Biochimica et Biophysica Acta, 642 (1981) 135—148

BBA 79143

INCORPORATION OF THE TRANSMEMBRANE HYDROPHOBIC DOMAIN OF GLYCOPHORIN INTO SMALL UNILAMELLAR PHOSPHOLIPID VESICLES

ION FLUX STUDIES

ALICE Y. ROMANS *, THERESA M. ALLEN **, WILLIAM MECKES, ROBERT CHIOVETTI, Jr. ***, LULU SHENG, HENRI KERCRET * and JERE P. SEGREST x

Departments of Pathology, Biochemistry and Microbiology, Institute of Dental Research and Comprehensive Cancer Center, University of Alabama in Birmingham Medical Center, Birmingham, AL 35294 (U.S.A.)

(Received October 29th, 1980)

Key words: Ion flux; Glycophorin; Membrane protein; Trypsin; Liposome; Phosphatidylcholine; Hydrophobic domain

Summary

Human erythrocyte glycophorin is one of the best characterized integral membrane proteins. Reconstitution of the membrane-spanning hydrophobic segment of glycophorin (the tryptic insoluble peptide released when glycophorin is treated with trypsin) with liposomes results in the production of freeze-fracture intrabilayer particles of 80 Å diameter (Segrest, J.P., Gulik-Krzywicki, T. and Sardet, C. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3294—3298), with particles appearing at or above a tryptic insoluble peptide concentration of 4 mmol per mol phosphatidylcholine. In the present study, increasing concentrations of tryptic insoluble peptide were added to sonicated small unilamellar egg phosphatidylcholine vesicles and the rate of efflux of $^{22}Na^{+}$

* Present address: Kimberly-Clark Corporation, Neenah, WI 54956, U.S.A.
** Present address: Department of Pharmacology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada.
*** Present address: Abteilung Mikrobiologie, Biozentrum, Universität Basel, CH-4056 Basel, Switzerland.
* Present address: Laboratoire de Neurobiologie Moleculaire, Campus de Beaulier, 35042 Rennes, Cedex, France.
x To whom correspondence should be addressed.
Abbreviation: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

was examined by rapid (30 s) gel filtration on Sephadex G-50. Below a concentration of 3—5 mmol tryptic insoluble peptide/mol phosphatidylcholine, $^{22}Na^+$ efflux occurs at a constant slow rate at given tryptic insoluble peptide concentrations. Above a concentration of 3—5 mM, the rate of efflux is biphasic at given tryptic insoluble peptide concentrations, exhibiting both an initial fast and a subsequent slow component. On the basis of graphic and computer curve-fitting analysis, with increasing tryptic insoluble peptide concentration, the rate of the slow component reaches a plateau at a tryptic insoluble peptide concentration of 3—5 mM and remains essentially constant until much higher concentrations are reached; the fast component increases linearly with increasing tryptic insoluble peptide concentration well beyond 5 mM. The most consistent interpretation of this data is as follows. The slow $^{22}Na^+$ efflux component is due to perturbations of small unilamellar vesicle integrity by tryptic insoluble peptide monomers. At a tryptic insoluble peptide concentration of 3—5 mmol/mol, a critical concentration is reached following which there is intrabilayer tryptic insoluble peptide self-association. The fast $^{22}Na^+$ efflux component is due to the increasing presence of tryptic insoluble peptide self-associated multimers (the 80-Å particles seen by freeze-fracture electron microscopy) which results in a significantly larger bilayer defect than do tryptic insoluble peptide monomers. The failure of complete saturation of efflux by the fast component is ascribed to the presence of two populations of small unilamellar vesicles, some of which contain tryptic insoluble peptide multimers and some of which do not.

Addition of cholesterol to the tryptic insoluble peptide/phosphatidylcholine vesicles decreases the rate of $^{22}Na^+$ efflux by inhibiting primarily the fast component. Freeze-fracture electron microscopy indicates that the presence of cholesterol has no effect on the size, number or distribution of 80-Å intrabilayer particles in the tryptic insoluble peptide/phosphatidylcholine vesicles. These results are consistent with a mechanism to explain the fast Na^+ efflux component involving protein-lipid boundary perturbations.

Efflux of $^{45}Ca^{2+}$ from phosphatidylcholine vesicles is also enhanced by incorporation of tryptic insoluble peptide, but only if divalent cations (Ca^{2+} or Mg^{2+}) are present in the external bathing media as well as inside the sonicated vesicles. If monovalent Na^+ only is present in the bathing media no $^{45}Ca^{2+}$ efflux is seen. Under conditions where $^{45}Ca^{2+}$ efflux is seen, both a fast and a slow component are present, although both appear lower than corresponding rate constants for $^{22}Na^+$ efflux. These results suggest a coordinated mechanism for ion efflux induced by tryptic insoluble peptide and, together with the $^{22}Na^+$ efflux studies, may have mechanistic implications for the transbilayer phospholipid exchange (flip-flop) suggested to be induced at glycophorin/phospholipid interfaces (de Kruijff, B., van Zoelen, E.J.J. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 509, 537—542).

Introduction

Protein-lipid interactions play a major role in determining the spatial and temporal properties of biological membranes. An understanding of the basic

principles of protein-lipid association is certain to be important to the ultimate conceptualization of many biological phenomena, such as membrane transport, membrane receptors and membrane-cytoskeleton interactions. Most intact biological membranes are sufficiently complex such that they severely limit our understanding of basic membrane mechanisms. One possible means of limiting the variables is to isolate a single structurally well characterized membrane protein and to study its interactions with biologically relevant lipids in a reconstituted membrane preparation.

