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The ion selectivity of nonelectrogenic ionophores measured on a bilayer lipid membrane: nigericin, monensin, A23187 and lasalocid A

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A new method for measuring ion selectivity of nonelectrogenic ionophores on a planar bilayer lipid membrane (BLM) has been developed. This method is based on the phenomenon of formation of local gradients of ion concentration arising in the unstirred layers near the BLM in the course of transmembrane ion fluxes. The method gives rise to reproducible values of ion selectivity, independent of the conditions employed. In the case of equal charges of cations, measurable selectivity is determined by a combination of the binding and the translocation rate constants for two cations. The following values have been obtained for cation selectivity: nigericin, K^+/Na^+ 25 ± 4; monensin, Na^+/K^+ 16 ± 4; A23187, Ca^{2+}/Mg^{2+} 14 ± 2; lasalocid A, K^+/Na^+ 12 ± 1, Ca^{2+}/Mg^{2+} 17 ± 2. The transport sequence of lasalocid A for cations is $K^+ > Na^+ > Ca^{2+} > Mg^{2+}$. The data obtained show that, for monovalent cations, the selectivity of the antibiotic used depends on the cation binding constants on the membrane surface. For the transport of divalent cations by ionophores A23187 and lasalocid A, their selectivity values must also depend on the translocation rate constants of ionophore-cation complex.

Introduction

The carboxylic polyether ionophores, nigericin, monensin, A23187 and lasalocid A (X537A), are widely employed in biochemistry for analyzing the effect of transmembrane cation gradients on the functions of cells and their organelles [1,2]. The ability of these ionophores to induce electroneutral fluxes of mono- and divalent cations across model membranes has been investigated [3–15]. The ion selectivity of these nonelectrogenic ionophores was studied earlier by measuring cation binding in solutions and in biphasic systems [10,16–21]. However, correct quantitative measurements of their cation transport selectivity have not yet been carried out because of the laborious methods for measuring electroneutral ion fluxes across the membrane. In the present work, we have applied our earlier method of measuring nonelectrogenic cation fluxes across the BLM. This method is based on the phenomenon of local gradients of ion concentration formation in the course of transmembrane ion fluxes in the unstirred layers near the BLM [23–25]. This method was used for investigating the selectivity of nigericin, monensin, calcium ionophore A23187 and lasalocid A for cationic pairs: potassium and sodium cations, and also calcium and magnesium cations. We also studied the transport sequence of

Abbreviations: BLM, bilayer lipid membrane; J_H, hydrogen ion electroneutral flux; TTFB, tetrachlorotrifluoromethylbenzimidazole; TH, protonated carrier; T, carrier; Mes, 4-morpholineethanesulphonic acid.

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lasalocid A for these four biologically important cations.

Materials and Methods

The BLM was formed on a Teflon partition, 0.4 mm in diameter, by a conventional method [22]. A membrane-forming solution contained 20 mg phosphatidylcholine from soya beans (Sigma) and 20 mg cholesterol (Boehringer), in 1 ml of *n*-decane. The thinning of the BLM was observed both visually and by measuring its capacity [23]. The experiments were carried out at room temperature.

The hydrogen ion electroneutral flux (J_H) was measured by the method based on the formation of local pH gradients in the unstirred layer near the BLM, during cation/proton exchange on the BLM in a solution with low buffer capacity [23–25]. The pH gradient was determined from the difference in the electrical potentials ($\Delta\Psi$) on BLM in the presence of a protonophore in the open-circuit mode. It was shown that, under these conditions, J_H is proportional to the membrane potential [24]. The protonophore, tetrachlorotrifluoromethylbenzimidazole (TTFB) and the antibiotics, nigericin, monensin, A23187 and lasalocid A (all of them from Calbiochem) were added on both sides of the BLM. In some experiments (see Fig. 3), the antibiotics were added to the membrane-forming solution of the phospholipid. J_H was calibrated against the potential by adding the increasing concentrations of sodium acetate, as described earlier [24].

