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KINETICS OF A Ca^{2+}-TRIGGERED MEMBRANE AGGREGATION REACTION OF PHOSPHOLIPID MEMBRANES

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SUMMARY

Ca^{2+} and other divalent cations can trigger aggregation of phospholipid vesicles containing phosphatidic acid or phosphatidylserine. The reaction, which can be detected by an increase in light scattering, has a critical dependence on the Ca^{2+} concentration, with a threshold near 4 mM Ca^{2+}. This is the concentration for half-saturation of the polar head groups and for full neutralization of the membrane surface charge. The aggregation proceeds as a "polymerization" reaction, eventually forming such large aggregates that the vesicles precipitate. The stopped-flow rapid mixing technique was used to study the vesicle dimerization reaction which is the first step in the overall aggregation process. Vesicle dimerization resulted in a doubling of light scattering and had a vesicle concentration-dependent time constant ($t_{\frac{1}{2}}$) which varied between 0.4 and 2.0 s under the conditions of the study. Analysis of the dependence of the reaction amplitude and $1/t_{\frac{1}{2}}$ on the concentrations of vesicles and Ca^{2+} showed that the Ca^{2+} binding is fast, and that the dimerization proceeds by a mechanism in which the vesicles first collide to form an encounter complex followed by a slower conversion of the encounter complex to a stable complex. For phosphatidic acid vesicles, about 200–700 collisions are necessary to achieve a stable dimer. The rate-limiting step in the overall reaction is thus the transformation of the encounter complex into a stable complex, requiring 0.5 and 1.0 ms. The above-mentioned results are relatively insensitive to the type of divalent cation or to the choice of negatively charged lipid (phosphatidic acid or phosphatidylserine).

Evidence is given that the stable complex is effected by Ca^{2+}-mediated salt bridges between the two membranes and that the rate constant of the transformation step derives from the statistics of the distribution and the rate of redistribution of Ca^{2+}-occupied polar head groups on the membrane surfaces. The relevance of these results to the problem of Ca^{2+}-induced fusion of biological membranes is discussed.

INTRODUCTION

Since the inception of the phospholipid vesicle as a membrane model [1], a large number of important correlations have been made between its properties and the properties of biological membranes. A most important similarity was estab-

lished in studies which have shown that thermal phase transition behavior, characteristic of phospholipid systems [2], can be produced in biological membranes of similar phospholipid compositions [3]. Furthermore, the studies of Overath et al. [4] have shown that phase transitions can influence the permeability of cell membranes. Since these phase changes involve changes in the conformation of the fatty acid chains of the phospholipids (cf. ref. 5), these studies constitute the first example of how changes in the physical state of phospholipid regions of biological membranes can affect the transport properties of the cell.

Recent work of Papahadjopoulos et al. [6] has not only served to underline the relevance of the phospholipid vesicle as a model for the biological membrane, but has also demonstrated its use as a tool for the manipulation of biological membranes. These workers showed that fusion of mammalian cells can be induced by incubation with phospholipid vesicles containing phosphatidylglycerol or phosphatidylserine. In a subsequent study, this group [7] showed that vesicles of similar composition can undergo a Ca^{2+}-dependent fusion reaction having a time course of hours. These findings imply that similar processes are involved in the cell fusion reaction.

The present communication investigates a Ca^{2+}-induced vesicle aggregation reaction, one of the partial steps in the vesicle fusion reaction. We observed our first evidence for this aggregation reaction in the course of our studies of divalent cation binding to vesicles containing phosphatidic acid [8, 9]. Divalent cation binding was accompanied by an increase in 90° light scattering and optical absorbance to an extent larger than could be explained by the effect of the bound cation on the refractive index of the membrane surface. Millipore filtration experiments suggested that this was the effect of aggregation. The present study investigates the kinetics of this aggregation reaction, using the stopped-flow rapid mixing technique.

MATERIALS AND METHODS

The phospholipids were purchased from Koch-Light Laboratories phosphatidylserine, batch 48376; phosphatidic acid, batch 60306, Na^+ salt; dimyristoyl-L-phosphatidylcholine. Vesicles were prepared by sonicating approx. 30 mg of lipid for approx. 10 min in 30 ml of medium consisting of 10 mM NaCl, 10 mM Tris · HCl, pH 7.4. The sonication was carried out in a vessel thermostatted at 30 °C, using a Heat Systems W185D Sonifier at stage 10 (nominal power output 150 W). As a precaution, the vesicle suspension was centrifuged for 10 min at $12\,000 \times g$ to remove any macroscopic aggregates.