Protein-protein interactions have been postulated to play key roles in determining membrane functions such as transport and mediation of transmembrane messages [3,4]. We previously reported that the membrane-associating domain of glycophorin [5,6], represented by the insoluble tryptic fragment, T(is), which is released when glycophorin is treated with trypsin, induces an increased ion flux through black lipid membranes when incorporated into the membranes [7]. In addition, freeze-fracture electron microscopic data suggests that protein-protein interactions occur between T(is) peptides incorporated into liposomes [1]. In the present communication, we present studies of ion fluxes from small unilamellar phosphatidylcholine vesicles measured as a function of concentration of incorporated T(is).

Materials and Methods

Lipids. Egg phosphatidylcholine and cholesterol were obtained from Avanti Biochemicals (Birmingham, AL) and were chromatographically pure as shown on thin-layer chromatography in chloroform/methanol/acetic acid/water and petroleum ether/diethyl ether/acetic acid systems, respectively. [^{14}C]Phosphatidylcholine, labeled in the choline head group, was obtained from New England Corp. All lipids were stored at −70°C under nitrogen. All lipid concentrations were determined by established procedures [8,9].

Chemicals. Tes and L-histidine were obtained from Sigma Chemical Co. Sephadex G-50 (medium) was purchased from Pharmacia. $^{22}Na^+$ and $^{45}Ca^{2+}$ were obtained from New England Nuclear Corp. Other chemicals were reagent grade. Deionized water was glass-distilled before use. Trifluoroethanol was obtained from Aldrich.

Proteins. Glycophorin, the MN-glycoprotein of human red cell membranes [10], was isolated following lithium diiodosalicylate disruption of red cell ghosts [11]. The hydrophobic segment T(is) was isolated from glycophorin by trypsin treatment followed by established isolation procedures [1], and its concentration determined by amino acid analysis on a Beckman 121-M amino acid analyzer with computing integrator. Glycophorin was lipid-depleted by washing with $CHCl_3/CH_3OH$ (2 : 1, v/v) followed by dialysis against distilled water.

Preparation of proteoliposomes and liposomes. Liposomes containing specific lipids or lipid mixtures were prepared by rotary evaporation and overnight lyophilization from solvents in which the lipids or lipid mixtures were soluble. The lipid film was then hydrated by vortex mixing at 23°C for 2 h in an aqueous buffer (2 mM Tes, 2 mM histidine, 100 mM NaCl or 100 mM $CaCl_2$, and 0.1 mM EDTA) containing approx. 0.5 μCi of the radioactive cation or anion. After hydration the multilamellar liposomes were sonicated in a bath-

type sonicator (Laboratory Supplies, Ltd., Hicksville, NY) for 1 h at 23°C. The liposome solutions were then kept for 24 h at 23°C before the ion permeability experiments were begun to allow the unilamellar liposomes to anneal. However, for the lipids used in this study, no difference was seen in efflux rates for experiments begun at 0, 24 or 48 h after sonication.

Proteoliposomes were prepared in essentially the same manner except that T(is) in trifluoroethanol was added to the lipids before the final solvent evaporation. Rehydration and sonication proceeded as described above. Phosphorous and protein analyses indicated that the T(is)/phosphatidylcholine ratio in the proteoliposomes was unchanged from the ratio present in the original mixture.

Sonicated egg phosphatidylcholine liposomes and sonicated proteoliposomes (10 mM T(is) per mol phosphatidylcholine, M/M) at a concentration of 1 mg phosphatidylcholine/ml, each containing tracer amounts of [^{14}C]phosphatidylcholine, were subjected to gel filtration on a 40 × 0.6 cm column of Sepharose 4B and the fractions measured for radioactivity with a Packard 460C Tricarb liquid scintillation counter.

Permeability measurements. The sonicated liposomes containing entrapped radioactive ions were initially separated from extraliposomal radioactive ions by passage over a 10 × 0.5 cm column of Sephadex G-50 (medium) in the same buffer minus tracer radioactivity. The column was presaturated with 5 mg multilamellar egg phosphatidylcholine. The Sephadex columns had been precalibrated with liposomes containing ^{14}C-labeled phosphatidylcholine as well as buffer containing ^{22}NaCl so that liposomes free of any non-incorporated radioactive ions could be collected in the void volume quickly, accurately, and reproducibly over the course of each experiment. The liposomes were then diluted into a larger volume of the nonradioactive buffer ($t = 0$) to initiate monitoring of the efflux of the ion under study. At specified time intervals thereafter, a 0.5 ml aliquot of the diluted liposome solution was withdrawn from the incubation vessel and passed over the calibrated Sephadex column to separate again the liposomes from leaked extraliposomal tracer ions. The liposomes were collected and the radioactivity of entrapped ions was determined on a Searle 1197 gamma counter or a Packard 460C Tricarb liquid scintillation counter. Radioactivity for each aliquot during the first time interval varied between 3200 and 2500 cpm for $^{22}Na^+$ experiments. Phosphatidylcholine liposomes and proteoliposomes generally were within 200 cpm of each other at the first time interval.

The results were expressed as a percentage of the radioactivity at $t = 0$ remaining as a function of time. The radioactivity at $t = 0$ was extrapolated from four sequential experimental measurements taken within the first 30 min of $t = 0$. The experimental protocol included variations in the T(is) concentration, the chemical nature of the radioactively labeled ion, the liposomal lipids, and the temperature.

