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BINDING OF MONOVALENT CATIONS TO PHOSPHATIDYLSERINE AND MODULATION OF Ca^{2+}- AND Mg^{2+}-INDUCED VESICLE FUSION

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The effect of several monovalent cations on the Ca^{2+}-induced aggregation and fusion of sonicated phosphatidylserine (PS) vesicles is studied by monitoring the mixing of internal compartments of the fusing vesicles using the Tb/dipicolinic acid assay. The dissociation of the fluorescent Tb-dipicolinate complex which accompanies Ca^{2+}-induced vesicle fusion is determined directly and is due to leakage of contents and entry of medium into vesicles. PS vesicles do not fuse when the medium contains only monovalent cations (at pH 7.4), regardless of the cation concentration or whether there is aggregation of the vesicles. A mass-action kinetic analysis of the data provides estimates for the rate of aggregation, C_{11}, and for the rate of fusion per se, f_{11}. Values of f_{11} increase dramatically with reduction in monovalent cation concentration and are primarily determined by binding ratios of Ca^{2+} or Mg^{2+} per PS. With 300 mM of monovalent cations, the fusion per se is essentially rate-limiting to the overall fusion process and values of f_{11} are significantly larger with the monovalent cations which bind the least, i.e., according to the sequence tetramethylammonium $> K^+ > Na^+ > Li^+$. With monovalent cations in concentrations of 100 mM or less, the aggregation is rate-limiting to the fusion and the overall initial fusion rates are determined by an interplay between aggregation and fusion rates. Under conditions of fast aggregation, the Ca^{2+}-induced fusion of small PS vesicles can occur within milliseconds or less.

Introduction

Binding of cations to acidic phospholipid vesicles results in charge neutralization and in vesicle aggregation. Divalent cations, such as Ca^{2+} and Mg^{2+}, can also induce the fusion of most acidic phospholipid vesicles and of mixed (acidic plus neutral) phospholipid vesicles [1–16]. It is known that Na^+ (500 mM) alone can induce the aggregation of phosphatidylserine (PS) sonicated unilamellar vesicles [17] and that this aggregation produces no fusion, but is fully reversible with respect to Na^+ concentration and temperature changes [18]. Furthermore, Na^+ concentrations above 100 mM inhibit Ca^{2+} or Mg^{2+} induced fusion of PS vesicles [3,19,20] and mixed PS/PC (phosphatidylcholine) vesicles [3].

Abramson et al. [21] noted that excess Na^+ could produce the release of H^+ from PS vesicles, and Puskin [22], using electron paramagnetic resonance spectroscopy, found that monovalent cat-

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Abbreviations: Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; PS, phosphatidylserine.

ions having smaller nonhydrated radii were more effective in displacing Mn^{2+} from PS vesicles. Specific binding of Na^+ to PS vesicles was proposed by Nir et al. [23], who determined a binding constant of $K_{Na} = 0.8\ (\pm 0.2)\ M^{-1}$ from the analysis of the amount of Ca^{2+} and Mg^{2+} bound in dialysis experiments in solutions of various concentrations of Ca^{2+}, Mg^{2+} and Na^+. Kurland et al. [24] studied the same system by measuring relaxation rates of $^{23}Na^+$ in nuclear magnetic resonance studies, and found values of K_{Na} from 0.6 to 1.2 M^{-1}. while the binding of the bulkier ion tetraethylammonium was negligible. Eisenberg et al. [25] studied the binding of monovalent cations using microelectrophoresis of large multilamellar PS vesicles. Their results gave a value of $K_{Na} = 0.6$ M^{-1} and no binding of tetramethylammonium to PS. From measurements of surface potential of PS monolayers, Ohki and Kurland [26] deduced binding constant values of 0.6 and 0.05 M^{-1} for Na^+ and tetramethylammonium, respectively. The sequence of binding constants to PS, $Li^+ > Na^+ > K^+ >$ tetramethylammonium obtained from the electrokinetic measurements [25] is consistent with the results of Puskin [22] for Mn^{2+} displacement from PS small unilamellar vesicles.

We examine here whether the effect of the monovalent cations in the above sequences is similar to that of Na^+, which inhibits the Ca^{2+}- or Mg^{2+}-induced fusion of small PS vesicles when it is present in large solution concentrations. The explanation for this inhibitory effect of Na^+ has been that increasing its solution concentration can reduce the binding ratios of divalent cations to PS below a critical threshold value which is required for membrane destabilization [3,19]. By employing the direct fusion assay of Wilschut and Papahadjopoulos [9] and Wilschut et al. [10], we can correlate the binding strength of monovalent cations with fusion inhibition and with the kinetics and extent of vesicle aggregation. As will be explained later, recent developments in the analysis of the kinetics of aggregation and fusion enable us to deduce separately rates of aggregation and rates of fusion and to demonstrate that the rate of fusion per se is crucially dependent on the binding ratio of divalent cation per PS.

Materials and Methods

Bovine brain phosphatidylserine was purified as described [6,27] or purchased from Avanti Polar Lipids (Birmingham, AL) and stored under argon at $-40°C$ in sealed ampules until use. $TbCl_3$ was obtained from Alfa (Danvers, MA), dipicolinic acid, Tes, L-histidine and LiCl from Sigma, NaCl from Mallinckrodt (Paris, KY), KCl, $CaCl_2$, $MgCl_2$ from Fisher, and tetramethylammonium chloride from K&K Laboratories (Plainview, NY). Cholic acid was purchased from Calbiochem (La Jolla, CA) and recrystallized twice.

Small unilamellar vesicles were prepared by sonication [28]. The lipids were mixed in chloroform, dried in a rotary evaporator and then in high vacuum for 30 min, suspended in the various aqueous media to be entrapped inside the vesicles, and sonicated for 1 h in a bath-type sonicator under argon. The temperature was maintained around 20°C by circulating the water in the bath. The resulting clear suspension was then centrifuged at 25°C for 1 h at $115000 \times g$ to eliminate any large or multilamellar vesicles; the upper 4/5 of the supernatant was collected for use in the experiments.

