Biochimica et Biophysica Acta, 944 (1988) 279–284
Elsevier

BBA 74134

The monensin-mediated transport of Na^+ and K^+ through phospholipid bilayers studied by ^{23}Na- and ^{39}K-NMR

Frank G. Riddell *, S. Arumugam * and Brian G. Cox

Department of Chemistry, The University of Stirling, Stirling (U.K.)

(Received 17 February 1988)

Key words: Phospholipid bilayer; Ion transport; Monensin; NMR, ^{23}Na-; NMR, ^{39}K-

Addition of monesin to preparations of large unilamellar vesicles made from egg yolk phosphatidylcholine (EPC) in sodium or potassium chloride solution and from dioleoylphosphatidylcholine (DOPC) in sodium chloride solutions gives rise to dynamic ^{23}Na- and ^{39}K-NMR spectra. The dynamic spectra arise from the monensin-mediated transport of the metal ions through the membrane. The kinetics of the transport are followed as a function of monensin and metal ion concentrations and are compatible with a model in which one monensin molecule transports one metal ion. Rate constants for the association and dissociation of the monensin-metal complex in the membrane/water interface are extracted and the stability constants for complex formation are evaluated. The rate constants in DOPC are similar to those in EPC, confirming that diffusion is not rate-limiting in the transport process and that dissociation of the complex is the rate-limiting step. Although potassium on its own is transported more rapidly, sodium forms the more stable complex and is therefore transported preferentially in competition with potassium.

Introduction

One of the most important processes for the maintenance of viability in living cells is the ability to transport materials through their limiting membranes [1]. Cells must import their raw materials and export their waste products and other finished materials. Maintenance of ionic balance and membrane potential also requires efficient transport processes for ions, notably Na^+ and K^+, which are quantitatively the most important metal ions in living cells. The membrane-bound transport systems that perform these processes are mainly enzymes such as the Na^+/K^+ pumping enzyme Na^+/K^+-ATPase [2].

Ionophores offer an alternative mode of transport for metal ions [3–7]. Ionophoric materials are efficient transporters of metal ions and H^+ through the limiting membranes of living cells. Many ionophores, notably monensin, nigericin and valinomycin, are active as antibiotics [8] and have other striking physiological effects. These properties presumably arise from dissipation of transmembrane ion gradients. It is believed that ionophores transport ions through membranes in the form of their complex with the metal [9].

We have previously demonstrated that ^{23}Na- and ^{39}K-NMR can be used to study the ionophore-mediated transport of Na^+ and K^+ ions through phospholipid bilayers [10,11]. Briefly, the experiment involves the preparation of phosphatidylcholine vesicles with equal concentrations of metal ions inside and outside the vesicle, the establishment of a chemical shift difference by use of an aquous shift reagent for the metal ions and the obtaining of dynamic NMR spectra as the

* Present address: Department of Chemistry, The University, St Andrews, Fife, U.K.
Correspondence: F.G. Riddell, Department of Chemistry, The University, St Andrews, Fife, KY16 9ST, U.K.

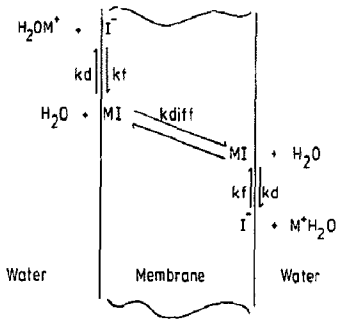

Fig. 1. Model used for ionic transport.

ionophore is added. The dynamic spectra allow rate constants to be measured for various concentrations of ionophore and of metal ion.

We also demonstrated [11] that the classical model of a mobile carrier system presented by Painter and Pressman [9] is satisfactory to account for the observed kinetics. This model is presented in Fig. 1. Three important phases are required for a metal ion to traverse the membrane. Firstly the ion must be complexed by the ionophore (k_f), then the ionophore-metal complex diffuses across the membrane (k_{diff}) and finally the the complex dissociates at the other membrane surface (k_d). Although both of the surface processes undoubtedly have many steps, they are conveniently represented by simple overall rate constants. The rate equations for transport derived from this model were presented and were shown to account satisfactorily for the observed kinetics of nigericin-mediated Na^+ and K^+ transport.