Rapid mixing experiments were performed with an Aminco-Morrow Stopped-Flow Apparatus (Cat. No. 4-8409) either in the light scattering or in the transmittance mode. Mixing was achieved within 10 ms. The progress curve for the change in scattering or transmittance (absorbance) for 0.5 mg/ml vesicles in the time range 10 ms–20 s could be expressed as the sum of two processes having half-times, $t_{\frac{1}{2}}$, of approx. 0.5 s and approx. 7 s. Only the first process was studied quantitatively. The beginning of the second process interfered with the evaluation of the first at times greater then four times $t_{\frac{1}{2}}$. The analysis was thus confined to shorter times, with routine measurements at 0.5 $t_{\frac{1}{2}}$, $t_{\frac{1}{2}}$ and 2 $t_{\frac{1}{2}}$. The end point was determined at 4 $t_{\frac{1}{2}}$ and the contribution of the second process tends to compensate for the approx. 20 % non-completion of the first reaction. The goodness of fit of the endpoint was deter-

mined from the constancy of the kinetics constants obtained for the three multiples of $t_{\frac{1}{2}}$ determined according to the mechanistic assumptions given in the next section. When the change in transmittance was large, it was necessary to recalculate the stopped-flow progress curve into absorbance for the purpose of calculating $t_{\frac{1}{2}}$.

RESULTS AND DISCUSSION

A substantial decrease in transmittance (increase in light scattering) is observed within a fraction of a second after mixing phosphatidic acid or phosphatidylserine vesicles together with Ca^{2+} or other divalent cations at a concentration of 1 mM or higher. Fig. 1 shows an oscilloscope trace of a stopped-flow experiment in which phosphatidic acid vesicles are mixed with 0.5 M Ca^{2+}. Following the rapid mixing (approx. 10 ms) a process is observed with $t_{\frac{1}{2}} = 0.7$ s, followed by a second process with $t_{\frac{1}{2}} \approx 7$ s. These are followed by a series of other slower processes which eventually result in precipitation of the total lipid material. Control experiments mixing the vesicles with distilled water or variable concentrations of NaCl indicated that the effect was not due to osmotic perturbation. The aggregation effects of divalent cation addition can be completely reversed by addition of an excess of EDTA during the first several minutes of aggregation. The divalent cation effect was not observed with lecithin vesicles, and is greatly reduced in preparations from phosphatidic acid/lecithin mixtures.

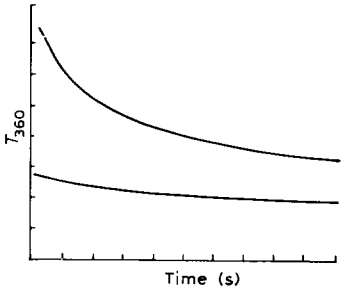

Fig. 1. Retrace of oscillograph record of rapid mixing experiment. Syringe A contained phosphatidic acid vesicles (0.7 mg/ml) in 10 mM NaCl, 10 mM Tris · HCl, pH 7.4: Syringe B contained 0.5 M $CaCl_2$. The vertical axis represents optical transmittance at 360 nm with a sensitivity of 6.2 % T per scale division; the horizontal sensitivity was 0.5 s per scale division for the first (top) sweep and 2 s per scale division on the second (bottom) sweep. The end point of the first reaction was considered to have been reached 3.0 s after mixing.

In order to determine which processes were responsible for the light scattering change, experiments were carried out in which the concentrations of Ca^{2+} and vesicles were varied. Fig. 2 shows the effect of Ca^{2+} on the rate and amplitude of the light scattering change corresponding to the first process. The amplitude increases very abruptly to its maximal value in the Ca^{2+} concentration range 1 to 6 mM. The rate of the reaction, as measured by $1/t_{\frac{1}{2}}$, increases also, but above the threshold it shows only a small variation over a 100-fold range of Ca^{2+} concentration. If the rate of the light scattering change were reporting the Ca^{2+} binding kinetics, then $1/t_{\frac{1}{2}}$ would

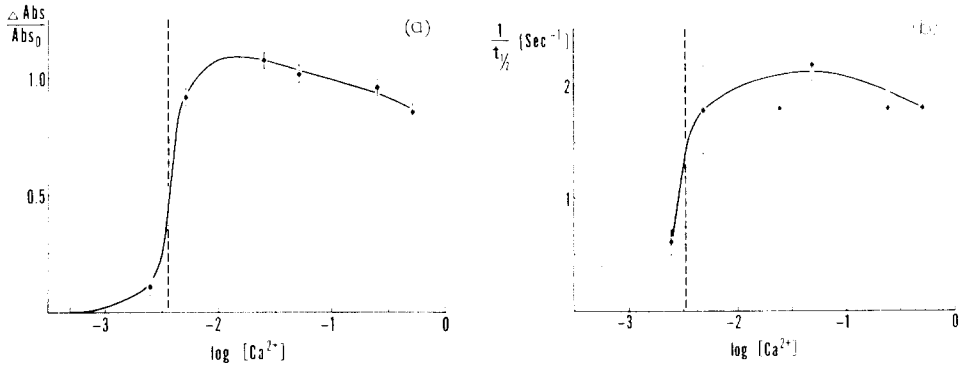

Fig. 2. The dependence of reaction amplitude and $t_{\frac{1}{2}}$ on Ca^{2+} concentration. The experimental conditions were identical to those of Fig. 1. A, amplitude; B, $1/t_{\frac{1}{2}}$.