Results

Phosphatidylcholine vesicles without incorporated protein are not appreciably permeable to Na^+ when the permeability is measured immediately after

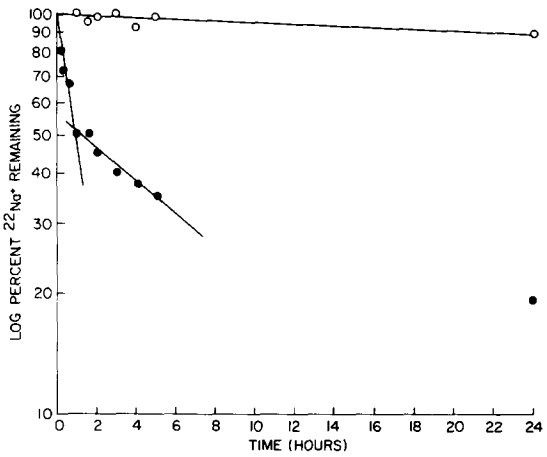


Fig. 1. Efflux of $^{22}Na^+$ from phosphatidylcholine liposomes (○———○) and T(is)/phosphatidylcholine liposomes (●———●) as a function of time. The T(is)/phosphatidylcholine liposomes contained 10 mmol T(is) per mol phosphatidylcholine and were prepared as described in the text. Partial equilibration of the intra- and extraliposomal spaces after long incubation is the likely explanation of the displacement of the 24 h point away from the straight line representing the slow efflux component.

sonication or after the liposomes have been held at 23°C for 24 or 48 h before efflux experiments are begun. After 24 h at 23°C, 90—95% of the initially trapped $^{22}Na^+$ remains inside the liposomes as shown in Fig. 1.

Effect of temperature on liposome permeability

Changes in the temperature at which the efflux experiments are carried out affect the rate of diffusion of Na^+ through the bilayer. As the temperature increases the rate of diffusion of Na^+ increases; after 6 h 99% of the liposomally entrapped $^{22}Na^+$ remains entrapped at 10°C as compared to 69% at 40°C.

Effect of T(is) on liposome permeability

The association of T(is) with small unilamellar phosphatidylcholine vesicles increases the permeability of the liposomes to Na^+; permeability increases as the T(is) concentration is increased. For example, in liposomes containing 10 mmol T(is) per mol phosphatidylcholine, only 20% of the liposomally entrapped Na^+ remains after 24 h as shown in Fig. 1. This T(is)-induced Na^+ efflux is also temperature dependent in liposomes containing 10 mmol T(is)/mol phosphatidylcholine; after 6 h 60% of the proteoliposomally entrapped $^{22}Na^+$ remains entrapped at 10°C as against 25% at 40°C.

As seen in Fig. 1, at 10 mmol T(is) per mol phosphatidylcholine, the efflux of $^{22}Na^+$ from small unilamellar proteoliposomes appears to be biphasic; there is an initial rapid efflux followed by a slower efflux of cations from the liposomes. Estimating the slow and fast components by linear regression and plotting the slopes of the two components as functions of T(is) concentration produces the results shown in Fig. 2A and B. The slow component increases with T(is) concentration until it reaches a maximum at a T(is) concentration of 2—3 mmol per mol phosphatidylcholine. The fast component is difficult to measure at low T(is) concentrations but clearly increases linearly with increased T(is) concen-

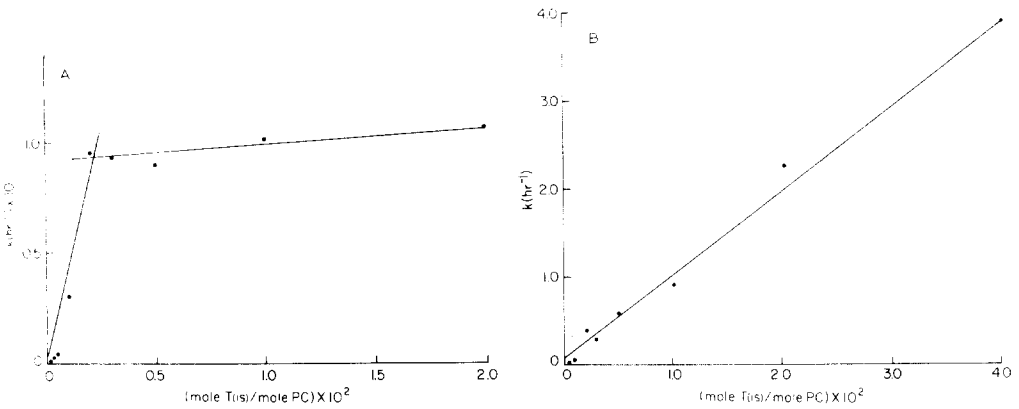


Fig. 2. (A) The apparent rate constant as a function of the molar ratio of T(is)/phosphatidylcholine (PC) for the slow component of the biphasic efflux of $^{22}Na^+$ from liposomes containing varying amounts of T(is). (B) The apparent rate constant as a function of the molar ratio of T(is)/phosphatidylcholine for the fast component of the biphasic efflux of $^{22}Na^+$ from liposomes containing varying amounts of T(is).

trations above 3 mmol T(is) per mol phosphatidylcholine.

Treating the $^{22}Na^+$ efflux curves, generated as T(is) concentration increases, as the sum of two exponential rate expressions, the data were fitted, using a non-linear least-squares computer program based on the Marquardt algorithm [12], to the following double exponential:

$$Y = B_1 e^{-B_2 t} + B_3 e^{-B_4 t}$$

where B_1 and B_3 are the proportions of slow and fast efflux components, respectively, contributing to the overall rate expression and B_2 and B_4 are the rate constants for the slow and fast components, respectively. The results of this analysis are shown in Table I as a function of T(is) concentration.

