MODULATION OF MEMBRANE FUSION BY CALCIUM-BINDING PROTEINS

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ABSTRACT The effects of several Ca^{2+}-binding proteins (calmodulin, prothrombin, and synexin) on the kinetics of Ca^{2+}-induced membrane fusion were examined. Membrane fusion was assayed by following the mixing of aqueous contents of phospholipid vesicles. Calmodulin inhibited slightly the fusion of phospholipid vesicles. Bovine prothrombin and its proteolytic fragment 1 had a strong inhibitory effect on fusion. Depending on the phospholipid composition, synexin could either facilitate or inhibit Ca^{2+}-induced fusion of vesicles. The effects of synexin were Ca^{2+} specific. 10 μM Ca^{2+} was sufficient to induce fusion of vesicles composed of phosphatidic acid/phosphatidylethanolamine (1:3) in the presence of synexin and 1 mM Mg^{2+}. We propose that synexin may be involved in intracellular membrane fusion events mediated by Ca^{2+}, such as exocytosis, and discuss possible mechanisms facilitating fusion.

INTRODUCTION

Ca^{2+}-mediated fusion of intracellular vesicles with the plasma membrane is believed to be the key step in exocytosis. Although the importance of Ca^{2+} in secretion is recognized, the central role of Ca^{2+} remains incompletely explained. Studies on the fusion of model membranes have shown that acidic phospholipids and divalent cations are important factors in membrane fusion (for reviews, see Papahadjopoulos et al., 1979 and Düzgüneş et al., 1980). The Ca^{2+}-induced fusion of phospholipid vesicles, such as phosphatidylserine (PS) (Wilschut et al., 1980), and mixtures of PS with phosphatidylethanolamine (PE) or phosphatidylcholine (PC) (Düzgüneş et al., 1981), have been studied extensively. The possible role of phosphate in membrane fusion has been proposed (Fraley et al., 1980). However, the level of Ca^{2+} required to initiate membrane fusion in these model systems is still much higher than that in the case of biological membranes in vitro (Dahl et al., 1979). Proteins are often considered to be involved in the fusion process. Synexin, a Ca^{2+}-binding protein originally isolated from bovine adrenal medulla, is thought to be the cytoplasmic Ca^{2+} receptor in the interaction of chromaffin granules with the plasma membrane at Ca^{2+} concentrations > 6 μM (Creutz et al., 1978).

We have used the fusion assay developed by Wilschut and Papahadjopoulos (1979) to study the kinetics of PS and PS/PE vesicle fusion in the presence of synexin. Synexin increases the rate of fusion appreciably in these two systems (Hong et al., 1981). This significant result has led us to examine further the effect of synexin and other Ca^{2+}-binding proteins on membrane fusion, with the aim of obtaining a membrane model which has the same sensitivity to Ca^{2+} as biological membranes with respect to fusion. We demonstrate here that synexin promotes fusion of phosphatidic acid (PA)/PE vesicles at < 10 μM Ca^{2+}, which is close to the intracellular Ca^{2+} levels necessary for exocytosis (Hodgkin and Keynes, 1957; Llinás and Nicholson, 1975; Baker et al., 1980). It seems likely that synexin may be playing a rather specific role in fusion, inasmuch as several other Ca^{2+}-binding proteins do not facilitate membrane fusion; on the contrary, prothrombin and its fragment 1 have a strong inhibitory effect.

MATERIALS AND METHODS

Lipids

PS was prepared from bovine brain as before (Papahadjopoulos and Miller, 1967; Papahadjopoulos et al., 1977). PA, phosphatidylinositol (PI), and PE transesterified from egg yolk PC were purchased from Avanti Polar Lipids (Birmingham, AL). Each lipid was stored as a chloroform solution in sealed ampules under argon at -40°C. Lipid phosphorus was determined by the method of Bartlett (1959). All the phospholipids used were shown to yield one spot (> 200 μg applied) by two-dimensional thin-layer chromatography on silica gel plates.

Chemicals

$TbCl_3$ was obtained from Alfa Div., Ventron Corp. (Danvers, MA), dipicolinic acid (DPA) from Sigma Chemical Co. (St. Louis, MO), and carboxyfluorescein (CF) from Eastman Kodak Co. (Rochester, NY). CF was purified by recrystallization and subsequently chromatographed on a Sephadex LH 20 column as the Na salt solution and eluted with water at neutral pH. All other chemicals were purchased in the highest purity available.

Preparation of Vesicles

Large unilamellar vesicles encapsulating the desired aqueous contents were prepared by the reverse-phase evaporation technique (Szoka and Papahadjopoulos, 1978) with minor modifications (Wilschut et al., 1980). The internal aqueous contents of the vesicles were as follows: (*a*) Tb vesicles: 2.5 mM $TbCl_3$, 50 mM sodium citrate or 40 mM sodium salt of nitrilotriacetic acid for PI- or PA- containing vesicles; (*b*) DPA

vesicles: 50 mM DPA, 20 mM NaCl; (*c*) carboxyfluorescein vesicles: 50 mM CF. In addition, the media contained 2 mM L-histidine and 2 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), pH 7.4, unless otherwise specified. Vesicles were separated from nonencapsulated material by gel filtration on Sephadex G-75 (elution buffer, 100 mM NaCl, 2 mM L-histidine, and 2 mM TES, pH 7.4). For Tb and DPA vesicles, 1 mM EDTA was included in the elution buffer. CF vesicles were also separated from nonencapsulated material in the same way, except that the EDTA concentration was 0.1 mM instead of 1 mM. The osmotic balance across the bilayers was maintained in all the experiments.