Assay for fusion

Fusion of the vesicles was monitored by means of the assay described previously [9,10] with some modifications. The vesicles were prepared in either of the following solutions: (A) 15 mM $TbCl_3$/150 mM sodium citrate/2 mM Tes/2 mM L-histidine (pH 7.4) or (B) 150 mM sodium dipicolinate/2 mM Tes/2 mM L-histidine (pH 7.4). The vesicles were separated from non-entrapped material by gel filtration on Sephadex G-75 (Pharmacia, Piscataway, NJ) by elution with 100 mM NaCl/2 mM L-histidine/2 mM Tes ('NaCl buffer') and 1 mM EDTA (pH 7.4). Lipid concentrations were determined by a phosphate assay. For the fusion assay, the Tb-vesicles and the dipicolinic acid-vesicles were mixed in a 1 : 1 molar ratio at a final lipid concentration of 50 μM in 1 ml NaCl buffer containing 0.1 mM EDTA. Fluorescence was measured in an SLM 4000 fluorometer by excitation at 276 nm and the emission was measured through a monochromator set at 545 nm (with the addition of a Corning 3-68 cutoff filter to eliminate the

scattering contribution to the fluorescence which in all cases is less than 1.5% of the full-scale fluorescence, see below). Full-scale (100%) fluorescence was set by the following procedure: a portion of the Tb-vesicles was passed through a Sephadex G-75 column equilibrated with NaCl buffer to eliminate the external EDTA, which interferes with the reaction of Tb^{3+} and dipicolinic acid. These vesicles were placed in 1 ml of NaCl buffer at a final concentration of 25 μM lipid, excess dipicolinic acid (20 μM) was introduced, and the vesicle contents were released by addition of 0.5% (w/v) sodium cholate and sonication for 5 min.

The fusion reaction was initiated by injecting an aliquot of a concentrated solution of the divalent ion (chloride salt) into the incubation mixture which was stirred constantly. The temperature was kept at 25°C.

The dissociation experiment

To determine the extent of the release of aqueous contents and the entry of Ca^{2+} and EDTA into the vesicles during fusion, the dissociation of the Tb-dipicolinate complex was measured in parallel experiments. 7.5 mM $TbCl_3$/75 mM sodium citrate/75 mM sodium dipicolinate/2 mM Tes, 2 mM L-histidine (pH 7.4) was encapsulated in the vesicles by the same method described above. The initial fluorescence of the vesicles was set to 100% and the quenching of the fluorescence subsequent to the injection of divalent cations was followed.

Vesicle aggregation

Light scattering at 276 nm was measured to follow the aggregation of the vesicles simultaneously during the fusion assay by means of a second photomultiplier channel at 90° to the excitation beam. A Corning 7-54 bandpass filter was used to eliminate the contribution from fluorescence. Aggregation of vesicles was also followed by turbidity measurements at 350 nm in a Beckman Model 34 spectrophotometer. Here, vesicles were injected into solutions of varying monovalent salt concentrations.

Results

Fig. 1 shows the fusion of PS vesicles induced by 1.5 mM Ca^{2+} as a function of Li^+ concentration. Fusion is expressed in terms of the percentage of maximum fluorescence (F), i.e., equal to the percentage of Tb which has complexed with dipicolinate. At 20 and 50 mM Li^+, we obtain essentially the same fusion curves, indicating that the fusion is not affected by the presence of Li^+ at these low concentrations. In 100 mM Li^+, the initial rate of fusion (defined as the initial slope of the fusion curve) is less than that of the lower Li^+ concentrations, but the extent of the fusion is greater. In 300 mM Li^+ there is very little or no fusion for several minutes. In 300 mM Li^+, limited aggregation of the vesicles can be induced with 2 mM Ca^{2+} (as measured by light scattering, data not shown), but still without significant fusion. Under these ionic conditions, the amount of Ca^{2+} bound to the membrane is not sufficient to produce bilayer destabilization.

In 20 mM of the other monovalent cations, and up to 1.5 mM Ca^{2+}, we have found that the early time-course of the fusion curves is essentially identical to that shown for 20 mM Li^+. We were especially interested in the apparent leveling off of

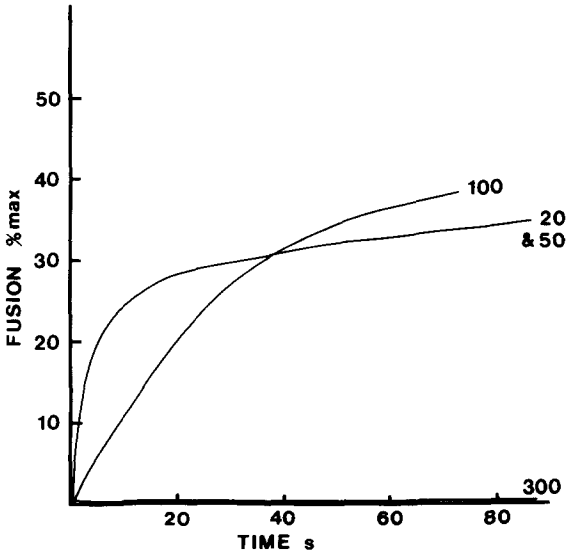

Fig. 1. Ca^{2+}-induced fusion of small vesicles. 1.5 mM Ca^{2+} was added at $t = 0$ to a vesicle suspension of 50 μM lipid. Maximal fluorescence (at 545 nm) was determined as described in Materials and Methods. Numbers on lines are mM Li^+. The uncertainty in fluorescence intensity is $\pm 1\%$. The contribution of light scattering to fluorescence intensity is less than 1.5% as determined by parallel experiments with vesicles made in NaCl buffer.

the fusion curve in all these cases, which could imply that the fusion ceases. In Fig. 2 we show the long-term behavior (up to 3 h) of fusion in 20 mM Na^+ and 1 mM Ca^{2+}. Curve a is the fusion curve over several hours and we see that the fluorescence does drop slightly from its peak value of 33% max F, i.e., where 33% of the encapsulated Tb is complexed with dipicolinate.