The results of this computer fit show that the efflux data can be reasonably well analyzed as the sum of two exponential curves. Furthermore, the results

TABLE I

CURVE FIT TO $Y = B_1 e^{-B_2 t} + B_3 e^{-B_4 t}$

K_{app}, apparent rate constant; PC, phosphatidylcholine.

mmol T(is)/mol PC	Slow		Fast	
	B_1(%)	K_{app} *	B_3(%)	K_{app}
1.0	97.9	0.01	—	—
1.5	94.0	0.03	—	—
2.0	96.4	0.01	2.71	1.52
5.0	94.9	0.04	8.7	3.52
10.0	73.2	0.10	33.2	4.73
15.0	78.5	0.09	25.1	2.46
25.0	74.7	0.09	29.4	3.21
50.0	55.4	0.12	49.1	3.92
100.0	33	0.10	89.8	7.76
200.0	8.8	0.28	106.8	4.62

closely correlate with the data of Fig. 2A and B. That is, the slow apparent rate constant levels off at approx. 5 mmol T(is) per mol phosphatidylcholine and remains constant until 200 mmol; the fast apparent rate constant seems to increase (albeit slowly) with increasing T(is) concentration beyond 5 mmol T(is) per mol phosphatidylcholine. More dramatically, the percent of the efflux due to the fast component (B_3) increases markedly beyond 5 mmol T(is). In the same way, the percent of the efflux due to the slow component (B_1) begins to decrease significantly beyond 5 mmol T(is) per mol phosphatidylcholine.

Effect of cholesterol on liposome permeability

Inclusion of both 10 and 25% by weight of cholesterol in the phosphatidylcholine liposomes significantly decreases the $^{22}Na^+$ efflux in the presence of 10 mmol T(is) per mol phosphatidylcholine, as shown in Fig. 3 for 25% cholesterol. Cholesterol/phosphatidylcholine liposomes leak more slowly than those without cholesterol at the T(is) concentrations studied. Visual inspection suggests that the decreased ^{22}Na efflux is due to the absence of a fast component; changes in the slow component are less obvious. Double exponential computer fitting confirms this impression (data not shown). The entire leakage profile is comparable to the slow phase of leakage in the presence of 10 mmol T(is) per mol phosphatidylcholine (cf. Figs. 1 and 3).

Freeze-fracture studies

In Fig. 4 the density of 80-Å freeze-fracture intrabilayer particles in T(is)/phosphatidylcholine multilamellar proteoliposomes is plotted against T(is) con-

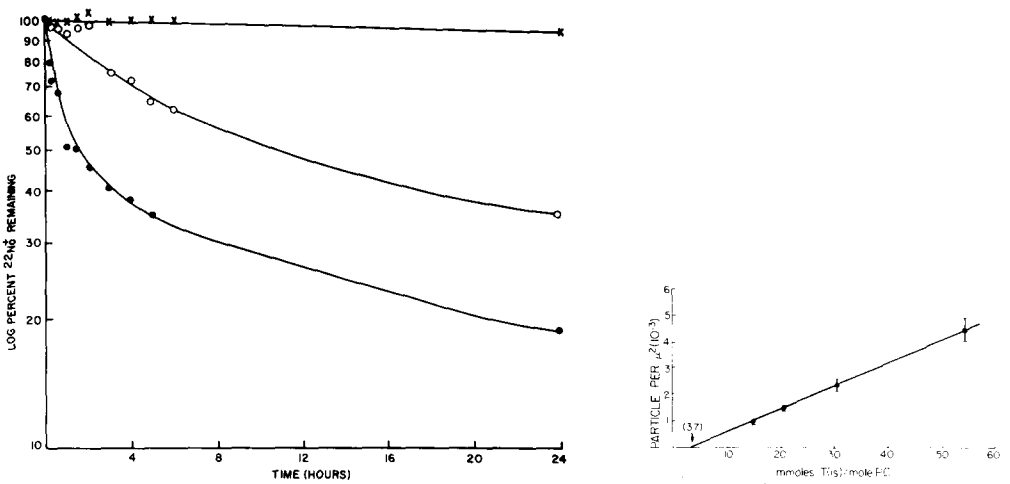
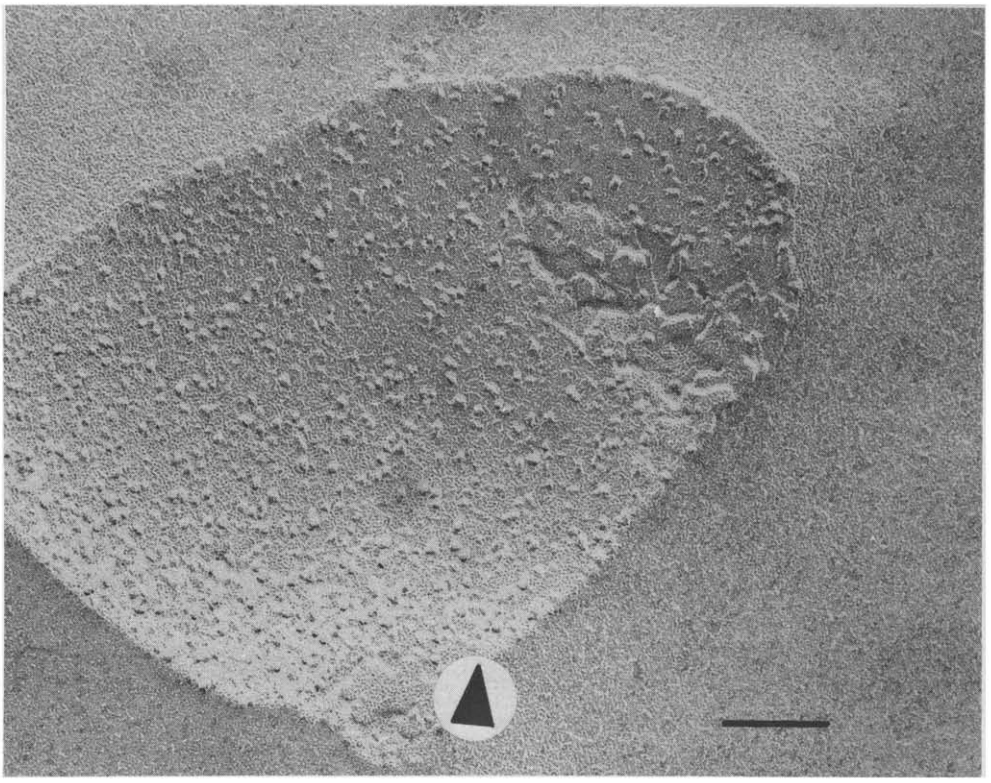
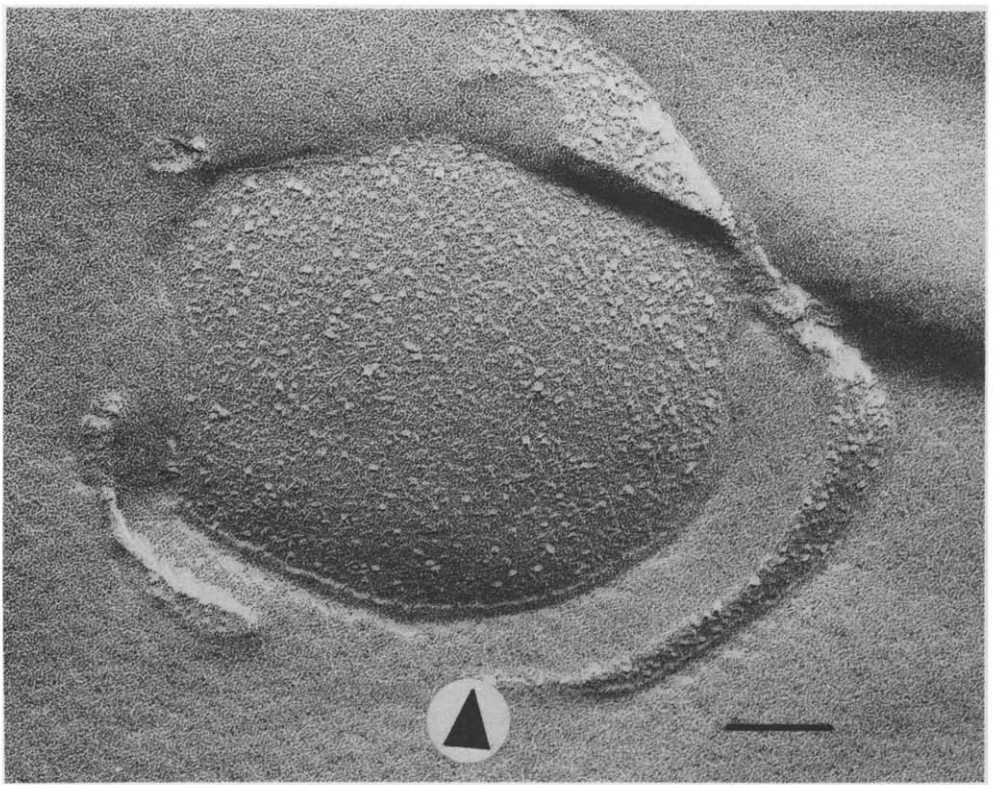