Measurements of Tb and CF Fluorescence

Membrane fusion was followed by measuring the interaction of Tb^{3+} and DPA initially encapsulated in separate populations of vesicles as described in detail by Wilschut et al. (1980). Fluorescence was measured in an SLM-4000 fluorimeter. The Tb/DPA complex was excited at 276 nm; fluorescence was measured through a Corning 3-68 cut-off filter (Corning Glass Works, Corning, NY). The measurements were carried out at 25°C in 1.0 ml of 100 mM NaCl, 2 mM L-histidine, 2 mM TES, and 0.1 mM EDTA. Total lipid concentration was 50 μM. Tb- and DPA-containing vesicles were mixed in a 1:1 ratio, 25 μM phospholipid each. Protein was added as a concentrated solution to the vesicle suspension prior to the introduction of Ca^{2+}. Small aliquots of Ca^{2+} were added as a concentrated solution at time 0. 100% Tb 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 DPA. CF release was monitored by following the fluorescence of CF entrapped initially at a self-quenched concentration (50 mM). The excitation wavelength was 430 nm and emission above 520 nm was detected by using the 3-68 Corning filter. Maximal CF fluorescence was determined by lysing the vesicles with 0.1% Triton X-100.

Proteins

Calmodulin from bovine heart and prothrombin from bovine plasma were obtained from Sigma. Prothrombin fragment 1 was prepared by the method of Owen et al. (1974) and was a generous gift from Dr. Craig M. Jackson of Washington University (St. Louis, MO). Proteins were dialyzed against the same buffer used for the fusion assay. Calmodulin was also isolated from bovine brain (Klee, 1977) and electroplax (Childers and Siegel, 1975). Synexin was isolated as described before (Creutz et al., 1978; Hong et al., 1981) and was stored at pH 6.5 in ice. Protein concentrations were determined by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as standard. Synexin used in most experiments was ~ 80% pure (indicated by SDS polyacrylamide gel electrophoresis).

Other Methods

Free Ca^{2+} concentrations were determined with a Ca^{2+}-selective electrode, operating with a neutral carrier incorporated in a poly (vinylchloride) membrane (Simon et al., 1978). The potential was measured with a digital pH meter (Fisher, Model 420), and millivolts were recorded. The electrode was calibrated in solutions of known Ca^{2+} concentrations.

Aggregation of the vesicles was followed by 90° light scattering at 276 nm or 430 nm during the fusion or release assays, respectively, by means of a second emission channel located opposite the fluorescence channel.

RESULTS

We have found previously that synexin enhanced the rate of Ca^{2+}-induced fusion of PS or PS/PE vesicles (Hong et al., 1981). The rate of fusion depended on the concentration of synexin in the incubation medium. The enhancement of fusion by synexin was Ca^{2+}-specific; Mg^{2+}-induced fusion of PS/PE vesicles (Düzgüneş et al., 1981) was not affected by synexin. We thought that other Ca^{2+}-binding proteins, such as calmodulin or prothrombin, could have a similar effect on membrane fusion. Bull et al. (1972) showed that bovine prothrombin (M_r = 68,900) binds to phospholipid vesicles in the presence of Ca^{2+}. We investigated the kinetics of Ca^{2+}-induced PS vesicle fusion in the presence of various amounts of prothrombin (Fig. 1 *A*). The inital rate and extent of fusion decreased as the concentration of prothrombin was increased. No fusion could be detected at prothrombin concentrations > 7.6 μg/ml.

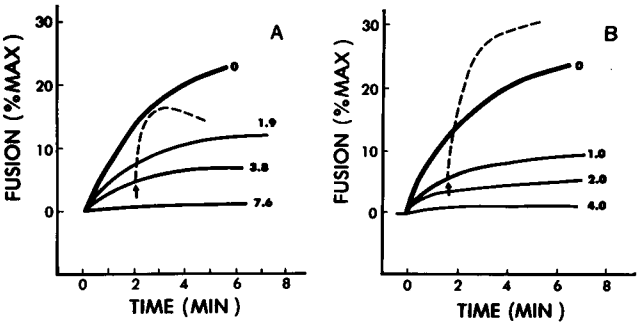

FIGURE 1 Inhibition of PS vesicle fusion by (*A*) bovine prothrombin and (*B*) fragment 1 of bovine prothrombin. Various quantities of protein (μg/ml) as indicated on the curves were added to the vesicle suspension prior to the addition of 2 mM Ca^{2+} Ca^{2+} was added at time = 0. More Ca^{2+} (2 mM) was added to overcome the inhibition at the times indicated by the arrows (broken lines).

Dombrose et al. (1979) demonstrated that bovine prothrombin fragment 1, the NH_2 terminus of prothrombin (M_r = 21,800), can also bind to small unilamellar phospholipid vesicles. We have found that its effect on fusion of PS vesicles was similar to that of prothrombin (Fig. 1 *B*). The same inhibitory effect of these two proteins was also obtained in PS/PE and PA/PE systems (data not shown).

When synexin was included in the fusion reaction, the inhibition of fusion by prothrombin or fragment 1 was less pronounced (Figs. 2 *A* and *B*). The decrease of fluorescence intensity at a later stage of fusion was the result of the release of contents and the entry of Ca^{2+} and EDTA into the vesicles due to the leakiness of the fusion product. The fluorescence intensity could be maintained at a particular level by the addition of EDTA (Fig. 3) to stop the fusion reaction (Wilschut et al., 1980).