We attribute this decline in the observed fluorescence to the dissociation of Tb-dipicolinate complex because of its leakage from the vesicles into the medium and the entry of medium into the vesicles. In both cases, the Tb-dipicolinate complex interacts with Ca^{2+} and EDTA, which dissociate the complex, and the fluorescence is diminished. This point was tested by pre-encapsulating the Tb-dipicolinate complex in the vesicles (fluorescence of the vesicles in buffer being set to 100% maximal Tb fluorescence) and then inducing their fusion with Ca^{2+}. The results of this dissociation experiment are shown in curve b of Fig. 2, where % max D gives the percent of dissociation of the pre-encapsulated Tb-dipicolinate complex. Clearly, over the long period (3 h) there is a significant transfer of material – either into or out of the vesicles – although initially there is no detectable dissociation.

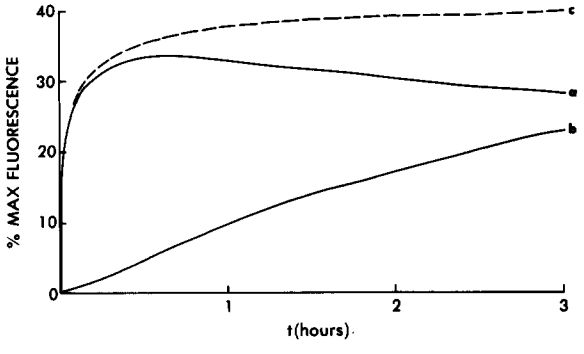

Fig. 2. The time-course of Ca^{2+}-induced fusion of PS small unilamellar vesicles in the presence of 20 mM Na^+ and 1 mM Ca^{2+}. For Li^+, K^+ and tetramethylammonium$^+$, the curves are similar to those with Na^+, at least initially. Curve (a) shows the fluorescence of the Tb-dipicolinate complex which arises due to intermixing of internal aqueous contents following the addition of Ca^{2+} at $t = 0$. Curve (b) shows the corresponding dissociation of the pre-encapsulated Tb-dipicolinate complex. Curve (c) represents the correction to the fusion values by the addition of 1/2 of the dissociation values as explained in the text.

We can combine the fusion and dissociation curves to obtain an estimate of the real extent of fusion, i.e., the value which the % max F (or just F for brevity) would have if there were no leakage of complex from the fused vesicles or influx of medium into the fused vesicles. This procedure has been described in detail before [31,45]. Initially, when there are only fused doublets, $0.5D$ (where D denotes % max D) equals the total amount of Tb-dipicolinate complex formed in the fusion experiment which has been dissociated, since only one-half of the fused doublets would contain the complex – the other half containing either Tb or dipicolinic acid. Later on, when there are higher order fusion products, D would have to be multiplied by a factor of between 0.5 and 1 in order to estimate the loss of Tb-dipicolinate complex in the fusion experiment (see Refs. 31,45 for the evaluation of this parameter). For our purposes, it is sufficient to take the sum $F + 0.5\ D$ as an estimate of the extent of fusion, i.e., this sum gives the fusion curve corrected for the loss of complex by dissociation (Fig. 2, curve c).

It is clear that the fusion of the vesicles continues in time, with new Tb-dipicolinate complex being formed by fusion and being dissociated due to leakage of complex and influx of Ca^{2+} and EDTA into the vesicles. The suspension here was clear at all times, indicating that even after 3 h the aggregates were not large.

In Fig. 3 we show the time-course of fusion induced by 2.5 mM Ca^{2+} in high monovalent cation concentrations, i.e., 300 mM tetramethylammonium, K^+, Na^+ and Li^+. Here we expect the aggregation to be fairly rapid [19,20], so that the overall fusion rate primarily reflects the bilayer destabilization between apposed vesicles.

Curve 3A shows the fusion kinetics uncorrected for dissociation. Fig. 3B shows the parallel dissociation experiments. The corrected fusion curves ($F + 0.5\ D$) are shown in Fig. 3C, where the inhibition of the fusion by the monovalent cations is shown even more clearly. It is clear that the monovalent cations inhibit the Ca^{2+}-induced fusion according to their sequence of binding constants [25], i.e., $Li^+ > Na^+ > K^+ >$ tetramethylammonium$^+$. It is interesting to note that for Li^+, Na^+ and K^+ the dissociation of complex per fusion event, roughly estimated by one-half of the

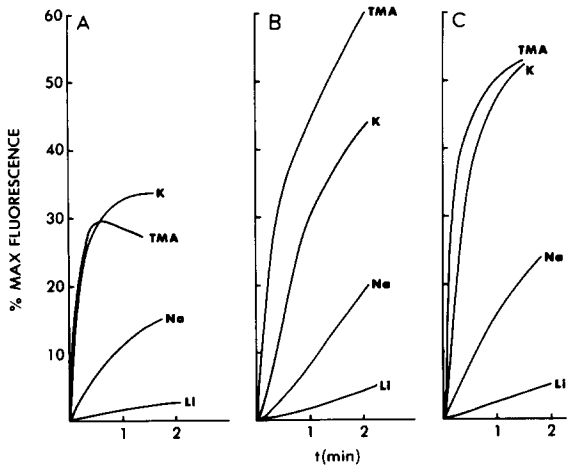

Fig. 3. The effect of monovalent cations (300 mM) on Ca^{2+}-induced fusion of small vesicles. 2.5 mM Ca^{2+} (A) Fusion (F). (B) Dissociation (D) of pre-encapsulated Tb-dipicolinate following initiation of fusion. (C) Percent of maximal fluorescence intensity corrected for the dissociation of the fluorescing complex during fusion. As explained in the text it is obtained by the addition of 1/2 of the percentage dissociation in B to the corresponding curve in A (thus, $F+0.5\ D$). Tetramethylammonium is denoted TMA.

percentage dissociation divided by the corrected fusion, is about the same, whereas for tetramethylammonium$^+$ it is larger.