Fig. 3. Efflux of $^{22}Na^+$ from phosphatidylcholine liposomes (X———X), T(is)/phosphatidylcholine liposomes (●———●), and T(is)/cholesterol/phosphatidylcholine liposomes (○———○). Both proteoliposome preparations contained 10 mmol T(is) per mol phosphatidylcholine. Cholesterol was present in the concentration of 0.33 mol per mol phosphatidylcholine. Liposomes were prepared as described in the text.

Fig. 4. Freeze-fracture intrabilayer particle densities of T(is)/phosphatidylcholine (PC) multimeric proteoliposomes plotted against T(is) concentration (see Ref. 1). The bars represent the standard deviations for each of five sets of points. The curve was plotted by the linear least-squares method using a Hewlett-Packard HP-55 electronic calculator.





centration. This figure represents a replot by linear regression of previously published data [1] in which the T(is) concentrations have been corrected for a systematic overestimation of T(is) concentration. The x-axis intercept by the particle density curve corresponds to a critical multimer concentration of 3.7 mmol T(is) per mol phosphatidylcholine.

In order to investigate the possible effect of cholesterol upon the intrabilayer association of T(is) monomers to form multimers, multilamellar egg phosphatidylcholine liposomes containing 67 mmol T(is) per mol phosphatidylcholine were prepared; a second preparation contained, in addition, 0.33 mol cholesterol/mol phosphatidylcholine. Freeze-fracture electron microscopic studies were performed on both preparations; Fig. 5 shows typical freeze-fracture electron micrographs. 80-Å intrabilayer particles are present in both preparations. Morphometric analysis of the two preparations and an analysis of variance applied to the results indicates that neither the particle counts per unit area nor the particle size are significantly different for the two multilamellar preparations; i.e., cholesterol has no measurable effect on intrabilayer particle density or size in multilamellar vesicles.

$^{45}Ca^{2+}$ *efflux*

The efflux of $^{45}Ca^{2+}$ from phosphatidylcholine vesicles is enhanced by T(is) only if Ca^{2+} or Mg^{2+} is present in the bathing media as well as inside the phosphatidylcholine vesicles. If monovalent Na^{+} only is present in the bathing media, no $^{45}Ca^{2+}$ efflux is seen (data not shown). From Fig. 6, $^{45}Ca^{2+}$ efflux from T(is)-containing phosphatidylcholine vesicles has both a fast and a slow component. However, the rate constants for the two components are lower than the corresponding rate constants for $^{22}Na^{+}$ efflux.

