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Biophysics

Synexin facilitates fusion of specific phospholipid membranes at divalent cation concentrations found intracellularly

(Ca^{2+}-binding protein/Ca^{2+}-induced membrane fusion/exocytosis/phospholipid vesicle)

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ABSTRACT The effect of synexin (an adrenal medullary protein) on the kinetics of Ca^{2+}- and Mg^{2+}-mediated membrane fusion was examined. Membrane fusion was studied by monitoring intermixing of the aqueous contents of phospholipid vesicles. Synexin facilitated Ca^{2+}-mediated, but not Mg^{2+}-mediated, fusion of phosphatidate/phosphatidylethanolamine (1:3) and phosphatidate/phosphatidylserine/phosphatidylethanolamine/cholesterol (1:2:3:2) vesicles. The threshold concentration of Ca^{2+} for fusion was decreased to $\approx$10 μM in the presence of synexin at 6 μg/ml and 1.5 mM Mg^{2+} in vesicle suspensions containing 50 μM lipid. This effect of synexin was drastically inhibited by including 25% phosphatidylcholine (mol/mol) in the vesicle membrane. It is proposed that the Ca^{2+}-dependent lipid-specific enhancement of membrane fusion by synexin contributes to an increase in the sensitivity of specific intracellular membranes to Ca^{2+} with respect to fusion.

Membrane fusion, believed to be the key event in exocytosis, is poorly understood due to the complexity of the membranes involved. Thus, a variety of model systems have been introduced for elucidating the molecular mechanism of Ca^{2+}-mediated membrane fusion. The most striking difference in the fusion behavior of secretory vesicles *in vitro* (1) compared with that of phospholipid vesicles (2–4) is the lower threshold concentration of Ca^{2+} needed to obtain measurable fusion (0.01–0.1 mM for the former compared with 1 mM for the latter). Indirect evidence suggests that proteins are involved in decreasing the Ca^{2+} concentration required for membrane fusion. To establish a specific role for proteins in relationship to Ca^{2+} concentration thresholds, we have studied the effects of several Ca^{2+}-binding proteins on phospholipid vesicle fusion by using a fluorescence assay to monitor the intermixing of aqueous vesicle contents (2, 5). Calmodulin and parvalbumin weakly inhibit the fusion of phospholipid vesicles (6, 7). Bovine prothrombin and its proteolytic fragment 1 have a strong inhibitory effect on fusion (7). Synexin, a Ca^{2+}-binding protein originally isolated from bovine adrenal medulla (8), can cause aggregation of isolated chromaffin granules at Ca^{2+} concentrations >6 μM. We have previously found that synexin increases appreciably the rate of fusion of phospholipid vesicles containing phosphatidylserine (PtdSer) and mixtures of PtdSer and phosphatidylethanolamine (PtdEtn) (6). To obtain a membrane model that has the same sensitivity to Ca^{2+} with respect to fusion as biological membranes, we have investigated further the effect of synexin on vesicles containing acidic phospholipids other than PtdSer. In this report, we demonstrate that synexin responds to Ca^{2+} but not to Mg^{2+} in promoting fusion of phosphatidate (PtdO)-containing vesicles. The lowest threshold of Ca^{2+} is $\approx$10 μM, which is in the range of intracellular Ca^{2+} levels necessary for exocytosis (9–12). In addition, we report that phosphatidylcholine (PtdCho) inhibits Ca^{2+}-induced fusion and discuss the possible role of synexin in exocytosis.

MATERIALS AND METHODS

Synexin. Synexin from bovine adrenal medullary tissue was isolated by ammonium sulfate precipitation and purified on Ultrogel AcA 34 (LKB) as described (6, 8). Purified synexin was concentrated and stored at 4°C, pH 6.5. Protein concentration was determined by the Bio-Rad assay using bovine serum albumin as the standard.

Lipids. PtdSer (from bovine brain), PtdO, and PtdEtn (transesterified from egg PtdCho) were purchased from Avanti Polar Lipids. PtdCho from egg yolk was prepared as before (13). Each lipid was stored as a chloroform solution in sealed ampoules under argon at −40°C. Lipid phosphorus was determined according to Bartlett (14). Cholesterol from Sigma was recrystallized twice before use.

Chemicals. $TbCl_3$ was obtained from Alfa-Ventron. Dipicolinic acid, L-histidine, sodium nitrilotriacetate, and *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes) were purchased from Sigma. All other chemicals used were of the highest purity available.