The results of fusion induced by 10 mM Mg^{2+} in the presence of 300 mM monovalent cations are qualitatively the same as those shown in Fig. 3 and are omitted for brevity. Here again, it is clear that the inhibition of divalent cation induced fusion by the monovalent cations is in the same sequence as that of their binding constants.

Fig. 4A shows the initial rate of fusion (i.e., the initial slope of the fusion curve in units of % max F per min) as a function of Ca^{2+} concentration in 300 mM of the monovalent cations. We note that the rate of fusion increases with the Ca^{2+} concentration and that in all cases the inhibition sequence is $Li^+ > Na^+ > K^+ >$ tetramethylammonium. The same inhibition is found in 500 and 900 mM monovalent monovalent cations (data not shown). We do not account for the dissociation reaction here, but as is clear from Fig. 2, this correction would increase the separation between these curves.

When the monovalent cation concentration is

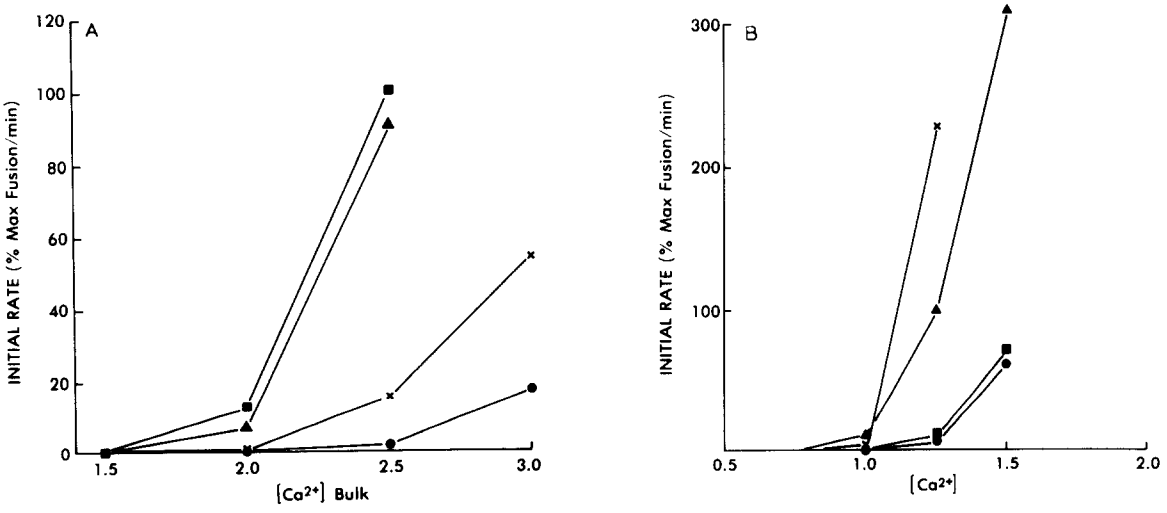

Fig. 4. Effect of monovalent cations and varying concentrations of Ca^{2+} on initial fusion rates. (See text for definition.) Concentrations of monovalent cations are 300 and 100 mM in A and B, respectively. ●, Li^+; ×, Na^+; ▲, K^+; ■, tetramethylammonium$^+$. The contribution of scattered light to initial fusion rates is less than 1.5% of maximal (100%) fluorescence per min. When initial rates are corrected for dissociation as explained in the text the initial rates with tetramethylammonium and K^+ in the presence of 2.5 mM Ca^{2+} increase by 47% max F/min and 8% max F/min, respectively, thus emphasizing the fact that tetramethylammonium$^+$ is less inhibitory to fusion than K^+.

reduced to 100 mM with more than 1.25 mM Ca^{2+}, the sequence of inhibition of fusion by the monovalent cations is Li^+ > tetramethylammonium > K^+ > Na^+ (Fig. 4B), although at 1.0 mM Ca^{2+} the order of Na^+ and K^+ is reversed. We interpret these inversions of the inhibition sequence to be due to the interplay between the kinetics of aggregation and fusion per se, as we shall elaborate later. In the presence of 20 mM of monovalent cations (6 mM Na^+ + 14 mM M^+) the initial rate of fusion is independent of which monovalent cation is used. These values are 40 and 150 (% max fusion/min) in the presence of 1 and 1.5 mM Ca^{2+}, respectively.

Analysis of results: aggregation and fusion rates

The overall fusion reaction is composed of two separate, but kinetically linked, steps. Two vesicles will dimerize according to aggregation kinetics, then the fusion of the apposed vesicles proceeds according to destabilization kinetics [32,45]. The mass action reactions which govern this process can be written as

$$V_1 + V_1 \underset{D_{11}}{\overset{C_{11}}{\rightleftharpoons}} V_2 \overset{f_{11}}{\rightarrow} F_2 \qquad (1)$$

and so on for aggregate-fusion products composed of three, four and larger numbers of vesicles. Here V_1 denotes the original vesicle monomer, V_2 denotes an aggregated dimer, and F_2 denotes a fused doublet. C_{11} is the dimerization rate constant and f_{11} is the rate constant for the fusion of the preformed dimer. The rate constant, f_{11}, accounts for both membrane destabilization and merging, which we denote as the fusion rate per se. The rate constant, D_{11}, accounts for the dissociation of dimers into monomers. The effects of higher order reactions are minimized by focusing on the initial stage of the fusion process. A detailed analysis [45] has shown that in most cases when $I\,(=0.5\,D+F)$ is less than 25%, the higher-order fusion products (F_3, etc.) contribute less than 2.5% of this value. It should be clear that the formation of fused doublets is the most biologically relevant process.

