Differential movement of ions in artificial phospholipid vesicles

Edward G. Sedgwick and Philip D. Bragg

Department of Biochemistry, University of British Columbia, 2146 Health Sciences Mall, Vancouver, BC, V6T 1W5, Canada

Received 13 June 1990; revised version received 6 August 1990

Pyranine was incorporated into sonicated unilamellar vesicles of soybean phosphatidylcholine to monitor changes in the internal pH of the vesicles. Dilution of soybean phosphatidylcholine vesicles loaded with 0.3 M KCl, KNO_3 or K_2SO_4 into salt-free buffer resulted in rapid exchange of K^+ and protons. A pseudoequilibrium distribution of ions was achieved, since addition of valinomycin, uncoupler or nigericin now caused a rapid alkalinization of the vesicle interior. Dilution into buffer containing NaCl gave a further exchange of Na^+ and protons following the initial K^+/proton exchange. Na^+ permeation was slower than that of K^+. A stable membrane potential was not generated by the ion movements. It is proposed that aqueous channels are formed through the phospholipid bilayers and that K^+ and Na^+ permeate through these channels as the hydrated ions.

Ion movement; Liposome; Aqueous channel; Pyranine; Vesicular pH

1. INTRODUCTION

Small inorganic ions such as Na^+ and K^+ do not readily cross model phospholipid membranes. Permeabilities are generally in the range of 10^{-12} to 10^{-14} cm/s [1–4]. The permeability for protons is much greater [5,6] and may arise by the formation of transient hydrogen-bonded chains of water molecules across the bilayer [6]. However, in contrast with these results, Scarpa and De Gier [7] have observed that multilamellar vesicles of egg phosphatidylcholine and of a highly unsaturated phosphatidylcholine, containing 70% linoleate, show significant permeability to Na^+ and K^+ but with limiting permeability to protons.

In this paper, we have shown that unilamellar vesicles of soybean phosphatidylcholine allow permeation by both Na^+ and K^+, but with discrimination between them, and, in contrast with Scarpa and De Gier, we find that permeation by protons is not rate-limiting. Furthermore, counterflow of K^+ and protons reaches a stable pseudoequilibrium which can be discharged by ionophores.

2. MATERIALS AND METHODS

2.1. *Preparation of phospholipid vesicles*

Soybean phosphatidylcholine (Sigma), purified by the procedure of Kagawa and Racker [8], was resuspended at a concentration of 50 mg/ml in 10 mM Hepes buffer containing the indicated salt at 0.3 M, and adjusted to the desired pH with NaOH or KOH. Pyranine (2.5 mM) was added and the mixture was then sonicated with a bath-type sonicator (Branson 1200) until it had cleared. The excess fluorescent dye was then removed by passing the sample through a 10 × 1 cm Sephadex G-50 (Fine) gel-filtration column equilibrated with the same buffer. Aliquots (0.05 ml) of these pyranine-loaded, column-processed vesicles were added directly to fluorescence assay systems. In experiments using the carbocyanine dye DISC(3)-5, it was added to vesicle suspensions as indicated to a final concentration of 5 μM. In some experiments large unilamellar vesicles were prepared by extrusion of soybean or egg yolk (Sigma Type V-E) phosphatidylcholine through polycarbonate filters of 100 nm pore size using an extrusion device (Lipex Biomembranes, Vancouver, BC). Where efflux of potassium was measured with a K^+-electrode (Orion) vesicles were prepared in 10 mM Hepes buffer, pH 7.0, containing 0.3 M KCl. Excess K^+ was removed by passing the sample through a 10 × 1 cm Sepharose CL 6B gel-filtration column equilibrated with 10 mM Hepes buffer, pH 7.0, containing 0.6 M sucrose. The diameter of the vesicles was determined by quasi-elastic light scattering using a Nicomp Model 270 submicron particle sizer [9].