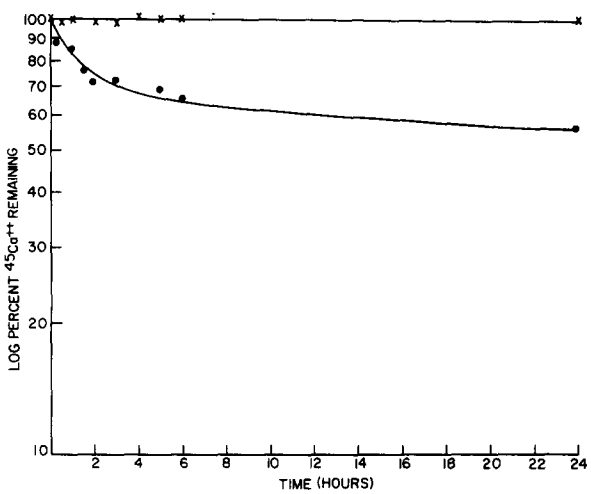


Fig. 6. Efflux of $^{45}Ca^{2+}$ from phosphatidylcholine liposomes (X———X) and T(is)/phosphatidylcholine liposomes (10 mmol/mol) (●———●) into buffer which is 0.10 M in $CaCl_2$ rather than 0.15 M in NaCl.

Fig. 5. Freeze-fracture electron micrographs of T(is)/phosphatidylcholine liposomes (10 mmol/mol) with (A) and without (B) 25% (w/w) cholesterol. Liposomes were prepared as described in the text and freeze-fractured with a Balzers BAF-300. Replicas were photographed with a Philips EM 400. Bar represents 1000 Å. Arrow heads indicate direction of shadow.

Discussion

The method of gel filtration on small Sephadex G-50 columns proved a rapid (30 s) and reproducible means of measuring ion efflux from small unilamellar phospholipid vesicles. The rate of column elution was increased by use of compressed nitrogen or argon.

The $^{22}Na^{+}$ efflux data are obviously complex and any interpretation of them runs the risk of being overly simplistic. However, the computer curve-fitting data are quite consistent with a two-component rate process. If true, this would seem to indicate that these are two populations of liposomes, one population with a fast rate of efflux (possibly masking a simultaneous slower efflux component in the same liposomes) and a second population having only a slower rate of efflux.

The appearance of a fast efflux component (superimposed upon a continuously present slow efflux) at and above a concentration of 3—5 mmol T(is) per mol phosphatidylcholine suggests that at 3—5 mmol T(is) there is a change in the physical state of one population of proteoliposomes, producing an increased cationic efflux. Furthermore, the percent represented by this population increases with increasing T(is) concentration. The change in state could involve T(is) peptide, phosphatidylcholine lipid, contaminants or a combination thereof.

We previously reported that T(is) incorporated into multilamellar vesicles forms 80-Å intrabilayer particles as seen by freeze-fracture electron microscopy and that each particle is formed by the self-association of multiple T(is) monomers [1,11]. A plot of particle density versus T(is) concentration (Fig. 4) suggests to us that this self-association is a micelle-like phenomenon, occurring at a critical multimer concentration of approx. 4 mmol T(is) per mol phosphatidylcholine.

A possible interpretation of the biphasic efflux data equates the slow cation efflux to minor bilayer perturbations induced by T(is) monomers and the fast cation efflux to major perturbations in phosphatidylcholine vesicle bilayer integrity induced by T(is) multimers. This hypothesis is consistent with our freeze-fracture studies (Refs. 1 and 11 and Fig. 4) and differential scanning calorimetric studies of T(is)/phosphatidylcholine recombinants [13].

Although we feel that the T(is) monomer-multimer hypothesis is an attractive one to explain the cation efflux kinetics, other possibilities, namely lipid- or contaminant-mediated changes in cation permeability, must be considered. Multilamellar, as well as large unilamellar, phosphatidylcholine liposomes are known to have slower cation efflux rates than unilamellar phosphatidylcholine liposomes (Refs. 14 and 15 and Allen, T.M., unpublished observations). However, the efflux kinetics seen in our studies cannot be explained on the basis of lipid structural geometry alone. Because both multilamellar and large unilamellar phosphatidylcholine vesicles have slower cation efflux rates than small unilamellar phosphatidylcholine vesicles and because there is an increase in the mean diameter of liposomes with increasing T(is) concentration (see Fig. 6), one would expect a progressive decrease in the rate of efflux of cations, if lipid geometry were the sole independent variable, not an increase as is observed.

Another possibility is that some contaminant present in the T(is) preparation

may have been responsible for the efflux changes seen. It is known that glycophorin prepared by the lithium diiodosalicylate-phenol procedure [11] contains significant amounts of lithium diiodosalicylate, the presence of this compound being independent of the lipid depletion of glycophorin by organic solvents [16]. T(is) prepared from these glycophorin preparations contains about 20% lithium diiodosalicylate by weight. However, when lithium diiodosalicylate was incorporated into small unilamellar phosphatidylcholine vesicles under conditions identical to those used to incorporate T(is), no significant increase in ^{22}Na efflux was seen. We conclude that lithium diiodosalicylate is not responsible for the efflux changes induced by T(is) incorporated into phosphatidylcholine vesicles.

The T(is) monomer-multimer interpretation of the biphasic efflux data supposes that the slow $^{22}Na^{+}$ efflux component is due to perturbations of phosphatidylcholine vesicle integrity by monomeric T(is) present in the bilayer; at a concentration of approx. 3—5 mmol T(is) per mol phosphatidylcholine, a critical concentration is reached, above which there is a subsequent self-association of T(is) to form discrete multimeric intrabilayer units. Consistent with this interpretation, above this critical concentration the slow efflux component remains constant in rate but progressively decreases in percent of the total efflux, from an initial 100% before and up to the putative critical concentration (Table I). Characteristically, in a micelle-like phenomenon the monomeric concentration of a given molecule remains essentially constant about the critical micelle concentration (corresponding here to the slow efflux rate) but the percent of the total molecules present as monomers progressively decreases as the total concentration of molecules increases.