The procedure [32,45] of simulating the data is based on obtaining experimental results over a wide range of lipid concentrations. Since for times of interest (up to $C_{11}X_0t = 1$, where X_0 is the molar concentration of vesicles i.e., $X_0 = 1.2 \cdot 10^{-8}$ M with 50 μM PS) the aggregation process is second order in vesicle concentration, whereas the fusion is first order, it can be anticipated that in very dilute vesicle concentrations the overall fusion reaction is rate limited by the aggregation. The parameter which determines the extent to which either the aggregation or the fusion will be rate-limiting to the overall process is $K = f_{11}/C_{11}X_0$. When $K > 100$, the aggregation is rate-limiting and the predicted fusion curves are insensitive to the particular value of f_{11}. In this case, the value of C_{11} can be well estimated from a single fusion curve (i.e., one lipid concentration), but only a lower bound on f_{11} can be deduced. When $K \leqslant 1$, then the fusion step per se is rate-limiting, while for intermediate values of K the rates of both aggregation and fusion per se affect the kinetics of the overall process.

In the current study, we deduce the information from one concentration only and we neglect the reversibility of aggregation. The reversibility of

TABLE I

SIMULATION OF FUSION KINETICS

The simulations are from Eqn. 2 in the Appendix using $C_{11} = 1.4 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ and $f_{11} = 1$ s^{-1} for case A, and $C_{11} = 6 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ and $f_{11} = 5$ s^{-1} for case B. The vesicle concentration $X_0 = 1.2 \cdot 10^{-8}$ M for a lipid concentration of 50 μM.

t(s)	Experimental (% max)			Calculated
	F	D	$F+1/2D$	$I(t)$
(A) PS small unilamellar versicles with 1.25 mM Ca^{2+} and 100 mM tetramethylammonium$^+$				
5	0.8	0	0.8	0.6
10	1.5	0	1.5	1.5
15	2.0	1.0	2.5	2.3
30	4.0	1.5	4.8	4.5
45	5.5	2.0	6.5	6.7
60	6.7	2.5	8.0	8.7
90	8.7	3.0	10.2	12.4
(B) PS small unilamellar vesicles with 1.0 mM Ca^{2+} and 20 mM Na^+				
2	1.8	0	1.8	1.3
4	3.4	0	3.4	2.6
6	4.4	0	4.4	3.9
8	5.5	0	5.5	5.2
10	6.0	0	6.0	6.4
15	7.5	0	7.5	9.3

TABLE II

ESTIMATES OF KINETIC RATE CONSTANTS AND CALCULATED AMOUNTS OF BOUND Ca^{2+}

	Li^+	Na^+	K^+	Tetramethyl-ammonium $^+$
(A) 300 mM M^+ + 2.5 mM Ca^{2+}				
$C_{11}(M^{-1}\cdot s^{-1})$	$>10^6$	10^7	$>10^6$	$>2\cdot10^6$
$f_{11}(s^{-1})$	0.001–0.002	0.01–0.1	>1	>1
K	<0.1	<1	– [b]	– [b]
$(Ca^{2+})_B/PS$ [a]	0.19	0.22	0.31	0.37
(B) 100 mM M^+ + 1.25 mM Ca^{2+}				
$C_{11}(M^{-1}\cdot s^{-1})$	10^5–10^7	$(1–1.5)\cdot10^6$	$(1–2)\cdot10^6$	$(1.2–1.4)\cdot10^5$
$f_{11}(s^{-1})$	0.001–0.5	>1	>1	>1
K	– [b]	>400	>400	>400
$(Ca^{2+})_B/PS$	0.28	0.31	0.36	0.40
(C) 20 mM M^+ + 1.0 mM Ca^{2+}				
$C_{11}(M^{-1}\cdot s^{-1})$	$6\cdot10^5$	$6\cdot10^5$	$6\cdot10^5$	$6\cdot10^5$
$f_{11}(s^{-1})$	>5	>5	>5	>5
K	>700	>700	>700	>700
$(Ca^{2+})_B/PS$	0.41	0.41	0.42	0.43

[a] This is the calculated amount of Ca^{2+} bound per PS headgroup using the binding constants of Eisenberg et al. [25] for the monovalent cations and of McLaughlin et al. [40] for Ca^{2+} binding 1-1 to the PS headgroup, i.e., 0.8 for Li^+, 0.6 for Na^+, 0.2 for K^+, 0.01 for tetramethylammonium$^+$ and 12 for Ca^{2+} in the units of M^{-1}. The calculations were performed as described before [34]. These values pertain only to the isolated vesicles before fusion, since it is known that after fusion new Ca^{2+}-PS complexes are formed which imply a greater extent of binding [47].

[b] The value of K is not estimated here due to the uncertainty in the value of f_{11}.

aggregation might show up with 100 mM Li^+ or tetramethylammonium or with 300 mM of any of the monovalent cations, in which case we can only make crude estimates from these data as described below.

The results of this analysis are given in Tables I and II. Table I illustrates the fit to the data obtained for two representative cases. The values of the predicted curve, I, agree with the measured total fusion curve, $F + 0.5\ D$, to within the $\pm 1\%$ experimental error. Table II shows the values of aggregation rate constants, C_{11}, fusion rate constants, f_{11} and K obtained for a variety of systems, as wells as the calculated values of Ca^{2+} bound per PS, Ca/PS.

The results demonstrate that when the monovalent cations exceed 100 mM in concentration, the fusion rate constant, f_{11}, is very sensitive to the identity of the monovalent cation as well as its concentration. f_{11} is significantly larger with K^+ and tetramethylammonium$^+$ than it is with either Na^+ or Li^+. With 20 mM monovalent cation, the overall fusion kinetics, and hence f_{11}, are independent of the particular monovalent cation used. The large amounts of bound Ca^{2+}, as shown in the table, evidently govern the aggregation and fusion completely. This dependence of f_{11} values on the monovalent cations correlates well with the binding ratios of Ca/PS, which in turn depend on both the binding constants of monovalent cations to PS and their solution concentrations. For instance, the value of f_{11} increases by at least 2 orders of magnitude when the concentration of Na^+ is reduced from 300 to 20 mM. Inspection of the values of $K = f_{11}/C_{11}X_0$ illustrates that with 100 mM or less of the monovalent cations, the aggregation is completely rate-limiting. With solution concentrations of 300 mM the fusion per se is nominally rate-limiting due to a reduction in the binding ratio Ca^{2+}/PS, in spite of the fact that the solution concentration of Ca^{2+} has been doubled compared to the case with 100 mM monovalent cations.