We were able to show for nigericin-mediated transport that diffusion was not rate-limiting and that consequently a plot of the reciprocal of the rate constant for transport vs. metal ion concentration should give both the rate constants for formation (intercept = $k_f'^{-1}$) and dissociation (slope = $k_d'^{-1}$) where the k' values incorporate terms in area and volume of the vesicles and in ionophore/lipid ratio. The apparent stability-constant for the metal-ionophore complex in the membrane is consequently given by slope/intercept. We were further able to demonstrate for nigericin that although sodium ions in isolation were transported faster by virtue of a faster dissociation step, potassium ions were transported preferentially in competition experiments due to the greater stability constant of the K^+ complex. This is in accord with the idea of nigericin being the anionic ionophore of preference for potassium.

We now present our results for the monensin-mediated transport of sodium and potassium through phosphatidylcholine bilayers. Monensin is an ionophoric antibiotic related to nigericin which is known to form complexes with all the alkali metal ions, the most stable of which in non-aqueous solvents is that with sodium. Monensin gives rise to dynamic ^{23}Na-, ^{39}K-, ^{87}Rb- and ^{7}Li-NMR spectra under our conditions. The rubidium results are difficult to quantify and the ^{7}Li results obtained by a magnetisation transfer technique will form the basis of separate reports [12,13].

Previous work on the transport properties of monensin have been reported by ourselves [10], by Sandeaux et al. [14,15] and by Degani and Elgavish [16]. Our results presented herein agree with these previous reports that monensin transports Na^+ by a 1:1 complex but now allow the extraction of rate constants for the reactions at the membrane surfaces and hence the stability constants for the M^+-monensin complex in the membrane surface.

Experimental

Large unilamellar vesicles were prepared from egg yolk phosphatidylcholine (EPC) and dioleoylphosphatidylcholine (DOPC) by a modification of the dialytic detergent removal technique introduced by Reynolds and co-workers [17] described in a previous paper [11]. In a typical preparation for sodium transport studies approx. 27 μmol of EPC together with 15 equivalents (0.405 mmol) of *n*-octyl-β-glucopyranoside were dissolved in 1.5 ml 100 mM NaCl. The solution was dialysed at 40°C against approx. 2 litres of 100 mM NaCl for 12 h. This dialysis was repeated three times by which time the vesicles had formed. The dialysis medium was then changed to 50 mM NaCl/10 mM Na_5PPP_i/20 mM choline chloride (PPP_i = linear tripolyphosphate) and two further dialyses were performed. For potassium the final dialysis medium was solely composed of K_5PPP_i and choline chloride. The amount of choline chloride added in all cases was such as to equalise the

osmotic pressure inside and outside the vesicles. All dialyses were performed under a constant stream of bubbled oxygen-free nitrogen and all dialysis solutions were presaturated with nitrogen at 40 °C for at least 12 h prior to use. Distilled water was used throughout.

The size of the vesicles is reflected by the percentage of the total volume enclosed. For the Na^+ and K^+ experiments with EPC the average internal volume was 11.5% in both cases. For the DOPC vesicles a smaller average size is indicated by the average internal volume being 6.6%. Because smaller vesicles give rise to shorter lifetimes of entrapped metal ions and the rate varies with r^{-1}, the rates for the DOPC vesicles have been reduced by a factor of $(11.5/x)^{1/3}$ where x is the percent Na^+ inside each vesicle preparation. The effect of this is to decrease k_f and k_d by approx. $(11.5/6.6)^{1/3}$. Details of vesicle sizes are given in a previous paper [11].