Vesicles containing acidic phospholipids other than PS have been shown to undergo divalent cation-induced fusion (Papahadjopoulos et al., 1976; Liao and Prestegard, 1979; Sundler and Papahadjopoulos, 1981) Fig. 3 shows that synexin also facilitated fusion of PA/PE vesicles in the presence of Ca^{2+}. Here the inital rate of fusion was enhanced by approximately three orders of magnitude. Furthermore, the threshold Ca^{2+} concentration was reduced to < 0.1 mM. The threshold level could be

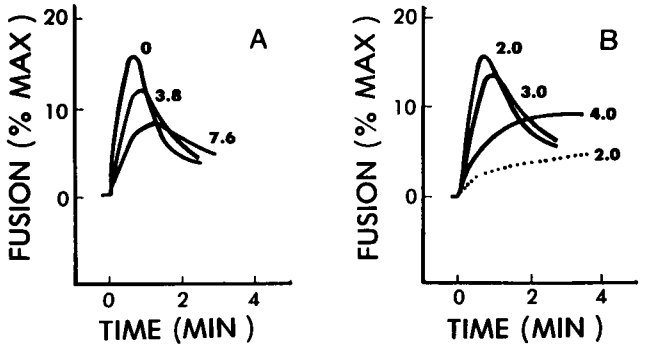

FIGURE 2 The effect of synexin on the fusion of PS vesicles in the presence of (*A*) bovine prothrombin or (*B*) fragment 1 of bovine prothrombin. 1.8 μg/ml synexin together with various quantities (μg/ml indicated on curves) of either prothrombin or fragment 1 were added before the introduction of 2 mM Ca^{2+}. Addition of Ca^{2+} was designated as time = 0. For comparison, fusion in the absence of synexin is shown as the dotted line.

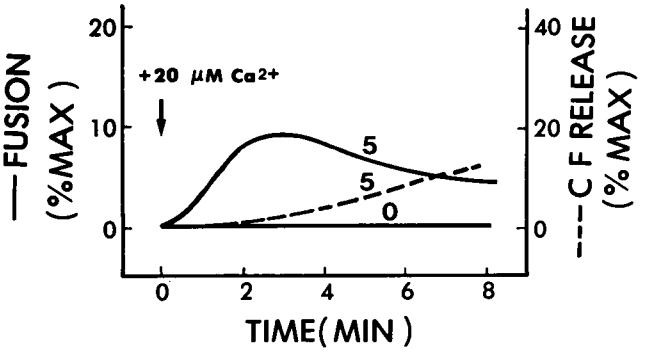

FIGURE 4 The effect of synexin on fusion of, and CF release from (broken line) PA/PE (1:3) vesicles in the presence of 1 mM Mg^{2+}. A low Ca^{2+} concentration (20 μM) was sufficient to initiate fusion in the presence of synexin (5 μg/ml). The final free Ca^{2+} concentration was determined by a Ca^{2+}-selective electrode. pH = 7.0.

reduced further by including Mg^{2+} at a concentration insufficient to induce fusion by itself.

Fig. 4 shows the kinetics of fusion upon addition of 20 μM Ca^{2+} to PA/PE vesicles preincubated with 1 mM Mg^{2+} and 5 μg/ml synexin. No increase in fluorescence intensity could be detected during the preincubation. Following the addition of Ca^{2+}, rapid fusion was obtained, indicated by the increase of Tb fluorescence. The decrease in the fluorescence signal after 3 min could be accounted for by the subsequent release of vesicle contents shown by the slow increase in CF fluorescence measured in parallel experiments (Fig. 4, broken line). Initial rates of fusion induced by different Ca^{2+} concentrations in this system are shown in Fig. 5. 1 mM Ca^{2+} was required to initiate fusion of vesicles at the rate of 2% maximum fluorescence/min, whereas in the presence of synexin < 20 μM Ca^{2+} was sufficient to reach the same rate. In contrast, calmodulin from bovine heart inhibited Ca^{2+}-induced fusion.

We have shown that pure PI vesicles only aggregated and did not fuse in the presence of Ca^{2+} (Sundler and Papahadjopoulos, 1981), but PI/PE vesicles fused at a concentration of Ca^{2+} above the threshold for aggregation (Sundler et al., 1981). Therefore PI was found to be unique among the acidic phospholipids in being resistant

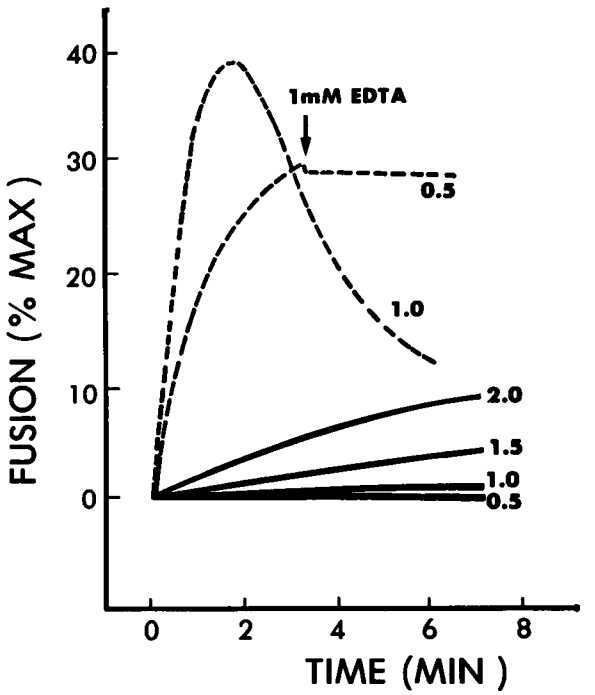

FIGURE 3 Time-course of Ca^{2+}-induced fusion of PA/PE (1:3) vesicles. The effect of various concentrations of Ca^{2+} (mM) on vesicle fusion in the presence (6.2 μ/ml, broken lines) and absence (solid lines) of synexin. One mM EDTA was added to stop the fusion reaction. Ca^{2+} was added at time = 0. pH = 7.0.