With 20 mM monovalent cations, the values of

f_{11} are at least 5 s^{-1}, which is the value previously found [32,45] for these vesicles with 100 mM Na^+ and 2 mM Ca^{2+}. However, we suspect that with 1 mM Ca^{2+} and 20 mM Na^+ the value of f_{11} might be larger than 5 s^{-1}, because here there is more bound Ca^{2+} than in the former case. With this value of f_{11} it is possible to deduce an upper bound on the time-course of fusion of these vesicles under most favorable conditions, i.e., when the rate of aggregation C_{11} is large, unlike the case with 20 mM Na^+. Assume that the vesicles are preaggregated by some means to dimers and then a sufficient amount of a fusogen is added, such that the value of f_{11} is 10 s^{-1}. At short times (see Appendix and Refs 32, 45), $F_2(t)/V_2(0) \approx f_{11}t$. Hence, the respective times for 10% and 1% fusion are 10 and 1 ms. Recent rapid freezing studies of Miller and Dahl [15] showed that 10 mM Ca^{2+} induced fusion of PS vesicles in less than 10 ms, which is the limit of resolution of their experimental system. Our prediction is that under most favorable conditions the actual fusion times are in fact shorter.

Although our previous work has shown that in theory the values of C_{11}, f_{11} and D_{11} can be independently fixed by using several lipid concentrations [45], for these electrolytes such experiments were not feasible. With 20 mM monovalent cation and 1.0 mM Ca^{2+}, we know that $f_{11} > 5$ s^{-1} and $C_{11} = 6.0 \cdot 10^5$ $M^{-1} \cdot s^{-1}$; hence, to measure f_{11} exactly we would need at least 1 mM lipid in order to induce the fusion step to be rate-limiting, i.e., $K < 50$. With this amount of lipid, the initial events of fusion would be over within a few seconds and could only be monitored by rapid mixing techniques. With 300 mM monovalent cations and 2.5 mM Ca^{2+}, the value of C_{11} could be specified only by lowering the lipid concentration to 1 μM or below, in order to induce aggregation rate-limiting kinetics. The fluorescence assay is not sufficiently sensitive at these lipid concentrations.

In Table III, we provide some calculated values of $I(t)$ for short times, up to 100 ms. This table shows the development of the fusion process when the large (but perhaps not be largest) value of $f_{11} = 10$ s^{-1} is employed together with a value of $C_{11} = 5 \cdot 10^8$ $M^{-1} \cdot s^{-1}$, which is already close to the value for diffusion controlled aggregation, $C_{11} = 3 \cdot 10^9$ $M^{-1} \cdot s^{-1}$. When the delay due to very fast aggregation is taken into account, the respective values of I are 0.3% and 1.7% with 50 and 500 μM PS, after 10 ms. It can also be noted that at very short times the value of I is proportional to the lipid concentration and to the square of the time.

TABLE III

CALCULATED VALUES OF $I(t)$, PERCENTAGE OF MAXIMAL FUSION, UNDER CONDITIONS OF VERY FAST AGGREGATION, $C_{11} = 5 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ AND FAST FUSION, $f_{11} = 10$ s^{-1}.

Calculations employ Eqns. 2 to 4. The vesicle concentrations are $X_0 = 1.2 \cdot 10^{-8}$ and $1.2 \cdot 10^{-7}$ M for lipid concentrations of 50 and 500 μM, respectively.

Time (s)	$I(t)$ calculated			
Lipid concentration (μM)	50	100	200	500
0.001	0.003	0.006	0.01	0.03
0.01	0.3	0.5	0.9	1.7
0.1	13.0	20.0 [a]	30.0 [a]	45.0 [a]

[a] These cases exceed the validity of the approximate solutions and are given only for illustration.

Discussion

The overall rate of fusion is determined by both the rate of aggregation and by the rate of the fusion reaction per se [31,32,45]. Previous kinetic studies on vesicle fusion [4,10,14] have used the quantity denoted initial rate of fusion, i.e., the initial slope of the fusion curve. While we have also expressed the results in terms of this quantity, the current study enables an evaluation of the rate of aggregation and the rate of fusion. Increasing the concentrations of divalent cations such as Ca^{2+} or Mg^{2+} results in enhancement of both the rate of aggregation and the rate of fusion, whereas increased concentrations of monovalent cations inhibit the fusion while enhancing the rate of aggregation. In the presence of large concentrations of Na^+ (over 500 mM) and other monovalent cations such as Li^+ or K^+ (at higher concentrations), PS vesicles aggregate but do not fuse [18,33]. In 300 mM Na^+ or Li^+, the addition of Ca^{2+} (up to 1.5 mM) or Mg^{2+} (up to 5 mM) produces aggregation with no significant fusion (results not shown). Hence, with these concentrations of

monovalent cations the aggregation is not rate-limiting but the fusion per se is inhibited completely, or becomes extremely low.

Table II presents calculated values of the binding ratios of Ca/PS and Mg/PS for several concentrations of combinations of monovalent and divalent cations [34]. These calculations demonstrate the decrease in the binding of divalent cations with increasing binding capacity and concentration of monovalent cations. This decrease is due to two effects. First there is competition between the divalent cations and the monovalent cations for binding sites. Second, increasing the concentrations of cations in solution, together with charge neutralization, decreases the magnitude of the surface potential, Ψ_0, which reduces the enhancement of the concentration of divalent cations close to the negatively charged vesicles' surfaces. This enhancement is given by $C_{++}(0) = C_{++}\exp(-2e\Psi_0/kT)$, in which C_{++} is the solution concentration of divalent cations, e is the magnitude of the charge of the electron, k is Boltzmann's constant and T is the absolute temperature.