Preparation of Vesicles. Large unilamellar vesicles encapsulating the desired aqueous contents were prepared by the reverse-phase evaporation technique as described (15) with minor modifications (2). The vesicles were made in either (*i*) 2.5 mM $TbCl_3$/40 mM sodium nitrilotriacetate or (*ii*) 50 mM sodium dipicolinate/20 mM NaCl. In addition, the media contained 2 mM L-histidine and 2 mM Tes, pH 7.4. Vesicles were separated from nonencapsulated material by gel filtration on Sephadex G-75 (elution buffer, 100 mM NaCl/2 mM L-histidine/2 mM Tes/1 mM EDTA, pH 7.4).

Fusion Assay. The fusion assay is based on the interaction of Tb^{3+} encapsulated in one population of vesicles with dipicolinic acid in another. Tb fluorescence intensity increases 10,000-fold when the Tb(dipicolinic acid)$_3^{3-}$ complex is formed inside the aqueous compartments of fusing vesicles (2). Fluorescence was measured with an SLM-4000 fluorometer. The Tb–dipicolinic acid complex was excited at 276 nm; fluorescence was measured through a Corning 3-68 cut-off filter. Tb- and dipicolinic acid-containing vesicles were mixed at a 1:1 ratio, 25 μM phospholipid each, in 1.0 ml of 100 mM NaCl/2 mM L-histidine/2 mM Tes/0.1 mM EDTA, pH 7.4. One hundred percent fluorescence was determined by lysing the Tb-containing vesicles (25 μM lipid, free of EDTA) with 1% sodium cholate in the presence of 20 μM dipicolinic acid.

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Abbreviations: PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PtdO, phosphatidate; PtdCho, phosphatidylcholine; Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid.

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Determination of Ca^{2+} Concentration. Free Ca^{2+} concentrations were determined with a Ca^{2+}-selective electrode operating with a neutral carrier incorporated in a polyvinylchloride membrane (16). The potential was measured in millivolts with a digital pH meter (Fisher model 420). The electrode was calibrated with solutions of known Ca^{2+} concentration and containing Mg^{2+} when appropriate.

RESULTS AND DISCUSSION

The initial rate of fusion of PtdO/PtdEtn (1:3) vesicles induced by divalent cations in the presence or absence of synexin is shown in Fig. 1*A*. Synexin was effective in enhancing the rate of Ca^{2+}-induced fusion (solid vs. open squares) and also in reducing the threshold level required for fusion but ineffective if Mg^{2+} was the only divalent cation present (solid vs. open circles). This is in agreement with previous observations that synexin facilitation of vesicle fusion (6, 7) and chromaffin granule aggregation (8) is Ca^{2+} specific. More than 1 mM Ca^{2+} was required to initiate fusion of vesicles at measurable rates whereas, in the presence of synexin, <200 μM Ca^{2+} was sufficient to reach the same rate. The threshold level could be reduced further by including Mg^{2+} at a concentration (1.5 mM in this case) not sufficient to induce fusion by itself. Under these conditions, the threshold concentration of Ca^{2+} was decreased by synexin to ≈10 μM (Fig. 1*A*, solid triangles), and a reasonably rapid rate of fusion was obtained at 25 μM Ca^{2+} (≈3% of maximum fluorescence/min). Ca^{2+} concentrations were determined for each sample by using Ca^{2+}-selective electrodes and therefore correspond to free Ca^{2+} in solution. Since the rate of fusion depends on the concentration of vesicles or synexin or both at a fixed divalent cation level (2, 6), the threshold concentration of Ca^{2+} for initiating fusion will be lower if the lipid and protein concentrations are increased. This observation is important physiologically because the minimal concentrations of Ca^{2+} and Mg^{2+} required for fusion of these vesicles are close to intracellular concentrations during exocytosis (9). The roles of Ca^{2+} and Mg^{2+} in inducing fusion of phospholipid vesicles have been studied in some detail (2, 4, 17–19). The role of Ca^{2+} seems to be related to the formation of "anhydrous" complexes, while the mechanism of action of Mg^{2+} is not fully understood at present. Mg^{2+} may participate in the reduction of electrostatic repulsive forces and thus reduce the intermembrane distance (17, 18). Synergistic effects between Ca^{2+} and Mg^{2+} have been observed before (4, 17, 18), even in the absence of synexin.

