+}-induced potential upon Mg^{2+} addition (Fig. 7) can be accounted for by the difference in the dissociation rate constants of A_2Ca and A_2Mg complexes. This explanation is in agreement with the data of the work [13] where the observed cation dissociation rate was higher for Ca^{2+} than for Mg^{2+} in $H_2O/CHCl_3/H_2O$ system. It may be concluded that the second version of the model agrees better with our experiments.

The value of the ratio of $J_H(Ca)$ and $J_H(Mg)$ estimated in our experiments as 5 is in good agreement with the values obtained for mitochondrial membranes (3 [31]) and liposomes (6 [22]). This ratio is sufficiently lower than the parameter of Ca/Mg selectivity $(S_{Ca/Mg})$ proposed in Ref. 21 and measured under the conditions of oppositely directed Ca^{2+} and Mg^{2+} gradients across the membrane. According to Ref. 21 and to Fig. 3, curve 2 this parameter is equal to 16. The simultaneous presence of Ca^{2+} and Mg^{2+} in the system provides the possibility of an additional process, Ca^{2+}/Mg^{2+}-exchange which can proceed via the reactions:

$$A_2Ca + Mg^{2+} \rightleftharpoons A_2Mg + Ca^{2+}$$

and

$$A_2Mg + Ca^{2+} \rightleftharpoons A_2Ca + Mg^{2+}$$
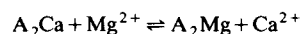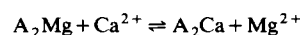
In the case when the rate of these reactions exceeds greatly the rate of the partial processes of $Ca^{2+}/2H^+$- and $Mg^{2+}/2H^+$-exchange these reactions may determine the calcimycin operation and the value of $S_{Ca/Mg}$.

The comparison of the kinetic properties of calcimycin- and nigericin-mediated cation/H^+-exchange shows that along with similarities there are considerable differences. Common features are: (1) Similar pH dependencies of J_H flux. (2) The increase of the cation K_m values with pH decrease. On the other hand, the pH dependencies of the ratio of Ca^{2+} and Mg^{2+} fluxes (calcimycin) and K^+ and Na^+ (nigericin) differ greatly. Besides, though the pH dependencies of the J_H flux are similar, the kinetic reasons of the effect of pH in the acidic pH range (from 5 to 7) are different. In the case of nigericin the decrease in J_H is brought about by the increase in K_m while in the case of calcimycin the saturating concentrations of Ca^{2+} are used (Fig. 4) and the reduction of J_H results from the decrease in V_{max} value. It is noteworthy that these two ionophores behave differently in the experiments presented in Fig. 7. In the case of nigericin the addition of sodium ions at the same BLM side where K^+ had been added had no effect on the membrane potential or even caused a slight increase of the potential (data not shown), while in the case of calcimycin a significant decrease was found. This observation directly showed the difference between the mechanisms of cation selectivity of these two ionophores.

Pohl and co-workers [22] in their work studied the effect of calcimycin concentration on the Ca^{2+} flux through membranes of liposomes at different pH. It was found that in the acidic pH range the variation was linear while in the alkaline pH range it was quadratic. This result contradicts our data. One of the reasons for this discrepancy may be the absence of the control on the pH inside vesicles which may differ substantially from the outside pH in the course of the transport. The study of the pH dependence of calcimycin-mediated Ca^{2+} flux through planar bilayer lipid membrane carried out in Ref. 17 showed that the flux increased with pH increase in the pH range from 5.5 to 8. The decrease in the Ca^{2+} flux was not found in the alkaline pH [17]. This difference in ionophore behavior can be attributed to the difference of the conditions of the measurements. In particular the measurements were carried out in the presence of arsenaso III [17] which is known to bind Ca^{2+} and according to Fig. 8 of the present paper could affect the flux.

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