Results

Fig. 1 shows the dependence of hydrogen ion flux across the BLM on the concentration of lasalocid A in the presence of transmembrane gradients of potassium (curve 1), and calcium (curve 2) ion concentrations. The graph represents values of steady-state fluxes which are attained 20–30 min after the addition of the antibiotic. This time is necessary to allow for lasalocid A incorporation into the BLM. The dependence is of a linear character. Thus, under our experimental conditions, the transfer of a monovalent cation (H^+,K^+) by the monomeric form of lasalocid A

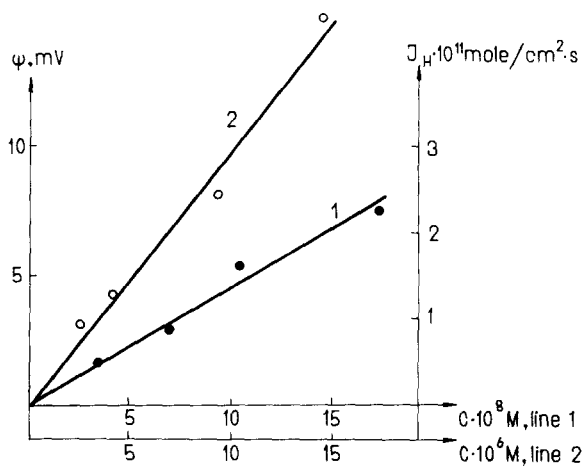

Fig. 1. Dependence of the BLM potential, or of the proportional electroneutral flux of H^+ across the BLM, on the concentration of lasalocid A (X537 A) with a KCl (curve 1) or $CaCl_2$ (curve 2) gradient on the membrane (10 mM on one side and 1 mM on the other side) in the presence of 10 μM TTFB. The solution was: 1 mM Tris (pH 7.0) 1 mM Mes/100 mM choline chloride. Positive is on the BLM side with the lower concentration of the cation.

must be the rate-limiting step of the process. Control experiments showed that, under our experimental conditions, in the absence of TTFB, the BLM electrical conductivity does not increase upon lasalocid A addition. Thus, the formation of a potential on the membrane is not connected with the previously described ability of this antibiotic to induce electrogenic fluxes across the BLM [26,27].

As seen from Fig. 1, one should add 0.23 μM lasalocid A to induce a flux equal to 30 pmol H^+/cm^2 per s. In our previous work, we measured hydrogen ion fluxes at different concentrations of nigericin (KCl gradient) and monensin (NaCl gradient) [24]. It was shown that the flux equal to 30 pmol H^+/cm^2 per s was induced at a nigericin concentration of 0.03 μM, and a monensin concentration of 0.15 μM. It is seen that regarding potassium-transfer efficiency, lasalocid A is inferior to nigericin and is similar to monensin.

Fig. 2 shows the dependence of an electroneutral flux of hydrogen ions across the BLM on the logarithm of lasalocid A concentration in the presence of equal gradients of KCl, NaCl, $CaCl_2$ and $MgCl_2$ concentrations on the membrane. Fig. 2

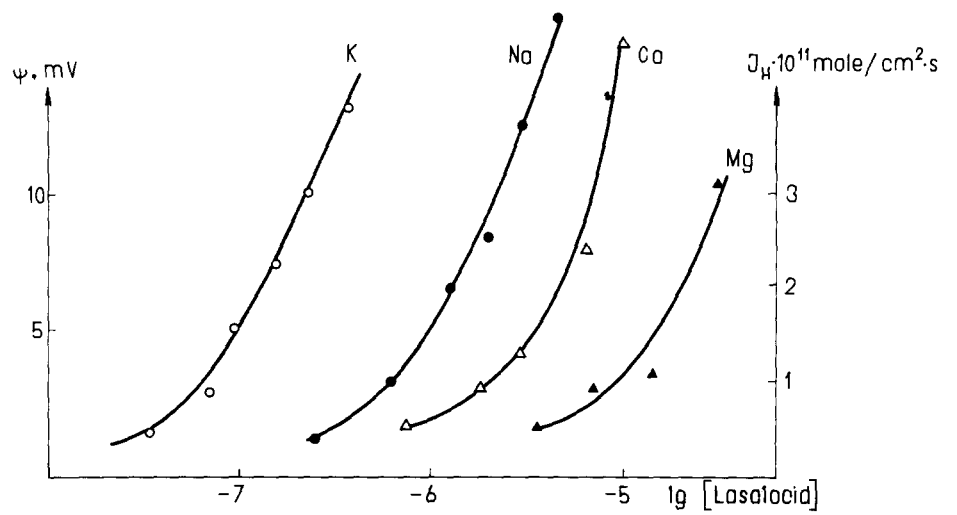

Fig. 2. Dependence of the BLM potential, or of the proportional electroneutral flux of H^+ across the BLM, on log. lasalocid A concentration at equal gradients of KCl, NaCl, $CaCl_2$ and $MgCl_2$ concentrations (10 mM on one side of the BLM and 1 mM on the other), in the presence of 10 μM TTFB. The solution was as in the legend to Fig. 1.

depicts the transport sequence of lasalocid A: $K^+ > Na^+ > Ca^{2+} > Mg^{2+}$.