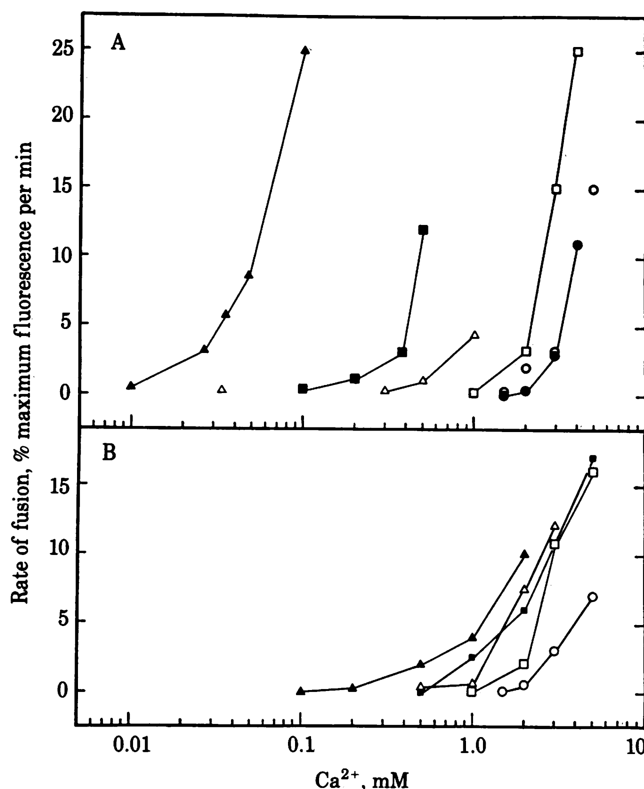

FIG. 1. Initial rate of fusion of PtdO/PtdEtn, 1:3 (mol/mol), (*A*) and PtdO/PtdEtn/PtdCho (1:2:1) (*B*) vesicles at various divalent cation concentrations. Synexin (final concentration, 6 μg/ml) was added as a concentrated solution to the vesicle suspension (50 μM lipid) prior to introduction of Ca^{2+}. Ca^{2+} was injected as a concentrated solution at time 0 with constant stirring. Experiments were carried out at 25°C. For those experiments in which both Ca^{2+} and Mg^{2+} were included, Mg^{2+} was added to the synexin/vesicle suspension before the introduction of Ca^{2+}. In experiments in which Mg^{2+} was the only divalent cation, the concentrations used are shown numerically by the Ca^{2+} concentration scale. ○, Mg^{2+} only; ●, synexin/Mg^{2+}; □, Ca^{2+} only; ■, synexin/Ca^{2+}; △, 1.5 mM Mg^{2+}/Ca^{2+}; ▲, synexin/1.5 mM Mg^{2+}/Ca^{2+}.

PtdCho is known to inhibit Ca^{2+}-induced fusion of vesicles containing PtdSer (4, 19) or PtdO (20) as the acidic phospholipid. We have therefore investigated the role of PtdCho in modulating the effect of synexin on fusion. The Ca^{2+} dependence of synexin-mediated fusion of PtdO/PtdEtn/PtdCho (1:2:1) vesicles is shown in Fig. 1*B*. Comparison of the fusion kinetics of PtdO/PtdEtn (1:3) and PtdO/PtdEtn/PtdCho (1:2:1) vesicles in the presence of Ca^{2+} alone (graphs based on the open squares in Fig. 1 *A* and *B*) shows that including 25% PtdCho inhibits fusion rates only slightly. A slight inhibition is also seen in the presence of both Ca^{2+} and Mg^{2+} (open triangles). The most remarkable difference between these two membrane systems was observed in the presence of synexin. The PtdCho-containing vesicles (Fig. 1*B*) were marginally affected by synexin, which stimulated the rates of fusion in the presence of either Ca^{2+} or Ca^{2+}/Mg^{2+} only slightly. Numerical comparison of these two systems showed that, for PtdO/PtdEtn/PtdCho vesicles, synexin reduced the Ca^{2+} concentration necessary for a fusion rate of 5% maximum fluorescence/min from 1.5 to 1.0 mM while, in the case of PtdO/PtdEtn vesicles (i.e., without PtdCho), this Ca^{2+} concentration threshold was reduced from 1.0 to 0.03 mM. These results indicate that PtdCho can diminish drastically the fusion-enhancing effect of synexin on phospholipid vesicles.

The low Ca^{2+} threshold necessary for fusion was maintained when the PtdO content of the vesicles was reduced. The effect of synexin on the fusion of PtdO/PtdSer/PtdEtn/cholesterol (1:2:3:2) vesicles is shown in Fig. 2. In the presence of synexin, PtdO/PtdSer/PtdEtn/cholesterol vesicles could be induced to fuse at Ca^{2+} concentrations similar to the threshold levels required for PtdO/PtdEtn (1:3) vesicles, even though membranes containing PtdSer as the only acidic phospholipid [such as PtdSer/PtdEtn (1:3) vesicles] require considerably higher Ca^{2+} concentrations for fusion (6). The kinetics of fusion in both systems were comparable. It should be noted that PtdO/PtdSer/PtdEtn/cholesterol (1:2:3:2) vesicles are not leaky during fusion. This was shown by adding Ca^{2+} to Tb-containing vesicles (free of EDTA) to initiate fusion in dipicolinic acid-containing medium and subsequently measuring the increase of Tb fluorescence. These findings reinforce our belief that large fusogenic domains are not necessary for fusion, provided that there are no inhibitory lipids such as PtdCho or phosphatidylinositol (21) in the potentially fusogenic sites. The results also indicate that cholesterol, which is one of the major lipids in biological membranes, does not affect synexin facilitation of Ca^{2+}-induced membrane fusion.