2.2. *Fluorescence measurements*

The fluorescence of pyranine was measured at 22°C with a Turner model 420 spectrofluorometer connected to a Linear chart recorder. The excitation and emission wavelengths were 460 nm and 520 nm, respectively. The fluorescence of DISC(3)-5 was measured in an SLM Aminco SPF 500C spectrofluorometer (Urbana, IL) interfaced to a Hewlett Packard Model 7470A plotter. Fluorescence was excited at 622 nm (5 nm band pass) and emission was measured at 670 nm (10 nm band pass). The fluorescence response of pyranine was calibrated in terms of pH by addition of acid or alkali to the external medium and observing the extent of the fluorescence change of the entrapped pyranine in the presence of uncoupler.

2.3. *Determination of vesicle volume*

To determine the trapped volume vesicles were prepared in 10 mM Hepes buffer, pH 7.0, containing 2 μCi/ml [^{14}C]sucrose. The excess sucrose was removed by passing the sample through a 10 × 1 cm Sephadex CL-6B gel filtration column equilibrated with 10 mM Hepes, pH 7.0. The amount of [^{14}C]sucrose retained in aliquots of these vesicles was determined by liquid scintillation counting. Phospholipid content was measured by the method of Osborne et al. [10].

Correspondence address: E.G. Sedgwick, Department of Biochemistry, University of British Columbia, 2146 Health Sciences Mall, Vancouver, BC, Canada V6T 1W5

Abbreviations: DISC(3)-5, 3',3'-diisopropylthiodicarbocyanine; TCS, 3,3',4',5-tetrachlorosalicylanilide

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50 © 1990 Federation of European Biochemical Societies

3. RESULTS

3.1. *Characteristics of soybean phosphatidylcholine vesicles*

The phospholipid vesicles were prepared by bath sonication of soybean phosphatidylcholine in the appropriate buffer (at pH 6.75). The vesicle population was sized by quasi-elastic light scattering using a Nicomp Model 270 submicron particle sizer. Two distinct populations were present: a mean diameter of 50 nm was shown by 33% of the population; the remainder had a mean diameter of 170 nm. The NMR spectra gave no indication of multilamellar vesicles. The trapped volume of vesicles was measured using [^{14}C]sucrose. A value of 0.87 μl/μmol soybean phosphatidylcholine was obtained. This can be compared with a trapped volume of 1.2 μl/μmol soybean phosphatidylcholine observed by others [9]. A value of 1.06 μl/μmol soybean phosphatidylcholine was obtained by measuring with a K^+-electrode the amount of K^+ released on dilution of vesicles loaded with 0.3 M KCl into buffer in the presence of valinomycin.

3.2. *Ion movements in vesicles of soybean phosphatidylcholine*

The fluorescent dye pyranine has been shown to be a reliable reporter of the pH changes within phospholipid vesicles [11]. Vesicles were loaded with pyranine, and the excess dye outside the vesicles was removed by column chromatography. Vesicles were loaded with different salts and diluted into buffer of the desired composition. The flux of other cations across the vesicle membrane was measured indirectly by determining the changes in the fluorescence of pyranine in response to counter movement of protons or hydroxyl ions. In some experiments efflux of K^+ was verified using a K^+ electrode.

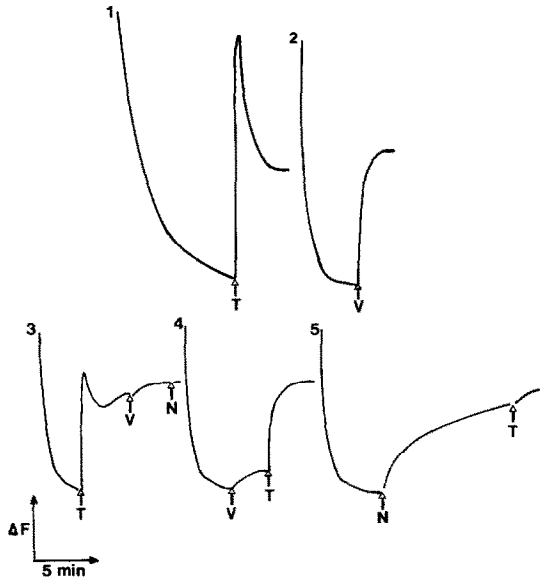

Fig. 1. Change in the fluorescence of entrapped pyranine in soybean phosphatidylcholine vesicles loaded with 0.3 M KNO_3 (panels 1, 2) or KCl (panels 3–5) on dilution into salt-free buffer. T, V and N: addition of 25 μM TCS, 5 ng valinomycin and 5 ng nigericin, respectively. Fluorescence is expressed in arbitrary units.