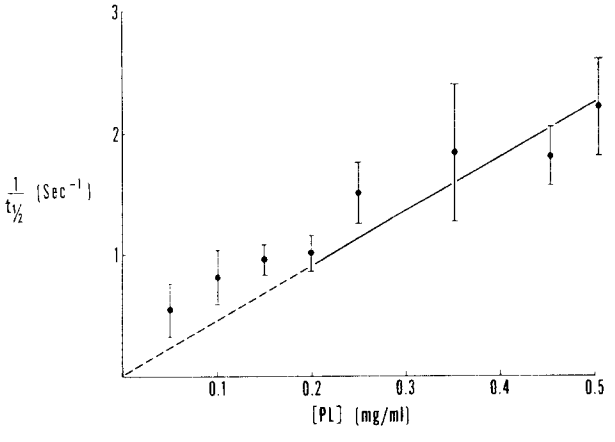

Fig. 3. Dependence of $1/t_{\frac{1}{2}}$ on phosphatidic acid vesicle concentration. The experimental conditions were identical to those of Fig. 1, except that syringe B contained 100 mM $CaCl_2$.

have been proportional to the Ca^{2+} concentration. That this is not observed indicates that the light scattering changes are reporting membrane events subsequent to the Ca^{2+} binding. Fig. 3 shows that the rate of the light scattering change increases with increasing vesicle concentration, indicating that we are dealing with a vesicle aggregation reaction.

Origin of light scattering change

The purpose of this section is to show that the fast light scattering change is due to vesicle dimerization. The probability of light scattering by a macromolecule or macromolecular aggregate is proportional to its concentration and molecular weight (cf. ref. 10, eqn 18 : 19) according to:

$$R_\theta = P(\theta)\,\kappa Mc \qquad (1)$$

where R_θ is a quantity proportional to the intensity scattered in the direction defined

by the angle θ, $P(\theta)$ is a constant approximately equal to one for small particles, κ is an optical constant involving the refractive index of the medium, differential refractive index of the particles and the wavelength of light, M is the molecular weight of the scattering particle and c is its concentration expressed in g/cm^3 of solution. Eqn 1 predicts that doubling of the molecular weight of the particles at constant weight concentration will double the light scattering intensity. Application of this simple theory to the phospholipid vesicles predicts that conversion of the vesicles to dimers should approximately double the optical absorbance of the solution*. Fig. 2 shows that this doubling of light scattering (Δ abs/abs$_0$ = 1.0) is actually observed, indicating that the reaction kinetics are indeed those of the dimerization process.

Mechanism of aggregation

The above information indicates that the vesicle aggregation reaction can be described according to a mechanism the first step of which is Ca^{2+} binding to the phosphate groups of the phosphatidic acid in a fast reaction:

$$n\ Ca^{2+} + \text{vesicle} \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} (\text{vesicle} \cdot n\ Ca^{2+}) \tag{2}$$

where n represents a number of Ca^{2+} per vesicle sufficient to promote aggregation. The dimerization of the vesicles then occurs in a slower step, with an apparent rate constant k_{app}

$$(\text{vesicle} \cdot n\ Ca^{2+}) + (\text{vesicle} \cdot n\ Ca^{2+}) \underset{k_{-app}}{\overset{k_{app}}{\rightleftharpoons}} (\text{vesicle} \cdot n\ Ca^{2+})_2 \tag{3}$$

In a recent study of Ca^{2+} binding to phosphatidic acid vesicles, we have shown that the phosphatidic acid binding sites are half-saturated at a Ca^{2+} concentration of approx. 3.6 mM [9]. Under this condition, the negative surface charge and surface potential of these vesicles are completely neutralized [9]. Comparison with the threshold effect on the aggregation reaction occurring at approx. 4 mM Ca^{2+} (Fig. 2) indicates that the number n corresponds to binding of one Ca^{2+} per 2.0 phosphatidic acid molecules and that it is this neutralization of surface charge which allows the vesicles to react with each other.

In addition to the dimerization reaction described in Eqn 3, reactions involving higher aggregated species are also possible:

$$(\text{vesicle} \cdot n\ Ca^{2+})m + (\text{vesicle} \cdot n\ Ca^{2+})p \underset{k_{-mp}}{\overset{k_{mp}}{\rightleftharpoons}} (\text{vesicle} \cdot n\ Ca^{2+})\, m+p \tag{4}$$

Precedents for this can be found in colloid chemistry and polymerization processes (cf. refs 10 and 11). The observation of slower light scattering increases on the time scale of seconds indicates that reactions of the type of Eqn 4 are indeed occurring. The discussion in this paper will be limited to the kinetics of the dimerization reaction.