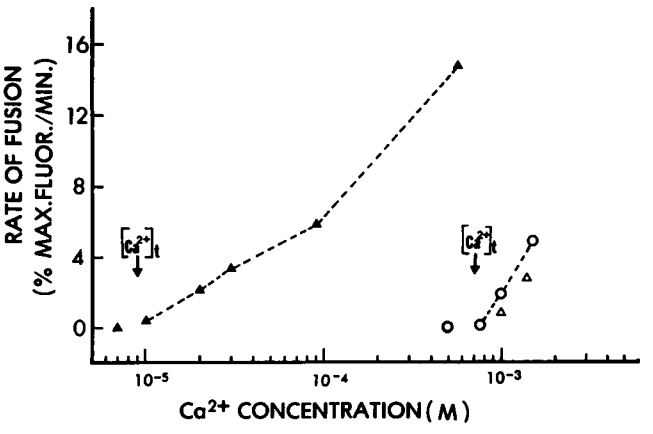

FIGURE 5 Initial rate of fusion of PA/PE (1:3) vesicles vs. the concentration of Ca^{2+} in the absence of protein (open circles). 1 mM Mg^{2+} was included in all experiments. Vesicles were preincubated with either 4 μg/ml calmodulin (open triangles) or 5 μg/ml synexin (solid triangles) in each experiment before the addition of various amounts of Ca^{2+} at time = 0. Free Ca^{2+} concentrations were determined by a Ca^{2+}-selective electrode. pH = 7.0.

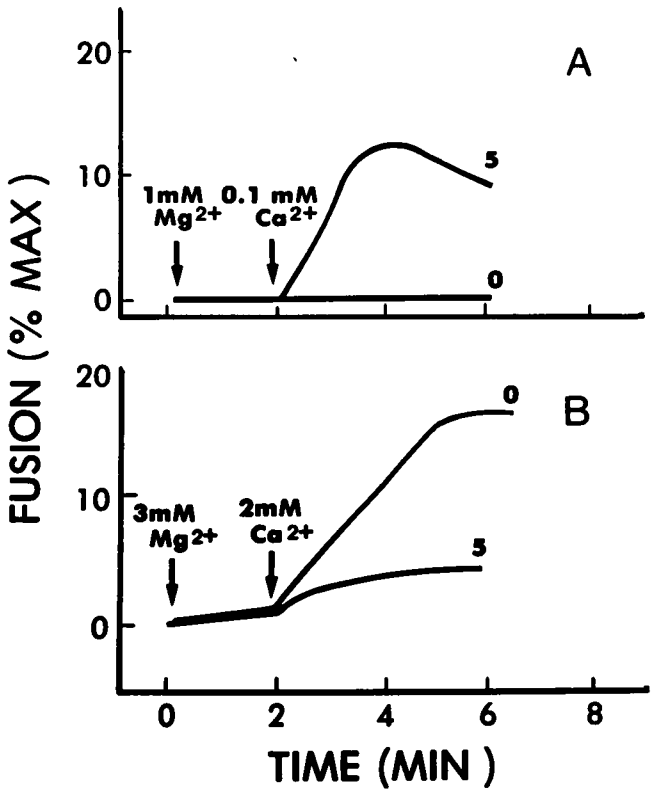

FIGURE 6 The effect of synexin (5 μg/ml) on fusion of (*A*) PA/PE (1:3) and (*B*) PI/PE (1:3) vesicles. Arrows indicate the order of the addition of divalent cations. pH = 7.0. Because there was 0.1 mM EDTA in the buffer, the free Ca^{2+} concentration in *A* was determined by C^{2+}-selective electrode as 0.03 mM.

to fusion. We investigated whether synexin facilitated the fusion of PI/PE (1:3) vesicles (Fig. 6). These vesicles exhibited a very slow rate of fusion when 3 mM Mg^{2+} was added. Upon introduction of 2 mM Ca^{2+}, rapid fusion was observed. When synexin was included in the incubation medium, however, the rate and extent of Ca^{2+}-induced fusion was drastically reduced (Fig. 6 *B*). In comparison, PA/PE vesicles exhibited an enhancement of fusion (Fig. 6 *A*). Lower concentrations of Ca^{2+} and Mg^{2+} were used here since fast fusion could take place in this system, even without synexin, at the divalent cation concentrations used for the PI/PE vesicles. The threshold concentrations of Ca^{2+} required for fusion in different membranes are summarized in Table I.

TABLE I
$[Ca^{2+}]_t$ FOR VARIOUS MEMBRANES*

Phospholipid composition	Ca^{2+} only		Ca^{2+} in presence of Mg^{2+}	
	−Synexin	+Synexin	−Synexin	+Synexin
PS	2.0	1.0	—	—
PS/PE (1:3)	2.5	1.0	1.0‡	0.5‡
PA/PE (1:3)	1.0	0.1	0.7§	0.09§
PI/PE (1:3)	2.0	3.0	1.0‡	2.0‡

*$[Ca^{2+}]_t$ is defined as the threshold concentration of Ca^{2+} (mM) required for fusion. $[Ca^{2+}]_t$ was obtained by extrapolating the lower section of the plot of fusion rate vs. Ca^{2+} concentration to zero rate (shown in Fig. 5). At this point, a small increment of Ca^{2+} would start the fusion reaction.
‡3 mM Mg^{2+} was present in the reaction.
§1 mM Mg^{2+} was present in the reaction.

DISCUSSION

Studies on isolated secretory vesicles have implied that proteins are involved in Ca^{2+}-dependent fusion processes (Dahl et al., 1979; Gratzl et al., 1980). Fusion of certain paramyxoviruses with cell membranes is thought to be mediated by a viral glycoprotein (Scheid et al., 1974). Synexin mediates the aggregation of isolated chromaffin granules at Ca^{2+} concentrations > 6 μM (Creutz et al., 1978). Synexin also induces the aggregation of PS vesicles (Morris and Hughes, 1979). In addition, we have demonstrated that synexin enhances the rate of fusion of PS or PS/PE vesicles (Hong et al., 1981). By fusion we refer to the communication between the internal aqueous space of two vesicles and not the pentalaminar structure observed in electron micrographs of secretory cells (Palade, 1975), which we consider as the first step toward fusion.

