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PROTEIN-MEDIATED INTERMEMBRANE CONTACT SPECIFICALLY ENHANCES Ca^{2+}-INDUCED FUSION OF PHOSPHATIDATE-CONTAINING MEMBRANES

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Ca^{2+}-induced fusion of glycolipid-phospholipid vesicles containing several different anionic phospholipids was investigated, with and without lectin-mediated intervesicle contact. In vesicles containing phosphatidylserine, phosphatidylinositol or its mono- or diphosphate as the anionic phospholipid fusion was induced only at 1–10 mM Ca^{2+} both in the absence and presence of lectin. In contrast, the Ca^{2+}-threshold for fusion of phosphatidate-containing vesicles was reduced to $\leqslant$ 0.1 mM Ca^{2+} by lectin-mediated intermembrane contact.

Membrane fusion occurs frequently, but under strict control, in the normal life of most mammalian cells; e.g. in the intracellular transfer of membranes between endoplasmic reticulum, the Golgi, lysosomes and the plasma membrane. The mechanism(s) by which these fusion events occur and are controlled is not well understood, although a requirement for Ca^{2+} has been established in certain cases, notably in exocytosis. The focal nature of membrane fusion, its high rate and the structural complexity of the membranes involved, have seriously hampered mechanistic studies. Simple model systems, such as the Ca^{2+}-induced fusion of anionic phospholipid membranes, are more amenable to study but the biological relevance of this model has been questioned since fusion of the extensively studied phosphatidylserine membranes requires millimolar concentrations of Ca^{2+} [1]. However, striking differences in Ca^{2+} sensitivity among the naturally occurring anionic phospholipids have recently been documented. Thus, pure phosphatidate membrane vesicles were found to undergo fusion at micromolar concentrations of Ca^{2+} while phosphatidylinositol vesicles were resistant to calcium-induced fusion [2].

In the model system referred to above [1,2] both intermembrane contact and membrane fusion have been induced by added calcium ions while in cellular membrane fusion specific mechanisms for intermembrane recognition and contact most likely occur. We have therefore investigated a model system consisting of glycolipid-phospholipid vesicles in which intermembrane contact (lectin-mediated) and fusion competence (calcium-mediated) can be independently controlled.

The phosphatidylethanolamine used in this study was isolated from egg yolk [3] and phosphatidate, phosphatidylserine and phosphatidylinositol were prepared as described [4]. The synthetic glycolipid phosphatidylethanol-lactobionamide (PELBA) was prepared by a modification of the procedure described for the coupling of lactobionic acid to alkylamines [5]. *Ricinus communis* agglutinin (RCA) was purchased from Boehringer-Mannheim Co. Large unilamellar vesicles were prepared [6] from lipid mixtures containing 10 mol% PELBA and < 0.1 mol%

Abbreviations: PELBA, phosphatidylethanol-lactobionamide; RCA, *Ricinus communis* agglutinin; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

[^{3}H]phosphatidylcholine and were then passed through a 0.1 μm Unipor-filter (Bio-Rad Lab.). Chelated terbium ions and dipicolinic acid (sodium salt) were encapsulated separately as described [2] and both vesicle populations were freed of non-encapsulated material by gel filtration.

A fluorimetric assay [1] which monitors the intermixing of separately encapsulated Tb and dipicolinate upon vesicle fusion was used. Tb- and dipicolinate-containing vesicles (75 nmol lipid each) were mixed in 1.5 ml of 0.2 M NaCl-2 mM Tes (pH 7.4) containing 0.15 mM EDTA. Into the sample was then injected small volumes of either a solution of $CaCl_2$ alone (usually 0.1 M) or a solution of RCA (4 mg/ml) followed 1 min later by $CaCl_2$. Fusion was monitored at 25°C in an Aminco-Bowman fluorimeter ($\lambda_{ex} = 278$ nm; $\lambda_{em} = 542$ nm) under continuous mixing of the sample. Tb-vesicles, free of EDTA, were lysed with cholate and mixed with an excess free dipicolinate for calibration of maximal Tb-dipicolinate fluorescence.

The lipid vesicles used in the present study contained 10 mol% of a glycolipid (PELBA) whose lectin-binding carbohydrate extends well out from the phospholipid headgroup layer. The vesicles were therefore rapidly and efficiently agglutinated by RCA both in the presence and absence of Ca^{2+} (unpublished observations).