After the rapid Ca^{2+} binding reaction of Eqn 2 has reached equilibrium,

* Eqn 18:19 of ref. 10 predicts that for vesicles with a radius of gyration of 250 Å and for light scattering at 360 nm, $P(\theta)$ = 0.97. Dimerization of the vesicles to double the radius of gyration would decrease $P(\theta)$ to 0.89.

the rate of destruction of the monomeric form of the vesicle (V) to form the dimer (V_2) will be given by:

$$-\mathrm{d}[V]/\mathrm{d}t = k_{app}[V]^2 - 2k_{-app}[V_2] \quad (5)$$

The small variation of the $\Delta abs/abs_0$ above the threshold Ca^{2+} concentration indicates the equilibrium of Eqn 3 lies far to the right and that $[V] > k_{-app}/k_{app} > [V]_{equilibrium}$ over the course of the rapid mixing experiment. This permits the back reaction term of Eqn 5 to be neglected for all but the lowest vesicle concentrations. The integrated rate equation then becomes:

$$[V]^{-1} - [V]_0^{-1} = 2k_{app} \cdot t \quad (6)$$

where $[V]_0$ denotes the concentration of the vesicle monomers at the instant of mixing. The degree of progress of the reaction α ($= 2[V_2]/[V]_0$) can be measured as the fraction of the maximal light scattering change $\Delta abs/\Delta abs_{max}$. Eqn 6 can thus be reformulated in terms of the experimentally measurable parameters such that:

$$k_{app}/N_{pl} = \frac{\alpha}{2(1-\alpha)[PL]t} \quad (7)$$

where N_{pl} is the number of phospholipid molecules per visicle and $[PL]$ is the concentration of phospholipid molecules (mol/l) in the medium. The first relaxation was found to obey Eqn 7 in the region of time during which the α value ($= \Delta abs/\Delta abs_{max}$) varied between 0.0 and 0.7. For routine evaluation of the rate constant k_{app}/N_{pl}, the value of $t_{\frac{1}{2}}$ ($\alpha = 0.5$) was determined from the stopped-flow trace by the procedure described in the previous section. Fig. 3 shows that $1/t_{\frac{1}{2}}$ of the reaction is roughly proportional to $[PL]$ as predicted by Eqn 7.

Dependence of lipid, cation and medium viscosity

Table I compares the measured $t_{\frac{1}{2}}$ values and reaction amplitude values for phosphatidic acid and phosphatidylserine with several divalent cations. Casual inspection reveals no pronounced differences in the behaviour of the phosphatidic acid and phosphatidylserine vesicles with the various cations aside from the observation that La^{3+} is effective at concentrations 10 times lower than those of the divalent cations. This can be attributed to the 10 times higher binding affinity [9] of La^{3+}. The rate of the aggregation reaction of the vesicles complexed with the cations is thus more of a property of the lipid membrane per se than of the complexing cation or type of negatively charged lipid.

A very important observation in this study was that dilution of the negatively charged lipid in a neutral lecithin matrix has a very pronounced effect on the overall equilibrium constant of the dimerization reaction, and has a considerable effect on the reaction kinetics. Table I shows that diluting the phosphatidic acid with lecithin to the extent of 50 % decreases the reaction amplitude to only 4 % and doubles the time necessary for half maximal extent of reaction. Assuming that 100 % dimerization would result in $\Delta abs/abs_0 = 1.0$ (cf. Eqn 1), this result indicates that the equilibrium constant for the dimerization reaction is reduced to less than 4 % of its original value by the incorporation of lecithin. This can be explained if the phosphatidic acid and its Ca^{2+} complex tend to distribute more or less randomly in the lecithin matrix [9].

TABLE I

THE EFFECT OF LIPID AND CATION ON $t_{\frac{1}{2}}$ AND REACTION AMPLITUDE

The relative amplitude was measured at 340 nm. The cation concentration was 50 mM, unless otherwise indicated. $T = 21$ °C.

Lipid	Lipid (mg/ml)	Cation	$t_{\frac{1}{2}}$ (s)	Δabs/abs$_0$
Phosphatidic acid	0.5	Ca^{2+}	0.43±0.10	1.16 ±0.02
Phosphatidic acid/lecithin (1:1)	0.5	Ca^{2+}	1.00±0.20*	0.044±0.022
Phosphatidic acid	0.5	Mg^{2+}	0.46±0.10	1.30 ±0.2
Phosphatidic acid	0.5	Mn^{2+} (5.0 mM)	0.45±0.07	1.3 ±0.1
Phosphatidic acid	0.5	Ba^{2+}	0.38±0.10	1.1 ±0.1
Phosphatidic acid	0.5	La^{3+} (0.5 mM)	0.56±0.02	1.2 ±0.5
Phosphatidylserine	0.3	Ca^{2+}	0.50±0.1	1.0 ±0.1
Phosphatidylserine	0.3	Mg^{2+}	0.67±0.1	1.0 ±0.1
Phosphatidylserine	0.3	Mn^{2+}	0.42±0.1	0.6 ±0.2
Phosphatidylserine	0.3	Sr^{2+}	0.35±0.1	0.6 ±0.2
Phosphatidylserine	0.3	Ba^{2+}	0.40±0.1	0.6 ±0.2
Phosphatidylserine	0.3	La^{3+} (0.5 mM)	0.35±0.1	0.6 ±0.2

* To be interpreted as time necessary for half-maximal change in α.