All preparations resulted in equal concentrations of metal ion inside and outside the vesicles and so the transport experiments involve no net movement of metal ions. A slight contraction of volume generally occurred upon dialysis and typically 1.45 ± 0.1 ml of vesicle suspension was recovered. To this suspension of large unilamellar vesicles was added sufficient of a 1 M solution of $DyCl_2$ (Na^+) or $TbNO_3$ (K^+) to generate a shift difference of approx. 12 ppm (Na^+) or 9 ppm (K^+), typically a few microlitres in both cases.

Commercial sodium and potassium tripolyphosphates were used throughout. The tripolyphosphates were shown by ^{31}P-NMR to contain approx. 85% triphosphate and 15% diphosphate. The errors in M^+ concentrations arising from this difference are less than 2%. Monensin was supplied by Sigma as its sodium salt (90–95% pure) and purified by dissolving in ethanol and precipitating with water. All phospholipids were supplied by Lipid Products.

Sodium transport studies were performed on a Bruker WP80 FT NMR spectrometer in Stirling operating at 21.19 MHz. Potassium transport studies were carried out on a Bruker WH360 FT NMR spectrometer in Edinburgh University operating at 16.8 MHz. In all cases the spectrometer was field/frequency locked on the ^{2}H resonance of ^2H_2O in the inner compartment of a coaxial tube. All measurements were performed at 303 K. Spectra were line-broaded, typically by 2 Hz (Na) or 5 Hz (K), to improve the signal-to-noise ratio. For ^{23}Na typically 4000 transients were collected into 512 data points with a spectral width of 1000 Hz, zero-filled and transformed in 4k data points. For ^{39}K typically 40000 transients were collected in 512 or 256 data points with a 2000 Hz spectral width, zero-filled and tranformed in 16k data points. Pulse widths of approx. 90° were used throughout and recycle times were several times T_1 in all cases.

Additional of small aliquots (microlitre amounts) of a standard solution of monensin in methanol 0.005–0.01 M caused a dynamic line broadening of both the in and out lines from the vesicle preparation. Lifetimes (τ) were followed by measuring the line broadening of the M^+_{in} peak using the relationship $(1/\tau) = \pi$ (line broadening) which applies near the slow exchange limit at which we are working. Rate constants (k) for efflux were determined from plots of $1/\tau$ vs. nigericin/PC molar ratio. Results are given in Tables I–III. Ratios of phosphatidylcholine to monensin used in this work range from approx. 6000 to 250. This corresponds to a concentration range of monensin in the membrane of approx. $0.65 \cdot 10^{-4}$ to $4.6 \cdot 10^{-3}$ M.

The activation energy for the overall process was determined by following the temperature variation of the linewidth of the Na^+_{in} signal. Sufficient monensin was added to a vesicle preparation at 100 mM Na^+ to establish a moderate rate (approx. 17 s^{-1}) at 303 K. The line broadenings at various temperatures were calculated from the observed linewidth minus the natural linewidth at that temperature (Table IV).

Results and Discussion

As we have previously observed for both monensin and nigericin, adding small aliquots of monensin to the vesicle preparation broadened both lines in the spectra in a fashion consistent with a dynamic exchange process between the M^+_{in} and M^+_{out} populations. The rates of exchange at different metal ion concentrations are recorded in Tables I–III. For every concentration of Na^+ and K^+ studied the transport rate in the direction in

TABLE I

RATE CONSTANTS FOR SODIUM TRANSPORT IN EPC

From the values presented in the table, $k_f' = (4.878 \pm 0.694) \cdot 10^4$ s^{-1}, $k_d' = (1.498 \pm 0.067) \cdot 10^3$ $M \cdot s^{-1}$, correlation coefficient = 0.983 and $K = 32.6 \pm 8.3$ M^{-1}.