Ca^{2+}-induced membrane fusion is critically dependent on the phospholipid composition (Papahadjopoulos et al., 1974; Düzgüneş et al., 1981). PC, but not PE, is extremely inhibitory in Ca^{2+}-induced fusion of PS-containing vesicles. Phospholipid specificity is also observed for the action of synexin. Synexin facilitates aggregation of most vesicles containing acidic phospholipids, but its effect on fusion varies from enhancement to inhibition, depending on the particular lipid composition (Table II).

Ca^{2+}-binding proteins other than synexin, for example, calmodulin (from bovine brain, electroplax, or bovine heart) and parvalbumin (from rabbit muscle), slightly inhibit Ca^{2+}-induced membrane fusion (Fig. 5 and Hong et al., 1981). Although calmodulin does not enhance Ca^{2+}-induced fusion of phospholipid vesicles, it could be modulating the activities of certain enzymes that may participate in membrane fusion. In this study we found that prothrombin and its proteolytic fragment 1, which retains the Ca^{2+}-binding activity of the parent protein, also reduce the initial rate and extent of membrane fusion to a larger degree than calmodulin (Fig. 1). Both prothrombin and fragment 1 have 10 γ-carboxylated glutamic acid residues which bind 6–8 Ca^{2+} per protein molecule.

TABLE II
THE EFFECTS OF SYNEXIN ON DIFFERENT PHOSPHOLIPID SYSTEMS

Phospholipid	Aggregation	Fusion
PS/PE, PA/PE, PS	Enhanced	Enhanced
PS/PC	Enhanced	No effect
PI/PE	Enhanced	Inhibited

Dombrose et al. (1979) have suggested that the Ca^{2+}-binding domains of these proteins may produce a high local charge density and thus provide a situation for electrostatic interaction with cations and other charged surfaces. This type of interaction is clearly not sufficient to induce fusion since these proteins are inhibitory. It appears that the orientation and surface structure of the protein, upon binding to the phospholipid membrane surface, may determine whether it will facilitate intermembrane contact, destablization of the membrane, and fusion.

The inhibition of fusion by prothrombin or fragment 1 (Fig. 1) may be rationalized by the supposition that the exposed surface of the protein after binding to one membrane is unable to interact with another membrane. Such monopolar configuration with respect to Ca- (and membrane-) binding is indicated by the primary structure of the protein and the observation that prothrombin or fragment 1 self-associates, probably in the form of dimers (Jackson et al., 1979). Moreover, the interaction of fragment 1 with phospholipid vesicles (Dombrose et al., 1979) does not result in vesicle aggregation.[1]

Synexin binds to isolated secretory vesicles and self-associates in the presence of Ca^{2+} in the form of extended rods (Creutz et al., 1979), indicating a bipolar configuration with respect to calcium-induced associations. Since the rate of fusion of PS/PE vesicles shows first-order dependency on synexin concentration (Hong et al., 1981), we conclude that synexin binds to (and induces aggregation and fusion of) phospholipid vesicles as a monomer. This conclusion is substantiated by the observation (Fig. 5) that synexin promotes fusion of PA/PE vesicles at a concentration of Ca^{2+} insufficient for self-association (Creutz et al., 1979). Following the above arguments, we envisage that the synexin monomer, due to its bipolar nature, can bind simultaneously to two vesicles, inducing their aggregation and fusion reaction.

In preliminary studies, we have found that > 90% of synexin binds to PS vesicles at Ca^{2+} concentrations above threshold. Assuming that the protein is spherical and is hexagonally close-packed on the surface of a 1,000 Å-diameter vesicle, it would cover an area equivalent to 23 phospholipid molecules. At a concentration of 5–10 μg/ml, synexin would cover 10–20% of the vesicle surface. This concentration range would correspond to ~ 100–200 synexin molecules per vesicle. Fusion is totally inhibited by prothrombin at a lipid:protein ratio ~ 500:1 (Fig. 1) at which point only 10% of the total surface of the vesicle will be covered by the protein. Addition of more Ca^{2+} (Fig. 1) or synexin (Fig. 2) can partially overcome this effect. Thus, synexin either competes with prothrombin for binding sites on the membrane surface or directly interacts with the available lipid surface. The inhibitory effect of prothrombin remains even in the presence of synexin. This would indicate that synexin cannot remove completely the bound prothrombin (or fragment 1) from the membrane surface.

At present, the molecular mechanism of synexin's effect in enhancing fusion is not known. Nevertheless, since synexin preserves the lipid specificity for Ca^{2+}-induced fusion of phospholipid vesicles, it is possible that a similar physicochemical principle underlies both phenomena. The enhancement of fusion could be dependent on the close proximity of the synexin-lipid interface, possibly involving anhydrous Ca^{2+} complexes. Such tight complexes between synexin and two interacting vesicles could reduce the energy of activation for the mixing of lipids in the domains immediately adjacent to these complexes. The chemistry of the phospholipid headgroup could determine whether synexin's association with the vesicles will simply enhance aggregation or, in addition, facilitate fusion. For instance, the phosphate groups of PI could be protected by the bulky and highly hydrated inositol groups from forming a complex with synexin favorable for fusion (Fig. 6). As synexin binds to PI/PE vesicles it may serve only as a spacer in between bilayers and inhibit fusion. In contrast to PI, the phosphate groups of PA are directly exposed on the membrane surface. The dramatic effect of synexin on PA/PE fusion may reflect a direct interaction of synexin with the phosphate groups of PA through Ca^{2+} binding, thus forming a complex highly favorable for fusion. The lipid specificity of synexin is also evident by the completely different effects on PS/PE and PS/PC vesicles. In the latter case, in which synexin induces aggregation but no fusion, the bulky choline groups of PC may be inhibiting the formation of a tight complex between synexin and the negatively charged groups of PS. Under these conditions, it is unlikely that synexin can induce a phase separation of PS from PC, because this would have resulted in fusion between these vesicles. Although the nature of Ca^{2+}-induced synexin-lipid interaction is not completely known, the conformation of synexin is critical inasmuch as heat denaturation (90°C for 2 min) totally eliminates the facilitation. The inhibitory effect of prothrombin and its fragment 1 is also reduced by the same heat treatment.