For 300 mM monovalent cation, the results of Table I together with Fig. 4A show that the initial rate of fusion, which mostly depends on bilayer destabilization since the aggregation is rapid, increases sharply with the amount of bound Ca^{2+}. Also, when the amount of bound Ca^{2+} is below 0.2 Ca/PS there is no appreciable fusion, i.e., the initial rate is much smaller than 10% max F/min. It is evident that the kinetics of the fusion step per se are controlled by the amount of bound Ca. Moreover, the monovalent cations in high concentrations inhibit the initial fusion process by competing with Ca^{2+} and Mg^{2+} for binding to the bilayer: both the inhibition sequence and the binding affinities decrease as $Li^+ > Na^+ > K^+ >$ tetramethylammonium$^+$.

When the concentration of monovalent cations is 100 mM or less (and the lipid concentration is 50 μM), the overall fusion reaction is rate-limited by the aggregation. This point has been elucidated by a detailed analysis of Ca^{2+} induced fusion of these vesicles in 100 mM NaCl [31]. Hence, in this case, the binding ratio of Ca/PS is not the main determinant of the initial rate of fusion. Consequently, the initial fusion rates follow other sequences (see Fig. 3B) and their values are determined by the various combinations of aggregation rates, C_{11}, and fusion rates, f_{11} (Table II).

Recent aggregation studies of vesicles, under conditions where there was no detectable fusion, have provided strong evidence that the reversibility of aggregation must be taken into account in the analysis of data [35]. The bulky cations tetramethylammonium or tetraethylammonium do not induce the aggregation of PS vesicles even when present in very large concentrations, above 1 M [36].

Calculations [19,37] indicate that at a distance of separation of $2/\kappa$, where $1/\kappa$ is the Debye length, the electrostatic potential barrier is not larger than a few kT when the concentration of a non-binding cation exceeds 1 M. Hence, the magnitude of the forward rate for aggregation (to a dimer state) is at most a few orders of magnitude smaller than that of a diffusion-controlled aggregation, and is not the factor prohibiting the aggregation of PS vesicles in the presence of tetramethylammonium. We have emphasized the importance of the depth of the potential well in the region of surface contact, i.e., in the region of primary minimum as the determinant of the equilibrium distribution of aggregation [17,19,20,35,37]. A shallow potential well would not yield a large degree of aggregation, even when the forward rate for aggregation is large, since the aggregates formed would be rapidly dispersing [35,37] due to the relatively large value of the backward rate. By this line of thought, we can understand the observation that Na^+ is more effective than Li^+ in inducing aggregation of PS vesicles. (Ho, J., unpublished data; Ohki et al. [33]). According to theory, the forward rates of aggregation should follow the same sequence as the binding constants. However, the shape of the potential minimum, which, depending on fine molecular details, cannot be predicted by macroscopic theories, may be deeper in Na-PS than in Li-PS vesicles. This possibility would imply that the extent of aggregation is larger in the presence of Na^+ than in Li^+. From detailed studies of Na^+-induced aggregation of PS vesicles it is known that the backward rate of deaggregation is significant [35,37]. Hence, a more shallow potential well may result in a larger backward rate, and consequently the threshold concentration for monovalent cation-induced aggregation may be

larger with Li^+ than with Na^+, in spite of the slightly larger binding constant of Li^+ relative to Na^+. The current results in Table II cannot yet provide the actual rate of aggregation, C_{11}, with Li^+. In contrast, with 100 mM Na^+, where the aggregation is rate limiting, the value of C_{11} is sharply defined between $1.1 \cdot 10^6$ and $1.5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. Hence, there is a possibility that the values of C_{11} with Li^+ are larger than those with Na^+. This question can be resolved only when fusion kinetics are examined in very dilute lipid suspensions, below 1 μM.

Except for the aggregation studies [33], which provided binding constants to PS according to the sequence $Na^+ > Li^+ > K^+ >$ tetramethylammonium$^+$, all other procedures, including the present study favor the sequence $Li^+ > Na^+ > K^+ >$ tetramethylammonium$^+$. This agreement between a variety of procedures also implies that binding of monovalent cations to membranes is quantitatively accounted for by macroscopic electrostatic equations [23,34,40]. The fact that the binding strength to PS increases with decreasing the ionic non-hydrated radii should not lead to conclusions regarding the order of binding strength in general. For instance, the binding constant of Ca^{2+} to PS, K_{Ca}, is significantly larger than K_{Mg} [1,23,26,39,40], although Mg^{2+} has a smaller non-hydrated ionic radius. According to the view of Szabo et al. [41] and Simon and Morf [42], the order of specificity is determined by subtracting the free energies of hydration from the free energies of interaction of the cations with the ligand. Krasne and Eisenman [43] indicated that the emerging sequences need not be monotonic with the non-hydrated ionic radii. Hence, the sequence found for the binding constants of cations to PS need not be the same in other phospholipids, as shown in the case of phosphatidylglycerol bilayers [44]. Studies on clays such as bentonite indicate a sequence of binding as $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ [48].

In this context it is of interest to mention recent differential scanning calorimetry and X-ray diffraction studies on 1,2-dimyristoyl-L-glycero-3-phospho-L-serine which showed that Li^+ (0.5 M) induces an isothermal crystallization of such bilayers, in contrast to NH_4^+, Na^+ or K^+ [46]. With bovine brain PS, all the monovalent cations we have studied seem to produce a stabilizing effect on the bilayer structures. However, with large concentrations of H^+ (below pH 4) significant structural changes of PS have been reported [6].

Concluding remarks

(1) With small concentrations of monovalent cations at room temperature, and with 50 μM of PS, the overall fusion is rate-limited by vesicle aggregation, whereas with monovalent cation concentrations of 300 mM or above, the actual fusion reaction is nominally rate-limiting.

(2) The binding strengths of monovalent cations to PS follow the sequence $Li^+ > Na^+ > K^+ >$ tetramethylammonium$^+$.

(3) The above monovalent cations inhibit the process of fusion per se by reducing the degree of membrane destabilization induced by divalent cations. The degree of fusion inhibition increases with the concentration of monovalent cations and with their binding capacity, and correlates well with the degree of reduction of the binding ratio of divalent cations to the membranes.