TABLE II

EFFECT OF SOLVENT VISCOSITY ON $t_{\frac{1}{2}}$

Experiment performed with phosphatidic acid vesicles, 0.5 mg/ml and 25 mM $CaCl_2$ at 23 °C.

Sucrose concentration (w/w)	η* (cP)	$t_{\frac{1}{2}}$ (s)	$t_{\frac{1}{2}}/\eta$ (s/cP)
0 %	0.937	0.45±0.05	0.48±0.05
21 %	1.875	0.77±0.05	0.41±0.03

* Value by interpolation of data for 20 and 25 °C (ref. 23).

and if it were necessary for a "patch" of phosphatidic acid molecules to segregate out for the dimerization to occur. The decrease in the equilibrium constant would result from the low probability of formation of such a patch and the increase in the reaction time might reflect the time necessary for the patch to come into being. Table II shows that the effect of medium viscosity (η) is to increase t and that the quotient $t_{\frac{1}{2}}/\eta$ is constant. This implies that a diffusion process is rate limiting. A calculation of k_{app} was made as an aid to the identification of this process.

Calculation of k_{app}

The aggregation would seem to be the result of a salt-bridging reaction which is not particularly sensitive to the type of divalent cation or negatively charged phospholipid but which does require microregions on the membrane surfaces containing only acidic phospholipids. The value of the rate constant for the overall reaction, k_{app}, can be calculated from experimental data as in Fig. 3 using Eqn 7 with an estimated value of N_{p1}. The limits of N_{p1} can be determined by using the vesicle radius

(R) to calculate the surface area ($= 4\pi R^2$) and by dividing by two times the surface area per phospholipid molecule (approx. 59 Å^2, cf. ref. 5). Using values of 250 Å [12] and 125 Å [13] (cf. ref. 14) as estimates of R allows the calculation of $2.66 \cdot 10^4$ and $6.67 \cdot 10^3$ as upper and lower limits of N_{pl}. As shown in Table II, the corresponding values of k_{app} are $3.9 \cdot 10^7$ and $9.9 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. The values for phosphatidylserine vesicles are about twice as large, but due to the uncertainty in the values of the parameters used above, we attach no great significance to this difference.

Does each vesicle collision lead to aggregation?

It is possible to make a calculation which shows whether the value of k_{app} is determined solely by the rate at which two vesicles can diffuse up to each other (diffusion control), or whether the rate of reaction is limited by slower processes occurring at the membrane surfaces. The expected relationship between the rate constant for bimolecular collision (k_1) of uncharged particles and on their diffusion constants (D) in the medium was determined over a half of a century ago by Smoluchowski [15, 16]. His treatment for spherical particles gives:

$$k_1 = 8\pi NRD \tag{8}$$

and

$$k_{-1} = 6D/R^2 \tag{9}$$

Where R represents the radii of the particles and where N is Avogadro's number. Interestingly enough, this equation was derived for application to the problem of the coagulation of colloidal suspensions. As shown by Debye [17], for the case of spherical particles, it is possible to combine this result with the Stokes-Einstein relationship:

$$D = \boldsymbol{k}T/(3\pi\eta R) \tag{10}$$

where η is the viscosity of the medium*.

Combination of these equations yields:

$$k_1 = 8\boldsymbol{k}TN/(3\eta) \tag{11}$$

predicting that the rate constant for collision will depend only on the absolute temperature and the viscosity of the medium, and that it will be independent of the particle size. Using this result with $\eta = 0.0097$ P we calculate a k_1 value of $6.8 \cdot 10^9$ $M^{-1} \cdot s^{-1}$. This value is two orders of magnitude greater than the value of k_{app} actually measured. It thus seems that processes occurring at the membrane surface represent the rate-limiting step in the aggregation reaction.

Detailed mechanism

The foregoing discussion indicates that the reaction of Eqn 3 is an oversimplification and that the minimal mechanism would be:

* The validity of the use of the Stokes-Einstein relationship (eqn 9) for the calculation of D is established in the study of Haung [13]. The diffusion constant of $(1.87 \pm 0.04) \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$ measured for his vesicle preparation is in excellent agreement with the value of $1.78 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$ obtained from eqn 10 using his diameter of 250 Å based on electron microscopy.

$$(\text{vesicle} \cdot n\ Ca^{2+}) + (\text{vesicle} \cdot n\ Ca^{2+}) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (\text{encounter complex}) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (\text{stable complex}) \quad (12)$$

where k_1 and k_{-1} are determined by the rate of diffusion (Eqns 9 and 10) and where k_2 is determined by processes occurring at the membrane surface. Since k_{app} describing the overall process is smaller than the calculated value of k_1, there must be rapid equilibration of the vesicle monomers and their encounter complexes (k_1, k_{-1}) followed by the slower step (k_2) of irreversible vesicle aggregations.