Mg^{2+} induces fusion of PS/PE (Düzgünes et al., 1981) or PA/PE (Sundler et al., 1981) vesicles at higher concentrations than Ca^{2+}. If Mg^{2+} is the only divalent cation present, synexin is ineffective in facilitating fusion in these membrane systems (Fig. 4 and Hong et al., 1981). This is in agreement with previous observations that synexin-induced chromaffin granule aggregation or synexin self-association is Ca^{2+}-specific (Creutz et al., 1978 and 1979). The observation that synexin lowers the Ca^{2+} threshold for PA/PE fusion to a larger extent in the presence of Mg^{2+} is important physiologically. This enhancing effect of Mg^{2+} could result from the reduction of electrostatic repulsive forces among vesicles or its direct participation in fusion. However, the mechanism of action of Mg^{2+} is not fully

[1]Hong, K., N. Düzgüneş, and D. Papahadjopoulos. Unpublished observations.

understood at present. Synergistic effects of Ca^{2+} and Mg^{2+} have been observed before, even in the absence of synexin (Portis et al., 1979; Düzgüneş et al., 1981; Wilschut et al., 1981).

In summary, synexin facilitates the fusion of certain types of phospholipid membranes, unlike the other Ca^{2+}-binding proteins we have studied. The degree of facilitation depends on the phospholipid composition of the membranes, being greater for PA/PE than for PS/PE vesicles. In contrast, synexin inhibits to the fusion of PI/PE membranes. Inasmuch as vesicle fusion can be induced by Ca^{2+} concentrations as low as 10 μM in the presence of synexin and Mg^{2+}, it seems likely that synexin is involved in Ca^{2+}-induced membrane fusion events in biological systems. The role of Ca^{2+} in exocytosis could therefore be ascribed to its interaction with synexin and membranes of specific phospholipid composition. The conversion of PI to PA during stimulation of secretory events, known as the "phospholipid effect" (Michell, 1975; Hawthorne and Pickard, 1979) could provide additional regulatory control for membrane fusion. As the intracellular Ca^{2+} concentration rises to a threshold level during stimulation of the cell, synexin would induce fusion of only those membranes that conform to the required lipid specificity (i.e. the cytoplasmic surfaces of the plasma membrane and the secretory vesicles). In addition, the frequency of fusion events at particular intracellular sites could be controlled by the proximity to local Ca^{2+} gradients and by other recognition ligands associated with the various membranes.

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DISCUSSION

Session Chairman: Franklyn G. Prendergast *Scribes*: Maria D. Suárez-Villafane and Debra A. Thompson

WEINSTEIN: Does current information on the primary, secondary, tertiary, and perhaps quaternary structure of synexin give any clues as to the mechanism by which fusion is promoted?

PAPAHADJOPOULOS: We are not able to say much about the mechanism at the moment. However, we have a couple of hints, which are the following: From the work of Creutz, Pazoles, and Pollard, it looks as though synexin aggregates with Ca^{2+} to form long rods, indicating a bipolar type of association, that is, a head-to-tail type of association. This associated form might interact with two individual vesicles, bringing them into closer contact than would normally be allowed by the electrostatic and hydration repulsive forces. By comparison, prothrombin, at least prothrombin fragments, forms dimers in the presence of Ca^{2+}; dimerization may indicate a monopolar type of protein structure, which could interact with one vesicle only.

Since we have been able to establish calcium-specific and lipid-specific roles for synexin, our observations might now induce enough enthusiasm to look into more details of the mechanism in the future.

CREUTZ: We don't have any information about amino-acid sequence. However, synexin is not a strongly basic protein, as one might have expected because you are seeing effects with acidic phospholipids while we are looking at negatively charged chromaffin granules. In terms of quaternary structure and the formation of rods, it is interesting that the polymerization is fairly temperature-insensitive, but the aggregation, at least in chromaffin granules, is strongly temperature-dependent. Have you noticed any temperature dependence of the phospholipid aggregation by synexin, and do you have any explanation for why chromaffin granules show such a strong temperature dependence?

PAPAHADJOPOULOS: No, we don't have an extensive temperature study.

In comparison with your work, the Ca^{2+} concentrations at which we observe effects on vesicle fusion would not induce aggregation between synexin molecules, so we feel that probably it is the monomer that interacts with vesicles. Would you agree?

CREUTZ: It doesn't appear that you have a curve complete enough to answer that. Your curves go off scale, but you do titrate over a broad range. When you initially see effects of synexin you are at ~10 μM Ca^{2+}, and you continue to see effects as you go through 100 μM Ca^{2+}. It looks as though you are titrating the same site on the synexin molecule as is titrated when the synexin molecule undergoes self-association. It seems to me you could have a small amount of the polymer form at those low Ca^{2+} concentrations, and that it may indeed be the polymer form that interacts.