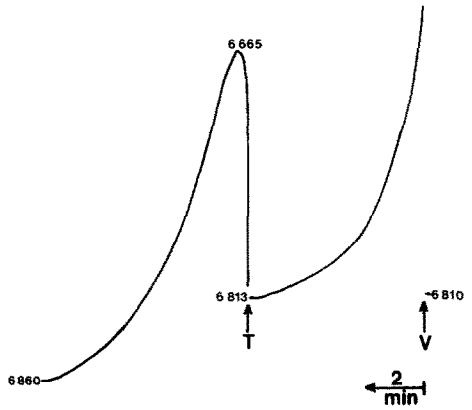

Fig. 2. Change in external pH on dilution of soybean phosphatidylcholine vesicles loaded with 0.3 M KCl into salt-free buffer. T, addition of 50 μM TCS; V, addition vesicles; pH was measured with a pH electrode. The pH of the external medium at various times during the experiment is given on the chart.

Fig. 1 shows the effect of adding phospholipid vesicles loaded with 0.3 M KNO_3 or KCl into buffer lacking these salts. A rapid decline in fluorescence towards a stable value was observed. The decline in fluorescence indicates an acidification of the vesicle interior as protons enter in exchange for K^+ effluxing from the vesicle down its concentration gradient. This acidification is not seen if the vesicles are diluted into buffer containing KNO_3 or KCl. Addition of the uncoupler TCS or valinomycin resulted in a very rapid alkalinization of the vesicle interior as protons effluxed from the vesicles. Proton movement in these experiments was confirmed by measuring the pH of the external medium with an electrode (Fig. 2).

Dilution of KNO_3- (Fig. 3, panels 1, 2), K_2SO_4- (panels 3, 4) or KCl-loaded vesicles (panels 5, 6) into

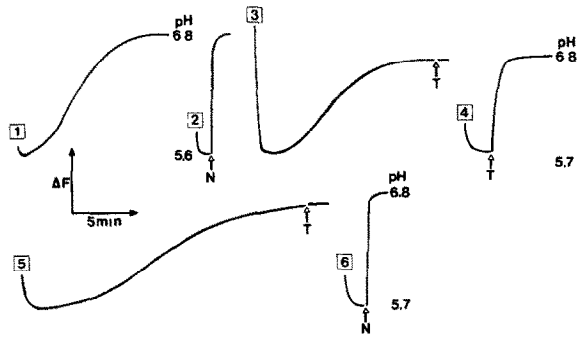

Fig. 3. Change in the fluorescence of entrapped pyranine in soybean phosphatidylcholine vesicles loaded with 0.3 M KNO_3 (panels 1, 2), K_2SO_4 (panels 3, 4) or KCl (panels 5, 6) on dilution into buffer containing 0.3 M NaCl. T and N: addition of 25 μM TCS and 5 ng nigericin, respectively. Fluorescence is expressed in arbitrary units.

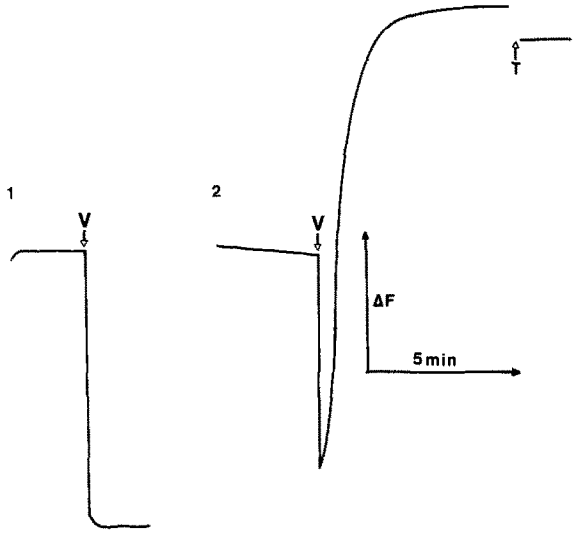

Fig. 4. Change in the fluorescence of entrapped pyranine in egg phosphatidylcholine vesicles loaded with 0.3 M KCl and diluted into salt-free buffer (panel 1) or buffer containing 0.3 M NaCl (panel 2). V, addition of 5 ng valinomycin. T, addition of 25 μM TCS. Fluorescence is expressed in arbitrary units.