Under these conditions, the value of k_{app} is given by:

$$k_{app} = (k_1/k_{-1})k_2 \quad (13)$$

Straightforward calculation using Eqns 8 and 9 gives $k_{-1} = (1.7\text{–}7.9) \cdot 10^5\ s^{-1}$ and $k_1/k_{-1} = (0.51\text{–}3.9) \cdot 10^4\ M^{-1} \cdot s^{-1}$ where the lower and upper limits are determined for $R = 250$ Å and $R = 125$ Å, respectively*. The value of k_2, the rate constant for the transformation of the encounter complex of the phosphatidic acid vesicle to a stable complex, is thus $(1.0\text{–}1.9) \cdot 10^3\ s^{-1}$. The fraction of the total number of collisions which are effective in producing a stable complex is thus only 0.0014–0.0059 ($= k_2/(k_{-1}+k_2)$). A second physical interpretation of this result is that two membranes which have bound Ca^{2+}, which have approx. 500 Å cross-sections and which are apposed within reaction distance of each other will require 0.5–1.0 ms ($1/k_2$) to aggregate. This 0.5–1.0 ms process will be discussed below in terms of rearrangement of the membrane surface structure. Table III, which gives the details of these calculations, shows that the results for phosphatidic acid and phosphatidylserine vesicles are quite similar.

TABLE III

CALCULATION OF KINETIC CONSTANTS FOR THE AGGREGATION REACTION

The values of "slope" were determined from plot of the type of Fig. 3, and the k_{app}/N_{p1} values were determined from them according to Eqn 6 using 643 and 710 as the molecular weights of the sodium salt of phosphatidic acid and of phosphatidylserine, respectively. This corresponds to fatty acid chain of 16 C length.

Lipid	Slope ($ml \cdot mg^{-1} \cdot s^{-1}$)	k_{app}/N_{p1} ($M^{-1} \cdot s^{-1}$)	k_{app} ($M^{-1} \cdot s^{-1}$)	k_1/k_{-1}* (M^{-1})	k_2** (s^{-1})
Phosphatidic acid	4.6	1.5×10^3	3.9×10^7 †	3.9×10^4 †	1.0×10^3 †
			9.9×10^6 ††	5.1×10^3 ††	1.9×10^3 ††
Phosphatidylserine	7.3	2.6×10^3	6.9×10^7 †	3.9×10^4 †	1.7×10^3 †
			1.7×10^7 ††	5.1×10^3 ††	3.4×10^3 ††

* Calculated using $k_1/k_{-1} = (4/3)\pi R^3 N \cdot 10^{-3}$ (M^{-1}).
** Calculated according to Eqn (12).
† Calculated using $R = 250$ Å; $N_{p1} = 2.66 \cdot 10^4$.
†† Calculated using $R = 125$ Å; $N_{p1} = 6.67 \cdot 10^3$.

* Since the aqueous volume of a vesicle with $R = 250$ Å and with a membrane thickness of 50 Å is only 50 %, deviations from a spherical shape can do no more than halve our estimate of the vesicle volume. The lower limit for k_1/k_{-1} would thus be greater or equal to half of the indicated k_1/k_{-1} values.

Model for aggregation reaction

Our previous studies [9] have shown that Ca^{2+} and other divalent and higher valent cations bind to phosphatidic acid containing membranes, whereas the monovalent cations do not. Although both monovalent and higher valent cations serve to decrease the negative surface potential due to the phosphatidic acid, the present study shows that only the higher valent cations can cause membrane aggregations. This is interpreted here as the combination of their ability to neutralize the membrane surface charge and to participate in ionic bonding which bridges the two membranes.

Figs 4A and 4B illustrate two possible modes of this salt-bridging interaction. Fig. 4A treats the interaction as the transformation of a 1 : 1 Ca^{2+}-(polar head group) complex into a 1 : 2 intermembrane complex. Evidence for the 1 : 1 interaction was given in a study of Ca^{2+} binding to membranes whose phosphatidic acid /lecithin content was varied [9]. Fig. 4B describes the membrane-membrane interaction as the conversion of a 1 : 2 Ca^{2+}-(polar head group) complex into a 1 : 4 intermembrane complex. Since both of the models share the same essential feature, that aggregation requires binding of divalent cations to the extent of one cation per two polar head groups, the present data do not allow us to distinguish between these two models.

Figs 4A and 4B show why Ca^{2+} binding and apposition of the two membranes are not in themselves sufficient to cause the membranes to aggregate. Salt bridging requires the positions of the Ca^{2+}-occupied and unoccupied polar head groups to be correlated, but such correlation of occupation of a large number of polar head groups is unlikely if their disposition is statistical. Aggregation thus requires rearrangement of the Ca^{2+} on the membrane surface. For phosphatidic acid and phosphatidylserine vesicles, this step (k_{2a}), and the subsequent aggregation of the membranes (k_{2b}) must require 0.3–1.0 ms.