$[Na^+]$ (M)	k (mol PC·mol monensin^{-1}·s^{-1})	S.D.	Correlation coefficient
0.025	$2.932 \cdot 10^4$	$0.662 \cdot 10^3$	0.995
0.375	$2.159 \cdot 10^4$	$0.362 \cdot 10^3$	0.997
0.050	$1.632 \cdot 10^4$	$0.255 \cdot 10^3$	0.998
0.075	$1.261 \cdot 10^4$	$0.145 \cdot 10^3$	0.999
0.100	$1.244 \cdot 10^4$	$0.409 \cdot 10^3$	0.987
0.100	$1.274 \cdot 10^4$	$0.192 \cdot 10^3$	0.998
0.125	$1.004 \cdot 10^4$	$0.211 \cdot 10^3$	0.995
0.150	$0.869 \cdot 10^4$	$0.321 \cdot 10^3$	0.983
0.200	$0.612 \cdot 10^4$	$0.656 \cdot 10^3$	0.928

TABLE II

RATE CONSTANTS FOR POTASSIUM TRANSPORT IN EPC

From the values presented in the table, $k_f' = (2.299 \pm 0.350) \cdot 10^4$ s^{-1}, $k_d' = (4.329 \pm 0.973) \cdot 10^3$ $M \cdot s^{-1}$, correlation coefficient = 0.814 and $K = 5.3 \pm 2.1$ M^{-1}.

$[Na^+]$ (M)	k (mol PC·mol monensin^{-1}·s^{-1})	S.D.	Correlation coefficient
0.075	$1.685 \cdot 10^4$	$0.412 \cdot 10^3$	0.994
0.100	$1.533 \cdot 10^4$	$0.540 \cdot 10^3$	0.979
0.125	$1.248 \cdot 10^4$	$0.303 \cdot 10^3$	0.997
0.150	$1.359 \cdot 10^4$	$0.789 \cdot 10^3$	0.980

TABLE III

RATE CONSTANTS FOR SODIUM TRANSPORT IN DOPC

From the values presented in the table, $k_f' = (1.307 \pm 0.212) \cdot 10^4$ s^{-1}, $k_d' = (0.949 \pm 0.089) \cdot 10^3$ $M \cdot s^{-1}$, correlation coefficient = 0.967, and $K = 13.8 \pm 5.4$ M^{-1}.

$[Na^+]$ (M)	k (mol PC·mol monensin^{-1}·s^{-1})	S.D.	Correlation coefficient
0.025	$1.201 \cdot 10^4$	$1.279 \cdot 10^3$	0.983
0.050	$0.640 \cdot 10^4$	$0.407 \cdot 10^3$	0.993
0.100	$0.593 \cdot 10^4$	$0.087 \cdot 10^3$	0.999
0.150	$0.402 \cdot 10^4$	$0.115 \cdot 10^3$	0.998
0.200	$0.358 \cdot 10^4$	$0.189 \cdot 10^3$	0.992

→ out varies linearly with monensin concentration, indicating a first-order relationship between monensin concentration and the transport rate.

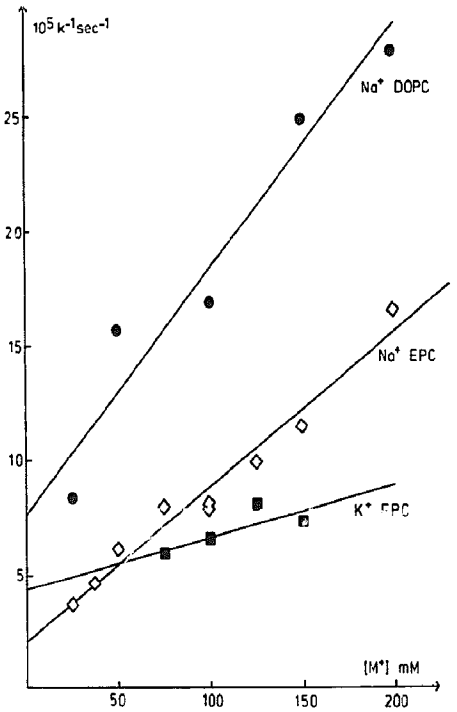

Fig. 2. Graph of k^{-1} vs. $[M^+]$ for Na^+ and K^+.