(4) With 20 mM Na^+ and 1 mM Ca^{2+}, the lowest bound on the value of the actual fusion rate is $f_{11} \approx 5\ s^{-1}$. Hence, under favorable conditions, the times required for the induction of 10% or 1% fusion of preaggregated small PS vesicles are shorter than 20, and 2 ms, respectively. When the delay due to (very fast) aggregation is taken into account, the corresponding times are 1–2 orders of magnitude larger, depending on lipid concentration.

(5) Since the rates of fusion, f_{11}, are most sensitive to the amount of divalent cations bound, and molecular diffusion is not expected to increase under those conditions, it is proposed that, at least at room temperature, membrane destabilization rather than membrane merging is the rate-limiting step in the actual fusion process.

Appendix

(i) Approximate solutions

The kinetic equations and solutions for the reactions described by Eqn. 1 are given in detail in Ref. 45. An approximate analytical solution [45], Eqns. 2–4 below, gives the value of $I(t)$, the

percentage of maximal fusion corrected for dissociation, within an error of 1% over a wide range of parameter values. The initial fusion stages are well simulated (see Table I) by:

$$I(t) = A(t)F(t) \times 100 \tag{2}$$

where t is the time

$$A(t) = (1+4C_{11}X_0t)^{1/4} - 1 \tag{3}$$

and

$$F(t) = 1 - [(1-\exp(-f_{11}t))/(f_{11}t)] \tag{4}$$

Eqn. 2 is very accurate as long as $C_{11}X_0t < 0.5$, where X_0 is the molar concentration of vesicles. In using Eqns. 2 and 4 we ignore the reversibility of aggregation, which is permitted when either $D_{11} \ll f_{11}$, or $D_{11} \ll C_{11}X_0$.

In the analysis of fusion of small PS vesicles with 2 mM Ca^{2+} and 100 mM NaCl, a conclusion has been reached that the reverse deaggregation reaction could be ignored in simulating the kinetics of their fusion. This conclusion was based [32,45] on an estimate that $D_{11} \ll f_{11}$. Values of D_{11} in the range of 0.02 to 0.2 s^{-1} were previously found by a detailed study of the aggregation of PS small unilamellar vesicles in the presence of NaCl [35,37]. In comparison, the value emerging for f_{11} was 5 s^{-1} [32,45]. If the dissociation of aggregates of PS vesicles in Ca^{2+} solutions does not occur at a faster rate than in Na^+ solutions, then the condition $D_{11} \ll f_{11}$ certainly holds, and ignoring the dissociation process is allowed. The same condition holds for the current results with 100 mM Na^+ and in particular with 20 mM of any of the monovalent cations, where the aggregation and fusion is determined by Ca^{2+}, being insensitive to the particular monovalent cation in solution. When the values of f_{11} are smaller ($f_{11} \leqslant D_{11}$), the neglect of the dissociation reaction can be permitted only with large lipid concentrations where the condition $C_{11}X_0 \gg D_{11}$ can be satisfied. Otherwise, the uncertainty in the values of f_{11} and C_{11} can be significant, despite an apparently satisfactory simulation of the experimental curves.

(ii) Determination of the parameters C_{11} and f_{11} from the simulation of kinetics of fusion

Inspection of Eqns. 2 and 4 indicates that when $f_{11}t \gg 1$, $F(t)$ approaches unity and consequently the analysis can only yield the value of C_{11}. Since we consider the early stages, i.e., $C_{11}X_0 < 0.5$, this condition also implies that $f_{11} \gg C_{11}X_0$. In such cases, the simulation can provide a reliable value of C_{11} and a reliable lower bound for f_{11}, i.e., a value whose further increase would not affect the simulation. This is the case with 100 mM of Na^+, K^+, tetramethylammonium$^+$ and with 20 mM of all the monovalent cations.

The procedure in Refs. 32 and 45 consists of determining the value of C_{11} from measurements on dilute vesicle suspensions, where the condition $f_{11} \gg C_{11}X_0$ holds, and then determining $f_{11} \approx C_{11}X_0$. In certain cases these parameters can be reasonably estimated from one lipid concentration by first determining C_{11} from the data at longer times and then determining f_{11} from the data at shorter times, where $F(t)$ (Eqns. 2 and 4) still increases with t.

This procedure has been utilized here, for several cases (see Tables I and II). The situation is more difficult when $f_{11} < C_{11}X_0$, which is the case with 300 mM of monovalent cations. However, even in these cases, an order-of-magnitude estimate for f_{11} can be obtained by another procedure.

Consider a situation where the vesicles are preaggregated up to a dimer state, such that initially the fusion process (see Eqn. 1) is given by

$$V_2 \xrightarrow{f_{11}} F_2 \tag{5}$$

$$\frac{dF_2}{dt} = f_{11}V_2 = -\frac{dV_2}{dt} \tag{6}$$

the solution of which is

$$F_2(t) = V_2(0)(1-\exp(-f_{11}t))$$

$$= \frac{X_0}{2}(1-\exp(1-f_{11}t)) \tag{7}$$

where $V_2(0) = X_0/2$.

Hence, since only half of the fused doublets contribute to $I(t)$, and the maximal value of I is proportional to $X_0/2$,

$$I(t) = \frac{100}{2}(1-\exp(-f_{11}t)) \tag{8}$$

In the presence of 300 mM of monovalent cations, the initial stages of the fusion could indeed by simulated by Eqn. 8. It should be noted that initially the vesicles are not fully preaggregated to a dimer state and there is some delay due to aggregation (see also Table III). Consequently, it may be expected that the values of f_{11} which satisfy this simulation with Eqn. 8 would be lower than the actual values. At later stages, when higher order aggregates, e.g., trimers, tetramers, etc., become more abundant, the actual values of $I(t)$ are larger than those given by Eqn. 8, and the values for f_{11} are overestimates. By employing Eqns. 2 to 4 in calculating $I(t)$ with a wide range of f_{11} and C_{11} values and then solving for f_{11} by the use of Eqn. 8, it follows that the error in f_{11} values due to the use of Eqn. 8 is less than an order of magnitude (see Table II).

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