The data of Fig. 2 show that the threshold in the Ca^{2+} concentration dependence of the aggregation coincides with the point of neutralization of the membrane surface charge. This can be readily understood in terms of Fig. 4, since low degrees of occupation of the phosphatidic acid binding sites would not result in sufficient proba-

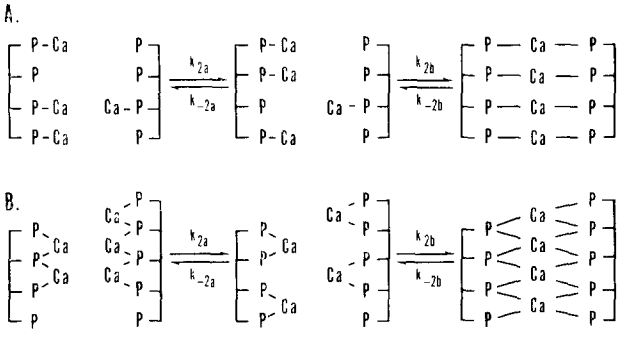

Fig. 4. Schematic model for the vesicle aggregation reaction. The membrane surfaces of two vesicles are depicted, with the symbol P denoting the charged polar head groups. The coordination of the cation is indicated by the solid lines. For visual clarity, the charges of the cation Ca^{2+} and the polar head group P^- are not given in the figure. (A) The bound cation is singly coordinated in the non-aggregated state (cf. ref. 9) and doubly coordinated in the aggregated state. (B) The bound cation is subject to higher degrees of intramembrane (cf. ref. 22) and transmembrane coordination.

bility of correlation of the positions of the occupied phosphatidic acid molecules for stable aggregation. The model of Fig. 4 also explains why the extent of the aggregation reaction is so drastically reduced by addition of lecithin to the phosphatidic acid. Aggregation of vesicles composed of lecithin is probably suppressed by short-range repulsive interactions resulting from the perpendicular orientation and dipolar character of the polar head groups. We would thus expect the formation of stable junctions between phosphatidic acid/lecithin mixed membranes to rely on "patches" composed of phosphatidic acid. For statistical distribution of phosphatidic acid and lecithin on the membrane surfaces of the encounter complex, the probability of occurrence of two apposed microregions containing only phosphatidic acid molecules would be $P = (f_{PA})^q$, where f_{PA} is the mole fraction of phosphatidic acid within the membrane and where q is the number of phosphatidic acid molecules within the two microregions. A value of q between 4 and 6 would be sufficient to explain the observed 25-fold reduction in k_2/k_{-2} for the 1 : 1 phosphatidic acid/lecithin mixture.

The irreversible step in the aggregation reaction requires 0.5–1.0 ms and involves the rearrangement of Ca^{2+} on the apposed surfaces of the two membranes. This rearrangement can occur by mechanisms involving movement of the vesicles, Ca^{2+} or phospholipids. (a) An unsuitable encounter complex can dissociate and then reassociate to form an encounter complex in which other areas of the membranes are brought into contact. The probability that this new complex is stable is $P = k_2/(k_2 + k_{-1})$. This process would be repeated until a stable complex is formed. (b) Rearrangement of Ca^{2+} on the membrane surface can occur in a process in which Ca^{2+} dissociates from one polar head group and associates with a second one. (c) A phospholipid molecule whose polar head group is occupied by Ca^{2+} can change places with an unoccupied neighbor. The observed decrease in k_{app} with increasing viscosity of the solution is predicted by mechanisms a, b and possibly c. The lack of dependence of the rate constant on the type of divalent cation and lipid polar head group might favor models a and c. It is concluded that all three mechanisms probably contribute to the overall process and that the present data do not allow us to determine their relative importance.

The analysis of the Ca^{2+} binding behaviour of lecithin/phosphatidic acid mixtures [9] gave rise to the prediction that at high Ca^{2+} concentrations the vesicles would bind more than one Ca^{2+} per two phosphatidic acid molecules and develop a net positive charge. The slight decrease in aggregation amplitude and $1/t_{\frac{1}{2}}$ with increasing Ca^{2+} concentration would seem to bear out this prediction, but the small size of this effect indicates that either a full 1 : 1 Ca^{2+}-polar head group stoichiometry is not achieved, even at high Ca^{2+} concentration, or that the aggregation reaction is much more complicated than our model for it.

Relationship of aggregation to membrane fusion

In a recent study, Papahadjopoulos et al. [7] showed that phosphatidylserine-containing vesicles will fuse in the presence of Ca^{2+}, allowing the phospholipids of the two vesicles to mix. This fusion reaction was observed for Ca^{2+} concentrations greater than 1 mM and for incubation times of about 1 h. The experimentally determined extent (most probably rate) of fusion increased with increasing Ca^{2+} with the half-maximal effect in the region of 2 mM Ca^{2+} (cf. Fig. 1, ref. 7). The Ca^{2+} effect decreased significantly with increasing lecithin content in lecithin/phosphatidylserine

mixtures, such that the amount of fusion observed with 60 % phosphatidylserine-40 % lecithin membranes was less than 10 % of that observed for 100 % phosphatidylserine membranes. There is thus a striking parallelism between the findings of the study of Papahadjopoulos et al. [7] on membrane fusion and findings of the present study on membrane aggregation. This leads us to suggest that the aggregation reaction occurring over a time course of seconds is the primary determinant of the fusion reaction. The latter occurs in the aggregated state, possibly by a mechanism similar to the micellarization model of Lucy [18], over a time course of approx. 1 h.