The linear relationship between k^{-1} and metal ion concentration (Fig. 2) is also in accord with our model. Together these results present strong confirmation of the validity of our model and of a 1:1 complex between M^+ and the transporting ionophore.

As with nigericin, the difference between the slopes of the lines for Na^+ and K^+ in Fig. 2 is strong evidence that diffusion is not the rate-limiting step. Our model predicts that if diffusion were rate-limiting the slopes would be given by $1/k_{diff}$. Since the Na^+ and K^+ complexes should have similar molecular volumes and shapes the diffusion rates should be similar for both metals. The 3-fold difference in slope for Na^+ and K^+ therefore argues strongly against diffusion being (wholly) rate-determining in the transport process for monensin. As reported earlier [11], incorporation of cholesterol into the membrane which is widely believed to stiffen the interior of biological membranes [18] had no appreciable effect on the monensin-mediated transport rates for sodium.

Further, a similar series of experiments in which Na^+ transport using vesicles of DOPC mediated by monensin was studied resulted in a k^{-1} vs. concentration plot almost parallel to the line for EPC but with a somewhat higher intercept. The incorporation of further unsaturation into the fatty acyl chains is believed to increase the mobility of the interior of the membrane and should lead to a faster diffusion rate through the membrane, i.e., different slope if diffusion is rate-limiting. In fact the main difference is in the intercept, indicating a slightly slower association rate. The slope of the DOPC line is slightly steeper than the EPC line, the wrong direction for a process with diffusion as the rate-limiting step, since it would indicate slower diffusion in the more mobile membrane. These three pieces of evidence point strongly to dissociation, and not diffusion, being rate-limiting.

Comparison of the rate data with that obtained for nigericin shows that the formation rates of the nigericin complexes are greater. The sodium complex of nigericin forms at about twice the rate of monensin and the potassium complex at about 4-times the rate. The dissociation rates show a reversed position for monensin compared to nigericin. The sodium-nigericin and potassium-monensin are the faster-dissociating complexes in each instance.

Application of the Arrhenius equation to the results presented in Table IV gives a small positive activation energy (7.8 kcal · mol^{-1}). This activation energy was obtained under almost identical conditions to those used for the same experiment on nigericin (monensin/EPC ≈ 1 : 690; nigericin/EPC ≈ 1 : 870) which showed a similar activation energy of 8.2 kcal · mol^{-1} [11]. These activation energies cannot be interpreted in detail as the temperature dependence of k arises from several individual rate constants. The results are, however, not unreasonable since the activation energies for the formation and dissociation of ionophore-metal complexes in homogeneous solution are small [19].

The ratio of slope to intercept gives the apparent stability constant for the ionophore-metal complex in the membrane surface. The values obtained for PC are 32.6 ± 8.3 M^{-1} (Na^+) and 5.3 ± 2.1 M^{-1} (K^+). For the DOPC vesicles the value obtained for Na^+ is 19.3 ± 5.4 M^{-1}. The Na^+ complex of monensin is thus considerably more stable in the membrane/water interface than the K^+ complex. This distinction also holds in homogeneous methanol solution but the absolute magnitudes of the stability constants are considerably larger: log K (methanol) = 6.48 (Na^+) and 4.97 (K^+) [21]. It is noticeable that the difference in stability constants for the Na^+ and K^+ complexes arises largely, as in the case of nigericin, from a difference in dissociation rate, with the association rates being very similar. This too is seen in homogeneous solution [21].

As we suggested before, part of the reduction in the stability constant on going from methanol to our membrane interface may be attributed to the higher solvation energy of the metal ions in water compared to methanol [22]. The remainder may come from interactions between the various participating species in the complex environment of the interface where the zwitterionic head group of the phospholipid, the carboxylate group of the ionophore, the metal ions, the anions and water all mingle.