Fig. 1 shows that when only Ca^{2+} was added to the glycolipid-containing phosphatidate-phosphatidylethanolamine vesicles a threshold concentration of approx. 1.5 mM had to be reached in order to initiate vesicle aggregation and fusion. This threshold is only slightly higher than that observed for glycolipid-free phosphatidate-phosphatidylethanolamine vesicles [7]. When intervesicle contact was established by the addition of lectin, we found that this was not in itself sufficient for vesicle fusion, but the subsequent addition of Ca^{2+} initiated fusion at a threshold concentration which was reduced several-fold (Fig. 1). We believe that the latter Ca^{2+}-threshold represents the Ca^{2+}-requirement for the actual fusion event. When Mg^{2+} was used instead of Ca^{2+}, no dramatic enhancement of the ion sensitivity was seen after lectin-mediated intervesicle contact (threshold: 2.5 mM versus 2.0 mM). This means that the selectivity for Ca^{2+} over Mg^{2+} in inducing fusion increased from < 2 to > 10 when the vesicles were first brought in contact by RCA.

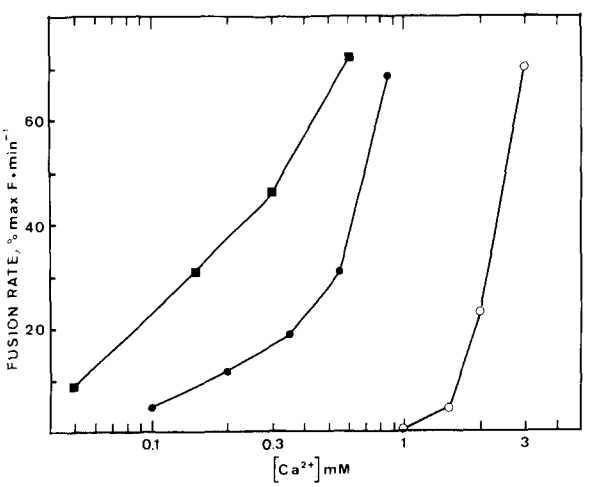

Fig. 1. Effect of lectin-mediated intermembrane contact on Ca^{2+}-induced fusion of PELBA/phosphatidate/phosphatidylethanolamine (10 : 20 : 70) vesicles. Additions: $CaCl_2$ alone (○); RCA (40 μg/ml) followed after 1 min by $CaCl_2$ (●) or RCA plus $MgCl_2$ (1 mM) followed 1 min later by $CaCl_2$ (■). Concentrations indicated represent total divalent ion added, corrected for the presence of EDTA (0.15 mM). Fusion rate represents the increase in Tb dipicolinate fluorescence (expressed as % of maximal fluorescence) per minute (see also Refs. 2 and 7). Experimental conditions were as described in the text.

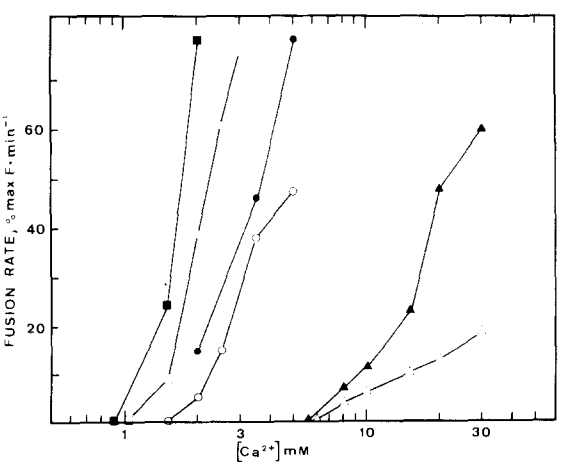

Fig. 2. Ca^{2+}-dependence of the fusion of PELBA/phosphatidylserine vesicles containing 5 (□, ■) or 10 (○, ●) mol% glycolipid and of PELBA/phosphatidylinositol/phosphatidylethanolamine (10:20:70) vesicles (△, ▲). Open symbols: $CaCl_2$ alone; filled symbols: $CaCl_2$ added 1 min after RCA (40 μg/ml).