HONG: We think synexin may self-associate at the higher Ca^{2+} concentrations in the absence of liposomes. Our recent experiments show that at 10–50 μM Ca^{2+}, 70% of synexin is associated with the vesicles, while at higher Ca^{++} almost 95% of synexin we added to the fusion sample associated with the membrane. Synexin does have a high affinity for acidic liposomes in the presence of Ca^{2+}.

CREUTZ: One mechanism that we have thought of is that the monomer binds first, and then it may be the polymerization reaction that leads to aggregation of the vesicles.

DÜZGÜNES: Regarding that point, if the synexin molecules stacked onto one another, would that act as a spacer between the vesicles, or would it bring them closer together?

CREUTZ: It would depend whether some of the synexin rod or polymer might be able to hide in the bilayer.

McINTYRE: You report the lipid concentrations as being 50 μM under these conditions and your protein concentration in micrograms per milliliter. What is the molar concentration of synexin and what is the number of synexin molecules/lipid vesicle?

HONG: The concentration of synexin used was in the range of 5–10 μg/ml. According to the average size of vesicles, the ratio of synexin to the number of vesicles is 100–200 synexin molecules/vesicle. For certain membrane systems we used ~2 μg of synexin/ml of 50 μM lipid suspension, so the ratio is 50 synexin molecules/vesicle. In terms of lipid:synexin, the ratio is ~1,200 lipid molecules/synexin.

POLLARD: One of the basic motifs I hear is the idea that Ca^{2+} is effecting some sort of dehydration, yet the original idea was that Ca^{2+} acts on synexin and the activated complex is doing the work.

How do you distinguish between the two possibilities?

PAPAHADJOPOULOS: First of all, I think that any mechansim of membrane fusion will have to take into account the hydration layers. I don't think we can get around that. Ca^{2+} would be an excellent agent for doing that.

What we are proposing is that Ca^{2+} is probably producing complexes between synexin and the phospholipid surface. In producing these complexes it is also dehydrating that region.

POLLARD: Yes, so if dehydration occurs at a relatively high concentration in the ambient, bulk phase, and if fusion is occurring at a relatively lower Ca^{2+} concentrations, then probably the observed hydration effect occurring in the test tube with lipids and Ca^{2+} is not happening when synexin is around.

PAPAHADJOPOULOS: Oh yes, there is "magic" involved there. It is not easy to explain that difference. The reason why we are optimistic is that a protein that acts as a catalyst creates local effects.

Hydration becomes a local phenomenon at the interface between the protein and lipid. There, the required concentration of Ca^{2+} is lower because of different binding properties of that particular domain. You could have the local dehydration effects under conditions where globally the lipid itself would not be dehydrated because of its lower affinity for Ca^{2+}.

MORRIS: Although Ca^{2+} works very well in your system with synexin at ~20 μM, what about the ~1.5 mM Mg^{2+} that allows the Ca^{2+} to work so well? Are you simply reducing the surface potential and charge on the membrane to allow the Ca^{2+}-mediated synexin binding to hold the particles together long enough to fuse them?

PAPAHADJOPOULOS: Your comment is apt, that is exactly what is happening. What we are saying is that Mg^{2+} works globally, i.e., it acts because of its high concentration with most of the phospholipid charges, and that effect is always also necessary to bring the vesicles close enough together. Still, only Ca^{2+} can operate locally by producing a new domain structure between the protein and the lipid.

DÜZGÜNES: In pure phospholipid systems you don't need to neutralize the overall surface charge totally in order to get fusion. In PS vesicles, for example, you can induce fusion at 1 –2 mM Ca^{2+} where the membrane still has a residual negative surface potential.

POLLARD: The Mg^{2+} data don't really mean that Mg^{2+} somehow reacts with synexin, or that it blocks surface charge. There is already a significant amount of buffering of ionic charges in your system. It is difficult to believe that ~1 mM Mg^{2+}, which is not reacting with synexin, is going to affect charges unaffected by your 100-mM buffer system.

PAPAHADJOPOULOS: The actual binding of Ca^{2+} and Mg^{2+} has been determined by us and by others, so we know exactly how much Mg^{2+} we have at the surface. Under these conditions a good majority of the negative charge would be neutralized by Mg^{2+}. The vesicles would be able to come into closer contact. There is no argument about that.

We have also seen this synergistic effect of Ca^{2+} and Mg^{2+} without the protein, so we are not surprised to see it in the presence of the proteins.

WEINSTEIN: Does the phospholipid head group specificity of the fusion promoted by synexin and calcium correlate well with the spontaneous Ca^{2+}-induced phase separation or spontaneous Ca^{2+}-induced fusion?

PAPAHADJOPOULOS: In all cases we have studied, synexin will not induce fusion between vesicles unless the vesicles themselves would fuse spontaneously at much higher Ca^{2+} concentrations. There is only a lower minimal Ca^{2+} concentration required. There is no change at all in the lipid specificity. Synexin is not introducing a new mechanism for lipid-lipid interactions.

POLLARD: It just dawned on me that PI is one of the phospholipids in the chromaffin granules and that specifically localized in those granules is an enzyme that phosphorylates PI to make phosphoinositides. This behavior may relate to the "phospholipid effect" mentioned in your paper. Is it possible that the acidic phosphatidylinositols can become the potential substrates for fusion?

PAPAHADJOPOULOS: We have begun some experiments to answer that question, but we don't have any answer yet.

BLUMENTHAL: According to Robert MacDonald, the Tb^{3+} and DPA rapidly come out of the vesicle to produce fluorescent energy transfer; according to him the slow unquenching of the carboxyfluorescein might be due to the slow diffusion out of the vesicle aggregate. What do you see if you add DPA outside the vesicle and the Tb^{3+} inside and then look at the rate of energy transfer under your fusion condition?