In the present work, we have applied a new method for measuring cation selectivity, formally analogous to the method of bi-ionic potentials used for electrogenic transport of ions. In experimental terms, the method consisted of the following. After the potential on the BLM had attained a steady-state value in the presence of a nonelectrogenic ionophore and a protonophore (given the existence of a concentration gradient of the cation), we formed a reverse gradient using the other cation and increased this gradient until the BLM potential reached zero. Under these conditions the ratio of transmembrane gradients of appropriate cations is a measure of cation selectivity of the ionophore.

This method was developed using two antibiotics, nigericin and monensin. Fig. 3 (curves 1, 4) represents the pH dependence of the observed selectivity of ionophores for sodium and potassium ions. It can be seen that this parameter does not depend on pH. For monensin it is equal to 16 ± 4, and, for nigericin, 25 ± 4. We also examined the dependence of the selectivity on the concentration of cations in the solution. We found that over a wide range of concentrations, the result does not depend on the concentration of the cations.

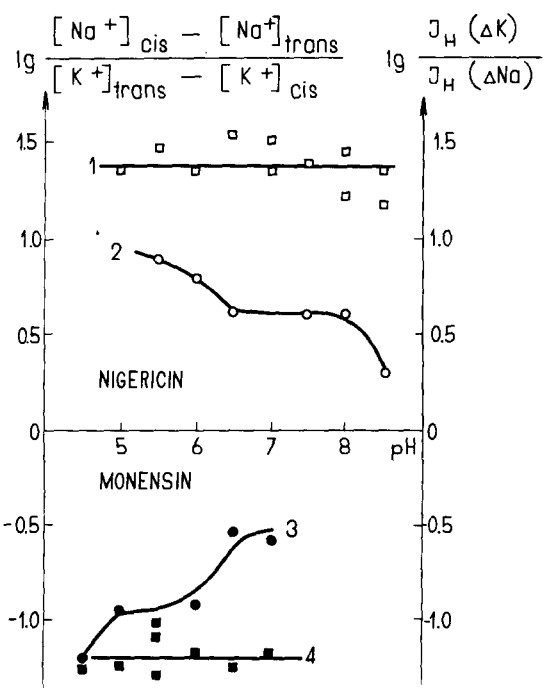

Fig. 3. Dependence of the ratio of K^+ and Na^+ concentrations at a zero flux of H^+ (curves 1, 4) and the ratio of the H^+ fluxes induced by these cations (curves 2, 3) on pH in the presence of 4.3 mM monensin (curves 3, 4) and 0.4 mM nigericin (curves 1, 2) in the membrane-forming phospholipid solution. The solution was as in the caption to Fig. 1, plus 1 mM β-alanine and 10 μM TTFB. Curves 2 and 3 represent data in the presence of 10 mM of the corresponding cation on one side of the BLM and 1 mM, on the other.

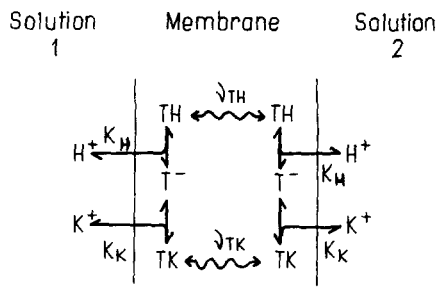

Fig. 4. Transfer of monovalent cations across the membrane by a nonelectrogenic ionophore. 1 and 2 represent different sides of the membrane, T is the carrier, straight arrow represents the chemical reaction, and the wavy arrow, the translocation across the membrane.