On the basis of this interpretation, the fast efflux component would be due to the increasing presence of T(is) multimers above the critical concentration as a function of increasing T(is) concentration, the presence of an intrabilayer T(is) multimeric unit producing a greater perturbation of phosphatidylcholine vesicle bilayer integrity than the sum of its individual monomeric parts. As already noted, the failure of the fast efflux component to obliterate totally the slow component seems likely to be due to two populations of vesicles, some of which contain multimeric T(is) intrabilayer units (plus masked monomers) and some of which contain only monomers.

The results of gel filtration studies of sonicated liposomes and proteoliposomes on Sepharose 4B (Fig. 7) show, that based on Stoke's radius calculations [17], the sonicated proteoliposomes, containing 10 mmol T(is) per mol phosphatidylcholine, have a larger mean diameter than the sonicated liposomes (approx. 350 as against 250 Å, respectively). In addition, there is a small fraction (4% of total [^{14}C]phosphatidylcholine) of proteoliposomes located near the void volume that have a diameter of approx. 600—1000 Å. Furthermore, these excluded 600—1000 Å proteoliposomes have a T(is) : phosphatidylcholine ratio that is 3.6 times higher than that of the included 350 Å proteoliposomes (data not shown). Although the 600—1000 Å proteoliposomes represent only 4% or so of the total [^{14}C]phosphatidylcholine, they represent at least 30% of the total entrapped aqueous compartment of the proteoliposomes. From Fig. 1 it is seen that proteoliposomes containing 10 mmol T(is) per mol phosphatidylcholine exhibit a rapid efflux component which accounts for 50%

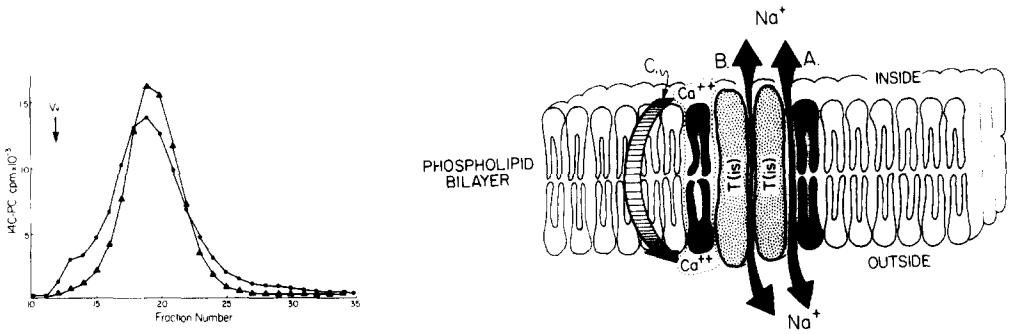


Fig. 7. Sepharose 4B chromatography of phosphatidylcholine liposomes and T(is)/phosphatidylcholine liposomes. The liposomes were equilibrated in Tes, NaCl, histidine, EDTA buffer as described in the text and sonicated for 1 h at 0°C under a nitrogen atmosphere in a bath-type sonicator. 0.5 ml of each sample was passed over the 40 X 0.6 cm column. Liposomes were comprised of phosphatidylcholine (with tracer amounts [^{14}C]phosphatidylcholine (^{14}C-PC) (▲——▲), and T(is)/[^{14}C]phosphatidylcholine (10 mmol/mol) (●——●).

Fig. 8. Possible mechanisms whereby T(is) multimeric units could induce the rapid cation efflux component observed in proteoliposomes. The figure represents a phospholipid bilayer containing an embedded T(is) multimer and is intended only as a schematic representation of possible molecular events. The dark phospholipid molecules adjacent to the T(is) multimer represent boundary (immobilized or otherwise perturbed) phosphatidylcholine molecules. (A) Rapid $^{33}Na^+$ efflux via defects created by a protein/boundary lipid interface. (B) Rapid $^{22}Na^+$ efflux via protein-protein channels created by T(is) multimers. The results of the cholesterol experiments shown in Figs. 3 and 4 make this an unlikely possibility. (C) One possible mechanism to explain the requirement that divalent cations be present on both sides of the proteoliposome bilayer for rapid efflux of $^{45}Ca^{2+}$ to occur. In this proposed mechanism, a transbilayer cluster of Ca^{2+} and boundary phosphatidylcholine molecules rotate about their sort axes normal to the T(is) multimer surface, resulting in the coordinated transbilayer exchange of one Ca^{2+} and two phospholipid groups from opposite sides of the bilayer. Steric hindrance produced by the restricted nature of the boundary lipid is one way to rationalize the proposed simultaneous exchange.

of the total $^{22}Na^+$ efflux. We suggest that, due to steric hindrance of the highly curved proteoliposome surface, the smaller 350 Å proteoliposomes contain T(is) monomers only, whereas the larger, less curved 600—1000 Å proteoliposomes contain T(is) multimers.

There seem to be two general mechanisms whereby an intrabilayer T(is) multimeric unit might produce a greater perturbation of small unilamellar phosphatidylcholine vesicle integrity to cationic flux than the sum of its individual monomeric components. The first possibility is that a protein-lined passage or channel is produced by T(is) protein-protein interaction. The second possibility is that a T(is) multimer produces an effective bilayer defect at its protein/lipid interface [2], leading to an increased flux of ions in this region (Fig. 8).