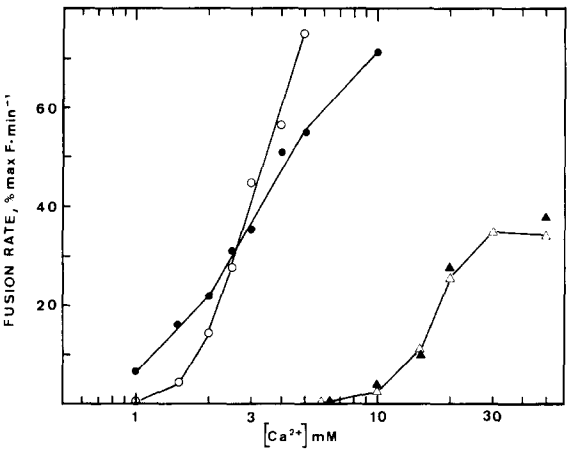

Fig. 3. Ca^{2+}-induced fusion of vesicles containing 10 mol% PELBA, 70 mol% phosphatidylethanolamine and 20 mol% of either phosphatidylinositol 4-phosphate (△, ▲) or phosphatidylinositol 4,5-diphosphate (○, ●). The latter two lipids were isolated from bovine brain [15] and their purity was confirmed by thin-layer chromatography [16]. Open symbols: $CaCl_2$ alone was added; filled symbols: $CaCl_2$ was added 1 min after RCA (40 μg/ml).

Furthermore, the sensitivity to Ca^{2+} was enhanced even more by the simultaneous presence of lectin and 1 mM Mg^{2+} (Fig. 1).

It should be noted that, considering the molecular size of the RCA, the vesicles would only be brought to a distance 60–80 Å apart by the lectin. This is not sufficient to interfere with the hydration shell of the vesicle surface [8], and therefore, probably not with the actual fusion event [4,7]. The possibility that RCA enhanced Ca^{2+}-induced fusion by clearing the area of vesicle contact from fusion-inhibiting glycolipid can be ruled out, since; (a) the Ca^{2+}-requirement for fusion in the presence of lectin is much lower than that for glycolipid-free vesicles [7], and (b) the amount of lectin used (0.66 nmol galactose-binding sites per ml) corresponds to only a small fraction of the surface-exposed glycolipid (5 nmol/ml). Furthermore, we found that the fusion of vesicles containing glycolipid in mixture with phosphatidylserine instead of phosphatidate and phosphatidylethanolamine was only minimally affected by lectin at the vesicle concentration (100 μM lipid) used here (Fig. 2). This agrees with a kinetic model put forward, according to which the establishment of intervesicle contact is rate-limiting in Ca^{2+}-induced aggregation and fusion of phosphatidylserine vesicles only at concentrations up to approximately 100 μM [9].

The phospholipid specificity of the lectin-mediated enhancement of fusion was further investigated by replacement of phosphatidate with inositol phospholipids. In glycolipid/phosphatidylinositol/phosphatidylethanolamine vesicles, the threshold for Ca^{2+}-induced fusion was approx. 10 mM (Fig. 2) and the presence of lectin did not lower this threshold although the rate of fusion increased above the threshold. Similar results were obtained when the vesicles contained phosphatidylinositol 4-phosphate instead of phosphatidylinositol (Fig. 3). In vesicles containing phosphatidylinositol 4,5-diphosphate the Ca^{2+} threshold was lower (Fig. 3) but the effect of lectin-induced intervesicle contact was also here very small.

When this work was completed, a study demonstrating facilitation of Ca^{2+}-induced fusion of phosphatidate-phosphatidylethanolamine vesicles by synexin, a protein isolated from the adrenal medulla, was presented [10]. The similarity between the effects of synexin and those of lectin-mediated intervesicle contact, reported here, may suggest that also synexin acts by establishing intermembrane contact. The similarity applies also to phosphatidylserine-containing vesicles (cf. Ref. 11 and Fig. 2, present paper), which supports this conclusion.

The present study and previous work [2] indicate that phosphatidate, in contrast to phosphatidylserine, phosphatidylinositol and the phosphoinositides, is sufficiently responsive to Ca^{2+} to be potentially involved in cellular, Ca^{2+}-induced, membrane fusion events. A rapid accumulation of phosphatidate has also been observed in certain cell types upon induction of exocytosis [12–14]. The results further show that protein-mediated intermembrane contact significantly increases not only the sensitivity to Ca^{2+} but also the Ca^{2+}/Mg^{2+} selectivity of the fusion reaction between phosphatidate-containing membranes.

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