Independent confirmation of the significantly greater stability of the Na^+ complex is given by the observation that an equimolar amount of K^+ does not significantly alter the transport rate of Na^+ [23]. The 6-fold greater stability of the Na^+ complex will ensure that in the presence of K^+ it monopolises the available monensin, leading to a negligible reduction in the Na^+ transport rate.

TABLE IV

TEMPERATURE VARIATION OF SODIUM TRANSPORT RATES [a,b]

Temperature (K)	k (s^{-1}) [c]
283	6.5
293	10.7
303	16.9
313	24.9
323	37.2
328	44.5

[a] Conditions: EPC, 100 mM Na^+, sufficient monensin to give a rate constant of 16.9 s^{-1} at 303 K.

[b] These results give an activation energy of 7.8±0.13 kcal · mol^{-1}.

[c] $k = 1/\tau_{M_{in}^+}$.

Acknowledgement

We thank the SERC for a grant to carry out this work and for an allocation of time on the high-field WH-360 spectrometer in Edinburgh.

References

1 Wright, J.K., Seckler, R. and Overath, P. (1986) Annu. Rev. Biochem. 55, 225–248.
2 Glynn, I.M. and Karlish, S.J.D. (1975) Annu. Rev. Physiol. 37, 13–55.
3 Topics Current Chem. (1981) 98, 1–41, 43–106; (1982) 101, 1–82, 83–110, 111–145.
4 Lehn, J.M. (1973) Struct. Bond. (Berlin) 16, 113–160.
5 Simon, W., Morf, W.E. and Meier, P.C. (1973) Struct. Bond. (Berlin) 16, 113–160.
6 Pressmann, B.C. (1976) Annu. Rev. Biochem. 45, 501–530.
7 Ovchinnikov, Y.A. (1979) Eur. J. Biochem. 94, 321–336.
8 Metal Ions in Biological Systems (1985) Vol. 19 (Sigel, H., ed.), Marcel Decker, New York.
9 Painter, G.R. and Pressman, B.C. (1982) Topics Current Chem. 101, 83–110.
10 Riddell, F.G. and Hayer, M.K. (1985) Biochim. Biophys. Acta 817, 313–317.
11 Riddell, F.G., Arumugam, S., Brophy, P.J., Cox, B.G., Payne, M.C.H. and Southon, T.E. (1988) J. Am. Chem. Soc. 110, 734–738.
12 Riddell, F.G., Arumugam, S. and Cox, B.G. (1987) Chem. Commun. 1890–1891.
13 Riddell, F.G., Arumugam, S. and Cox, B.G. (1988) in Li: Inorganic Pharmacology and uses in Psychiatry (Birch, N., ed.), IRL Press, Oxford.
14 Sandeaux, R., Seta, P., Jeninet, G., Allemand, M. and Gavach, C. (1978) Biochim. Biophys. Acta 511, 499–508.
15 Sandeaux, R., Sandeaux, J., Gavach, C. and Brun, B. (1982) Biochim. Biophys. Acta 684, 127–132.
16 Degani, H. and Elgavish, G.A. (1978) FEBS Lett. 90, 357.
17 Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds, J.A. (1981) Biochemistry 20, 833–840.
18 Shah, D.O. and Schulman, J.H. (1967) J. Lipid Res. 8, 215–226.
19 Blok, M.C., Van Deenen, L.L.M. and De Gier, J. (1977) Biochim. Biophys. Acta 464, 509–518.
20 Cheng, K.-H., Lepcock, J.R., Hui, S.W. and Yeagle, P.L. (1986) J. Biol. Chem. 261, 5081–5087.
21 Cox, B.G. (1985) Annu. Rep. Chem. Soc. C, 43–80.
22 Cox, B.G. (1973) Annu. Rep. Chem. Soc. A, 249–274.
23 Hayer, M.K. (1984) Ph.D. Thesis, Stirling University, Stirling, U.K.