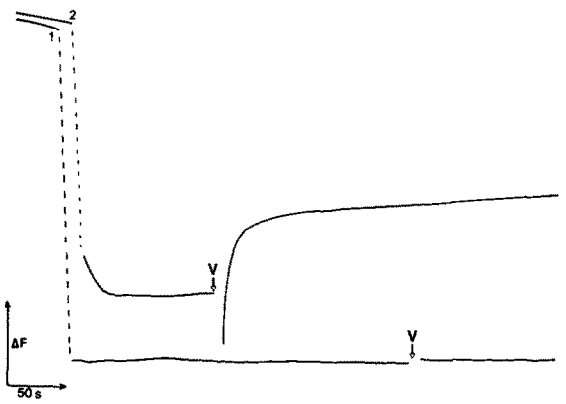

Fig. 5. Change in the fluorescence of DISC(3)-5 on addition of large unilamellar vesicles of soybean (1) or egg (2) phosphatidylcholine loaded with 0.1 M KCl to salt-free buffer. V, addition of 5 ng valinomycin. Fluorescence is expressed in arbitrary units.

0.3 M NaCl in buffer again gave a rapid fall in the internal pH from pH 6.75 to about pH 5.6 due to K^+/H^+ exchange. As in Fig. 1, addition of TCS or nigericin resulted in a rapid internal alkalinization to an equilibrium value as protons moved down their concentration gradient (with TCS) or re-exchanged with K^+ (in the presence of nigericin) (Fig. 3, panels 2, 4, 6). However, if the ionophore was not added a subsequent alkalinization was observed with vesicles loaded with any of the potassium salts. The alkalinization returned the internal pH to the same value (pH 6.8–6.9) obtained with the ionophore (Fig. 3, panels 1, 3, 5). This effect depended on the presence of external Na^+, and must therefore result from the partial discharge of the pH gradient, generated by the initial K^+/H^+ exchange, by exchange of Na^+ for protons. Note that the exchange of Na^+ was slower than that of K^+ for protons.

3.3. *Ion movements in vesicles of egg phosphatidylcholine*

Vesicles of egg phosphatidylcholine were prepared in an analogous manner to those of soybean phosphatidylcholine. Dilution of KCl-loaded vesicles into salt-free buffer did not result in rapid K^+/H^+ exchange, in contrast to the results with the soybean phospholipid (Fig. 4). Addition of valinomycin gave rapid acidification of the vesicle interior as K^+ effluxed from the vesicle to be replaced by protons.

Dilution of 0.3 M KCl-loaded vesicles into 0.3 M NaCl in buffer again showed little evidence of ion movement until valinomycin had been added (Fig. 4). Then, a rapid alkalinization reversed the initial acidification phase. The entry of Na^+ in exchange for internal protons is similar to that which occurs with vesicles of soybean phosphatidylcholine.

3.4. *Membrane potential in phospholipid vesicles*

The possibility that a membrane potential had a role in the movement of ions in the vesicles of soybean phosphatidylcholine was examined using the fluorescent carbocyanine potential probe DISC(3)-5. Dilution of large unilamellar vesicles of soybean phosphatidylcholine containing 0.1 M KCl, or vesicles formed by sonication, into salt-free buffer gave an immediate non-specific quenching of fluorescence. There was no further response following addition of valinomycin (or TCS, not shown) (Fig. 5). As a control, large unilamellar vesicles of egg phosphatidylcholine loaded with KCl were used. Addition of valinomycin generated a membrane potential, interior negative (Fig. 5).