The importance of lipid composition in defining the role of

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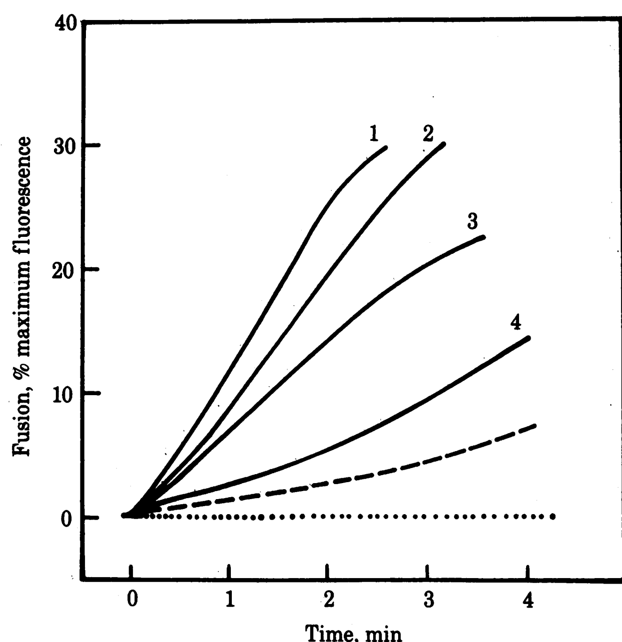

FIG. 2. Time course of Ca^{2+}-induced fusion of PtdO/PtdSer/PtdEtn/cholesterol (1:2:3:2) vesicles. ——, Fusion kinetics of vesicles at various concentrations of Ca^{2+} in the presence of 1.5 mM Mg^{2+} and synexin at 6 μg/ml. Curves: 1, 1.9 mM; 2, 0.4 mM; 3, 0.1 mM; 4, 0.03 mM. – – –, Fusion in the absence of synexin (1.5 mM Ca^{2+}); ······, typical representation of release of vesicle contents during fusion: Ca^{2+} (1.5 mM) was added to induce fusion of Tb-containing vesicles (50 μM lipid, free of EDTA) in the presence of 1.5 mM Mg^{2+}.

synexin was also shown earlier: studies with vesicles composed of various phospholipids indicated that synexin enhances aggregation but not fusion of PtdSer/PtdCho vesicles but enhances fusion of PtdSer/PtdEtn vesicles (6). Moreover, it has been shown that synexin inhibits the fusion of phosphatidylinositol/PtdEtn vesicles (7). Lipid composition, therefore, seems to determine the role of synexin in fusion phenomena. The evidence presented here indicates that, in the presence of Mg^{2+}, synexin is sensitive to lipid specificity even at micromolar concentrations of Ca^{2+}.

In summary, synexin has been shown to facilitate the fusion of phospholipid membranes containing PtdSer, PtdO, or both. This facilitation is Ca^{2+} specific and is evidenced by a drastic decrease in the minimal Ca^{2+} concentration required for fusion. Since vesicle fusion can be induced by Ca^{2+} at concentrations as low as 10 μM in the presence of synexin and Mg^{2+}, it appears that synexin may be involved in Ca^{2+}-induced intracellular membrane fusion events such as exocytosis. The observations reported here and elsewhere (7) on the importance of lipid specificity for expression of the role of synexin in membrane fusion imply that phospholipid domains of appropriate composition might be necessary for any such action of synexin. The potential sites for fusion appear to be determined not only by the intracellular Ca^{2+} concentration but also by synexin and the chemistry and composition of phospholipid polar groups of the membrane. For example, the fusion capacity of membranes containing uniformly distributed PtdCho would be lower than that of membranes that have less PtdCho or membranes in which PtdCho is segregated in domains separate from the potentially fusogenic lipids (PtdSer, PtdO, PtdEtn). The same could apply to membranes containing sphingomyelin instead of or in addition to PtdCho. The mechanism of the effect of synexin in enhancing fusion is not known at present. However, since synexin facilitates membrane fusion of only those phospholipids themselves susceptible to fusion by Ca^{2+}, it seems that synexin does not function by an entirely new mechanism but simply reduces the threshold Ca^{2+} concentration required for fusion. A more detailed study of the interaction of synexin with Ca^{2+} and phospholipids will help in elucidating the molecular mechanism of Ca^{2+}-induced membrane fusion and the role of Ca^{2+} in exocytosis.

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