Biological significance

The reactions discussed in the present study can be considered as a model for all reactions in which two membranes come in contact. Of considerable interest are reactions which possibly involve membrane fusion triggered by Ca^{2+}, as in the case of quantized acetylcholine release through the presynaptic membrane [19, 20]. A body of evidence supports the hypothesis that the quantized release is the result of the Ca^{2+}-induced fusion of approx. 500 Å diameter acetylcholine-containing vesicles with the presynaptic membrane [19–21]. We speculate here that this phenomenon involves a salt-bridging effect of Ca^{2+} between the membranes. However, proteins would probably serve as negatively charged sites rather than phospholipids since the latter show little Ca^{2+}/Mg^{2+} specificity. Furthermore, the vesicle theory of acetylcholine release would require a mechanism whereby aggregation would immediately result in fusion of the two membranes, such that the contents of the vesicle would be extruded into the synapse within 0.5–2.0 ms, the synaptic delay time [19].

In the present study, a 0.5–1.0 ms time constant is calculated for the process in which two juxtapositioned membranes with bound Ca^{2+} can react to form Ca^{2+} cross-linkages. It is thus tempting to compare this time constant with the synaptic delay time, visualizing a process in which fusion occurs after patches of negatively charged groups are formed on the synaptic vesicle and presynaptic membranes and are cross-linked by Ca^{2+}. The phospholipid vesicle aggregation reaction is certainly much simpler than the fusion reaction of the synaptic vesicles. Nevertheless, the maximal rates of these two reactions are ultimately determined by the rate of deployment of their building blocks, in this case phospholipids, protein and ions. Although we have only succeeded here in characterizing the kinetics of the interaction of two of the building blocks, phospholipids and ions, it is hoped that extensions of these kinetic investigations will give more information about the rates of the fundamental reactions upon which all triggering processes ultimately depend.

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REFERENCES

1 Bangham, A. C. (1968) Prog. Biophys. Mol. Biol. 18, 29–95
2 Chapman, D., Williams, R. M. and Ladbrooke, B. D. (1967) Chem. Phys. Lipids 1, 445–475
3 Reinert, J. L. and Steim, J. M. (1970) Science 168, 1580–1582
4 Overath, P., Schairer, H. U. and Stoffel, W. (1970) Proc. Natl. Acad. Sci. U.S. 67, 606–612

5 Träuble, H. and Haynes, D. H. (1971) Chem. Phys. Lipids 7, 324–335
6 Papahadjopoulos, D., Poste, G. and Schaeffer, B. E. (1973) Biochim. Biophys. Acta 323, 23–42
7 Papahadjopoulos, D., Poste, G., Schaeffer, B. E. and Vail, W. J. (1974) Biochim. Biophys. Acta 352, 10–28
8 Haynes, D. H. and Staerk, H. (1974) J. Membrane Biol. 17, 313–340
9 Haynes, D. H. (1974) J. Membrane Biol. 17, 341–366
10 Tanford, C. (1961) in Physical Chemistry of Macromolecules, pp. 275–316, John Wiley and Sons, New York
11 Tanford, C. (1961) in Physical Chemistry of Macromolecules, pp. 587–603, John Wiley and Sons, New York
12 Papahadjopoulos, D. and Miller, N. (1967) Biochim. Biophys. Acta 135, 624–638
13 Haung, C. (1969) Biochemistry 8, 344–352
14 Seufert, W. D. (1970) Biophysik 7, 60–73
15 Smoluchowski, M. (1916) Physik. Z. 17, 557–585
16 Smoluchowski, M. (1917) Z. Physik. Chem. 92, 129–168
17 Debye, P. (1942) Trans. Electrochem. Soc. 82, 265–272
18 Lucy, J. A. (1970) Nature 227, 814–817
19 Katz, B. (1969) in The Release of Neural Transmitter Substances, pp. 26–27, Charles C. Thomas, Springfield, Ill.
20 Hubbard, J. I. (1970) Prog. Biophys. Mol. Biol. 21, 33–124
21 Whittaker, V. C. (1969) in Neurosciences Research Symposium Summaries, (Schmitt, F. O., Melnichuk, T., Quarton, G. and Adelman, G., eds), Vol. III, pp. 555–565
22 Papahadjopoulos, D. (1968) Biochim. Biophys. Acta 163, 240–254
23 Handbook of Biochemistry Selected Data for Molecular Biology (1973) (Sober, H. A., ed.), pp. *f*288–*f*301, CRC Press, Cleveland, Ohio