4. DISCUSSION

Using pyranine to monitor the internal pH of sonicated unilamellar vesicles of soybean phosphatidylcholine, or a pH electrode to measure external pH, we have shown that both K^+ and Na^+ will cross the phospholipid bilayer. The movement of Na^+ was slower than that of K^+. The permeation by H^+ was rapid and did not evidently limit the movement of Na^+ and K^+. This result is in contrast to that of Scarpa and De Gier [7], and also with our experiments using vesicles of egg phosphatidylcholine (Fig. 4). In these cases movement of Na^+ and K^+ was limited by that of H^+. Thus, addition of uncoupler caused K^+ to exit from the vesicles down its concentration gradient with entry of protons. The reason for the difference in permeability between vesicles of soy and egg phosphatidylcholine is not clear at present. It is possible that the presence of other phospholipids in the

former preparation decreases the tightness of packing of the fatty acyl side-chains, thus permitting the formation of aqueous channels more readily (see below).

The driving force for ion movement in the experiments described above is the concentration gradient of K^+. However, it is puzzling that addition of the ionophores induced reversal of ion movements when the system had apparently come to equilibrium, and when the exit of K^+ and entry of protons was a 1:1 exchange, as indicated by the lack of generation of a membrane potential. It seems likely that the exit of K^+ from the vesicles resulted in a pseudoequilibrium which decayed on addition of ionophore. The 'overshoot' shown in Fig. 1, panels 1 and 3, also suggests that the vesicles studied here have a propensity to establish non-equilibrium states.

Our results are not due to ionophore-induced lysis of the vesicles. Ionophores did not cause the release of [^{14}C]sucrose from vesicles preloaded with this substance. It is possible that the swelling caused by dilution of salt-loaded vesicles into salt-free buffer favours the formation of aqueous channels across the bilayer. However, ion exchange does occur in the absence of an osmotic gradient, for example, on dilution of KCl-loaded vesicles of soybean phosphatidylcholine into isotonic sucrose or NaCl. Dilution of the vesicles into different concentrations of NaCl (range 0.02–0.3 M) did not apparently affect the rate of the initial K^+/H^+ exchange. The rate of the subsequent Na^+/H^+ exchange was diminished as the concentration of NaCl was lowered (results not shown). These results suggest that the osmotic gradient as such is not the primary driving force for rapid ion exchange. The more rapid movement of the hydrated K^+ (radius 3 Å) than hydrated Na^+ (radius 4.5 Å) would be consistent with the aqueous channel mechanism. (Non-hydrated ionic radii in crystals or K^+ and Na^+ are 1.33 Å and 0.95 Å, respectively, [12].) Movement of protons along the ordered hydrogen-bond chain of water in the channel would be rapid [6].

Acknowledgements: This research was supported by a grant from the Medical Research Council of Canada. Large unilamellar vesicles were prepared by Simon Eastman. Nuclear magnetic resonance measurements were carried out by Dr Kim Wong. Drs Vladimir Palaty and Kim Wong are thanked for helpful discussions.

REFERENCES

[1] Hauser, H., Phillips, M.C. and Stubbs, M. (1972) Nature 239, 342–344.
[2] Johnson, S.M. and Bangham, A.D. (1969) Biochim. Biophys. Acta 193, 82–91.
[3] Mimms, L.T., Zampighi, Y., Nozaki, Y., Tanford, C. and Reynolds, J.A. (1981) Biochemistry 20, 833–840.
[4] Pike, M.M., Simon, S.S., Balschi, J.A. and Springer, C.S. (1982) Proc. Natl. Acad. Sci. USA 79, 810–814.
[5] Perkins, W.R. and Cafiso, D.A. (1986) Biochemistry 25, 2270–2276.
[6] Deamer, D.W. and Nichols, J.W. (1989) J. Membrane Biol. 107, 91–103.
[7] Scarpa, A. and De Gier, J. (1971) Biochim. Biophys. Acta 241, 789–797.
[8] Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487.
[9] Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim. Biophys. Acta 812, 55–65.
[10] Osborn, M.J., Gander, J.E., Parisi, E. and Carson, J. (1972) J. Biol. Chem. 247, 3962–3972.
[11] Clement, N.R. and Gould, J.M. (1981) Biochemistry 20, 1534–1538.
[12] Netter, H. (1969) Theoretical Biochemistry, John Wiley, New York.