One possible way of distinguishing between the two mechanisms is to add cholesterol to the T(is)-phosphatidylcholine complex; cholesterol is known to tighten the packing of phosphatidylcholine bilayers [18] and thus might minimize the rapid efflux component we observe in our T(is)-phosphatidylcholine preparations if this component were due to a protein-lipid interfacial phenomenon. As seen in Fig. 3, the rapid efflux component is essentially wiped out by cholesterol addition to the T(is)-phosphatidylcholine preparations. Freeze-fracture electron microscopy indicates that the presence of cholesterol in the phosphatidylcholine vesicles has no measurable effect upon intrabilayer particle

formation by incorporated T(is). Although it is conceivable that cholesterol could inhibit the formation of T(is) particles in sonicated vesicles but not in multilamellar ones, we consider this an unlikely possibility. On the basis of the available data, we propose that T(is) induces $^{22}Na^+$ efflux from phosphatidylcholine vesicles by perturbation of the vesicle bilayer integrity, T(is) multimers having a geometrically greater effect than monomers. This conclusion is similar to that reached by van Zoelen et al. [19] in studies of intact glycophorin incorporated into phospholipid vesicles. Using ^{13}C-NMR, Gerritsen et al. [20] found that the permeability barrier to Dy^{3+} of single component phospholipid vesicles containing intact glycophorin could be restored by the addition of phosphatidylethanolamine or lysophosphatidylcholine, indicating the nature of the discontinuities in the bilayer area as a function of membrane lipid composition. In this regard, we find that phosphatidylserine liposomes containing T(is) lack a rapid efflux component, in a manner similar to phosphatidylcholine/cholesterol proteoliposomes (data not shown).

The model for T(is)-induced $^{22}Na^+$ efflux predicts that once all available phospholipid is incorporated into protein/lipid boundaries, essentially no free phosphatidylcholine would be available to form new protein/lipid boundaries upon the addition of incremental amounts of T(is) and thus there would be no additional increase in ion efflux. In fact, there is a saturation of the rapid efflux component above T(is) concentrations of 100 mmol T(is) per mol phosphatidylcholine (Table I).

The slower efflux of $^{45}Ca^{2+}$ compared to $^{22}Na^+$ is to be expected for passive phosphatidylcholine vesicle efflux in the absence of selectivity for bivalent cations. The biphasic nature of the $^{45}Ca^{2+}$ efflux is compatible with the monomer-multimer mechanism postulated for monovalent cation efflux. The requirement that Ca^{2+} or Mg^{2+} be present on both sides of the T(is)-containing phosphatidylcholine vesicle bilayer for $^{45}Ca^{2+}$ efflux to occur implies that some coordinate mechanism is involved in T(is)-induced bivalent cation efflux (i.e., movement of one bivalent cation out requires the movement of one bivalent cation in). It was previously reported by de Kruijff et al. [2] that intact glycophorin incorporated into phospholipid vesicles increases the rate of phospholipid flip-flop. It is reasonable to postulate then that phosphatidylcholine boundary lipid adjacent to the hydrophobic periphery of T(is) multimers may be more susceptible to transbilayer exchange (i.e., flip-flop), a suggestion also advanced by van Zoelen et al. [19]. This protein-induced transbilayer phosphatidylcholine exchange could involve a coordinated mechanism of ion-phosphatidylcholine headgroup interaction with simultaneous exchange of opposed ion-phosphatidylcholine pairs (Fig. 8). Such a model would explain the $^{45}Ca^{2+}$ efflux data.

Acknowledgements

This work was supported in part by NIH grants GM-23177, CA-22868 and DE-02670. A.Y.R. was supported by NIH Postdoctoral Training Grant GM-07561.

References

1 Segrest, J.P., Gulik-Krzywicki, T. and Sardet, C. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3294—3298
2 De Kruijff, B., van Zoelen, E.J.J. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 509, 537—542
3 Segrest, J.P. and Jackson, R.L. (1977) in Membrane Proteins and their Interaction with Lipids (Capaldi, R.A., ed.), pp. 21—45, Marcel Dekker, Inc., New York
4 Cuatrecasas, P. (1974) Annu. Rev. Biochem. 43, 169—214
5 Segrest, J.P., Jackson, R.L., Marchesi, V.T., Guyer, R.B. and Terry, W. (1972) Biochem. Biophys. Res. Commun. 49, 964—969
6 Segrest, J.P., Kahane, I., Jackson, R.L. and Marchesi, V.T. (1973) Arch. Biochem. Biophys. 155, 167—183
7 Lea, E.J.A., Rich, G.T. and Segrest, J.P. (1975) Biochim. Biophys. Acta 382, 41—50
8 Dittner, J.C. and Wells, M.A. (1969) Methods Enzymol. 14, 486
9 Ames, B.N. and Durbin, D.T. (1960) J. Biol. Chem. 235, 769—775
10 Segrest, J.P. (1977) in Mammalian Cell Membranes (Jamieson, G.A. and Robinson, D.M., eds.), Vol. 3, pp. 1—26, Butterworths, London
11 Marchesi, V.T. and Andrews, E.P. (1973) Science 174, 1247
12 Marquardt, D.W. (1963) J. Soc. Ind. Appl. Math. 11, 431—436
13 Romans, A.Y. and Segrest, J.P. (1977) in Cellular Neurobiology (Hall, Z., Kelley, R. and Fox, C.F., eds.), pp. 191—197, Alan, R. Liss, Inc., New York
14 Papahadjopoulos, D. and Watkins, J.C. (1967) Biochim. Biophys. Acta 135, 639—652
15 Lawaczeck, R., Kamosho, M. and Chan, S.I. (1976) Biochim. Biophys. Acta 443, 313—330
16 Romans, A.Y. and Segrest, J.P. (1978) Biochim. Biophys. Acta 511, 297—301
17 Ackers, G.K. (1967) J. Biol. Chem. 242, 3237—3238
18 Semer, R. and Gelerinter, E. (1979) Chem. Phys. Lipids 23, 201—211
19 Van Zoelen, E.J.J., van Dijck, P.W.M., de Kruijff, B., Verkleij, A.J. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 514, 9—24
20 Gerritsen, W.J., van Zoelen, E.J.J., Verkleij, A.J., de Kruijff, B. and van Deenen, L.L.M. (1979) Biochim. Biophys. Acta 551, 248—259