Also depicted in Fig. 3 (curves 2 and 3) is the pH dependence of the observed cation selectivity of the antibiotics measured under conditions, where the hydrogen ion flux across the BLM is not zero. Plotted along the ordinates is the ratio of J_H values at constant transmembrane gradients of potassium and sodium concentrations. It can be seen that the observed selectivity of monensin and nigericin, measured this way, was not constant, and decreased with the increasing pH. Experiments showed this ratio to depend not only on pH, but also on the selected range of potassium and sodium concentrations in the medium (with the ratio of concentrations of these cations on the opposite BLM sides being invariable).

This method was also applied in order to measure the cation selectivity of the other ionophores, A23187 and lasalocid A, at a zero flux of hydrogen ions. The measurements were carried out at pH 7.0 (for conditions, see legend to Fig. 1). For A23187, $[Mg^{2+}]/[Ca^{2+}] = 14 \pm 2$ ($N = 4$); for lasalocid A, $[Na^+]/[K^+] = 12 \pm 1$ ($N = 3$) and $[Mg^{2+}]/[Ca^{2+}] = 17 \pm 2$ ($N = 3$).

TABLE I

CORRELATION BETWEEN THE IONOPHORE CATION SELECTIVITY MEASURED FROM CATION TRANSPORT ON BLM AND THE BINDING OF THESE CATIONS IN THE MONOPHASIC AND BIPHASIC SYSTEMS

Ionophore	Selectivity	Ratio of binding constants for two cations	
		monophasic system	biphasic system
Nigericin			
K^+/Na^+	25 ± 4	50 [16]	45 [17]
Monensin			
Na^+/K^+	16 ± 4	25 [16] 32 [18]	10 [17]
A23187			
Ca^{2+}/Mg^{2+}	14 ± 2	2 [10] 1 [21]	3 [19]
Lasalocid A			
Ca^{2+}/Mg^{2+}	17 ± 2	6 [10] 5 [20]	2 [21]
K^+/Na^+	12 ± 1	10 [20] 7 [10]	–

Discussion

It follows from Fig. 2 that lasalocid A is a better potassium ionophore than calcium. These data are in accordance with the data on cation fluxes induced by lasalocid A in erythrocytes [28] and mitochondria [29]. Lasalocid A is usually used as a calcium ionophore in physiological experiments. Since it is a powerful potassium ionophore, one should be careful in the interpretation of the results of such experiments because the change of potassium gradient induced by lasalocid A can produce large regulatory effects.

If the nonelectrogenic cation/proton exchangers are in operation, the transmembrane flux of a cation in one direction is equal to the flux of hydrogen ions in the other. Accordingly, the value of the cation flux should depend not only on the cation concentration in the solution, but also on the hydrogen ion concentration. The value of selectivity of two cations, measured as the ratio of cation fluxes, should also depend on the pH of the medium. From Fig. 3 (curves 2 and 3), it can be seen that the cation selectivity measured in this way does decrease with the increasing pH for nigericin and monensin. A drop in the ratio of hydrogen ion fluxes caused by sodium and potassium cations is due to the decrease in the concentration of the protonated form of the carrier (TH) on the membrane surface. At high pH values, in conditions where translocation of the TH form is the rate-limiting step of the transport, the ratio of the fluxes should tend to 1.

It should be expected that the selectivity of the ionophore for two cations, measured when the flux of hydrogen ions across the membrane is

zero, should not depend on pH. As seen from Fig. 3 (curves 1, 4), the ratio of the concentrations of two cations resulting in a zero flux of hydrogen ions does not depend on either pH or the absolute concentrations of cations. This shows that the given criterion of cation selectivity depends only on parameters determining the cation selectivity of the ionophore, i.e., cation-to-ionophore binding constants and the translocation rate constants of these complexes.

The simplest scheme describing the process of nonelectrogenic transmembrane exchange of cations is given in Fig. 4. The model comprises two significant steps; the chelating of the cations (K^+ and H^+) by the carrier (T), and the translocation of the TK and TH complexes across the membrane. Let us suppose that (i) only the TK, TH and TNa complexes of the carrier can diffuse across the membrane and (ii) the reactions of the carrier with the metal cations and the proton are equilibrated at the interface. Proceeding from these two assumptions, parameters were found for the ratio of the concentrations of two different monovalent cations (say, K^+ and Na^+) under the condition of zero flux of hydrogen ions across the membrane:

$$J_H = \nu_{TH}([TH_2]-[TH_1]) = \nu_{TK}([TK_1]-[TK_2]) + \nu_{TNa}([TNa_1]-[TNa_2]) = 0 \quad (1)$$

where J_H is the flux of hydrogen ions across the BLM equal to the total flux of K^+ and Na^+ ions, with ν_{TH}, ν_{TK}, ν_{TNa} being the translocation rate constants of the complexes TH, TK and TNa, respectively, with symbols 1 and 2 designating the two sides of the membrane. From the equilibrium at the membrane interfaces it follows that:

$$K_{cation} = \frac{[T_1][cation_1]}{[Tcation_1]} = \frac{[T_2][cation_2]}{[Tcation_2]} \quad (2)$$

where the cation may be K^+, Na^+ or H^+. Substituting TH, TK and TNa from Eqn. 2 into Eqn. 1 and assuming that $[H_1^+] = [H_2^+]$, we obtain an expression for the cation selectivity of the ionophore at $J_H = 0$ which, as seen from Eqn. 3, is determined by a combination of the binding and the ionophore translocation rate constants.

$$\frac{[K_1^+]-[K_2^+]}{[Na_2^+]-[Na_1^+]} = \frac{\nu_{TNa}K_K}{\nu_{TK}K_{Na}} \quad (3)$$

Eqn. 3 also holds for the case of divalent cations. It is assumed that calcium and magnesium form complexes as a result of reactions $2T^- + Ca^{2+} = T_2Ca$, and $2T^- + Mg^{2+} = T_2Mg$; also, TH complexes are formed by the reaction $T^- + H^+ = TH$.

$$\frac{[Ca_1^{2+}]-[Ca_2^{2+}]}{[Mg_2^{2+}]-[Mg_1^{2+}]} = \frac{\nu_{T_2Mg}K_{Ca}}{\nu_{T_2Ca}K_{Mg}} \quad (4)$$

With respect to the selectivity of differently charged cations (say, K^+ and Ca^{2+}), Eqn. 5 corresponds to the condition $J_H = 0$

$$\frac{[Ca_1^{2+}]-[Ca_2^{2+}]}{[K_2^+]-[K_1^+]} = \frac{2\nu_{TK}K_{Ca}}{\nu_{T_2Ca}K_K[T^-]} \quad (5)$$

The concentration of T^- depends on the pH of the medium and on the cation concentration, and thus, in the case of differently charged cations, the selectivity, measured at $J_H = 0$, depends on the composition of the solution and may be used as an ionophore selectivity characteristic only under the given specific conditions.

It is seen from Eqns. 3 and 4 that if $J_H = 0$, the measured selectivity of the ionophore for two cations with equal charges is determined by a combination of constants: the cation-to-ionophore binding constants at the interface and the translocation rate constants for the salts thus formed. The binding constants of the ionophores studied by us have been reported in the literature [10,16–21]. This makes it possible to find out which of the parameters (ν or K) in Eqns. 3 and 4 accounts for the selectivity of ionophores on the BLM. Represented in Table I are data on ionophore selectivity, obtained with respect to transport in the present work (first column) and the literature data on cation binding in monophasic (second column) and biphasic (third column) systems. It can be seen from Table I that, in the case of monovalent cations, the numerical value of selectivity, determined from the transport in the BLM, and the ratio of cation binding constants, are close to each other. This similarity shows that the selectivity of nigericin, monensin and lasalocid A is determined by constants of metal ions binding to ionophore at the membrane/water interface.

At the same time, the Ca^{2+}/Mg^{2+} selectivity of A23187 and lasalocid A, according to transport, is considerably higher than the calculated ratio of binding constants (Table I). According to Eqn. 4, this must be due to large differences in the constants of the T_2Ca and T_2Mg translocation rates across the membrane. These data indicate the existence of different mechanisms for selectivity of nonelectrogenic ionophores; in one limiting case the selectivity is determined by the differences in cation-to-ionophore binding constants at the membrane/water interface, and in the other case, by the differences in membrane permeability for cation-ionophore complexes.

We must say in conclusion that, unlike selectivity values measured from the ratio of cation concentrations for a zero flux of hydrogen ions across the membrane, the ratio of fluxes of two cations under equal conditions is strongly dependent on the experimental conditions, and thus can be used as a qualitative measure of cation selectivity. Since only the latter criterion has been used thus far, it may be inferred that in the present work, the values of cation selectivity of nigericin, monensin, A23187 and lasalocid A in cation transport across the membrane have been measured correctly for the first time, to our knowledge.

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