PAPAHADJOPOULOS: These are experiments we have done recently in response to MacDonald's comments. Under the proper conditions the leakage of Tb^{3+} out to react with DPA adds negligibly to the fluorescence that you see when you have Tb^{3+} inside and DPA inside.

PARSEGIAN: The one parameter that is used in your assay is the maximum-fluorescence peak between the two rapid processes of the onset of fluorescence and its decay. The heights of such peaks are notoriously sensitive to very small changes in either contribution.

Yet it is virtually axiomatic in the way you describe the data that the height of that peak is synonymous with fusion as we understand it, that is, with mixing of vesicle contents. Are we ready for such an axiom?

PAPAHADJOPOULOS: No, actually we don't think we do that because of the same reason you mentioned.

We concentrate on initial rates and the very early rise in fluorescence. By following independently the release of contents we can systematically show that under these conditions the initial leakage of contents is not contributing very significantly. Also, by using EDTA to quench the reaction we find that at the initial times, after the addition of Ca^{2+}, the fluorescence is completely fixable with EDTA. This indicates that the reaction occurred inside the vesicles. The EDTA would otherwise quench the reaction. If you used the peak of fluorescence as an indication there would be trouble in leaky systems. You have to establish how leaky the system is. It turns out that in systems composed of a mixture of phospholipids, leakage is much less severe even at later times. Most of our calculations involve initial rates and under these conditions I am confident that the contribution of leakage is not substantial.

MORRIS: In reference to possible leakage of Tb^{3+} out of the vesicles, there have been numerous NMR studies employing lanthanides as differential shift reagents for inner and outer groups in vesicles which have revealed little or no leakage on a time-span that is much longer than that used in the kinetic studies described in this paper.

You report the effects of a variety of agents on the rate of vesicle fusion. What is the rate-limiting step in vesicle fusion?

PAPAHADJOPOULOS: In the systems we have been studying up to now, aggregation is rate-limiting and fusion is much faster than aggregation. It was an important point to establish because in some biologically-relevant fusion phenomena, for example, exocytosis at the synaptic nerve terminal, it is well known that fusion events follow within microseconds after membrane depolarization.

What we are interested in knowing is if vesicle fusion induced by Ca^{2+}

is a fast process or a relatively slow process. The answer at this time is that it is a fast process and that the rate-limiting step is in the rate of aggregation.

MORRIS: When Duncan Haynes and I studied aggregation rates of chromaffin granules, we found that they were practically diffusion-controlled, whereas those of the the PS vesicles are obviously not. Does the synexin improve the aggregation rates, or is it simply enhancing fusion after the particles are together? Have you done any experiments that address this question?

PAPAHADJOPOULOS: No, that's a very good question. We don't have the answer yet. It is possible to analyze the result kinetically and actually derive that answer. We are very interested in knowing whether synexin enhances the aggregation or whether it increases the actual rate of fusion.

WEINSTEIN: Are there major differences in the systems between small and large vesicles? That is, has much of the work been done with large reverse-phase-evaporation vesicles, or with the small sonicated ones?

PAPAHADJOPOULOS: We have progressed into the large unilamellar veiscles because they have advantages. However, the comparisons have been made now relating fusion kinetics and thresholds of Ca^{2+} concentration.

The most relevant comparison is seen looking at fusion of pure PS vesicles in the presence of Ca^{2+} and Mg^{2+}. Sonicated vesicles will aggregate and fuse at lower concentrations of Ca^{2+}. We feel that that is in reasonable agreement with the higher curvature allowing smaller vesicles to come into closer contact.

WEINSTEIN: So, qualitatively, are the specificities similar or are there major differences, i.e., in PA vs. PS findings?

PAPAHADJOPOULOS: The differences have not been studied in all the phospholipid systems. There is one spectacular difference: the effect of Mg^{2+} on the small vesicles. Small sonicated vesicles will fuse rather slowly with Mg^{2+} compared to their fusion with Ca^{2+}. The vesicles do fuse in the presence of Mg^{2+}, but that fusion process is relatively leaky compared to Ca^{2+}-induced fusion; and they will fuse only until they reach a size of ~700 –1,000 Å. Highly curved vesicles will fuse with Mg^{2+} to a limited degree but will finally stop fusing. Larger vesicles in Mg^{2+} will simply aggregate but there will be no fusion.

POLLARD: Let us consider whether osmotic gradients are involved in the fusion driving force. Can osmotic gradients be part of the driving force that you consider?

PAPAHADJOPOULOS: In our systems there is no osmotic difference so we have settled the question as to whether the osmotic difference is needed. We have not studied up to now any additional effect of an imposed osmotic change in our system.

T. THOMPSON: If there is a phase change on the lipid induced by the addition of Ca^{2+}, then there should be a shrinkage of the area/lipid molecule and that shrinks the internal volume by as much as 30%. Water can move freely across the bilayer, but if there is any salt involved it can't, so that there can be an osmotic gradient generated at that point.

We have just looked at this phenomenon using the carboxyfluorescein partial-quench system (D. Lichtenberg et al. 1982. *Biochim. Biophys. Acta.* In press). You can in fact force larger vesicles to go through the phase change and back and watch this internal volume change very easily. It comes to ~25% using this method, vs. a theoretical expectation of 30%.

There must be an osmotic pressure gradient generated in that instance in our systems.

BLUMENTHAL: Was that for small unilamellar vesicles?

T. THOMPSON: No, it was with 700-Å dipalmitoyl phosphatidylcholine vesicles. As far as I know, it doesn't work with small vesicles. You don't see this effect.

PRENDERGAST: Do you have any independent evidence from quasi-elastic scattering or any other physical measurement, that synexin associates with the membranes in the absence of divalent cations or with Mg^{2+}? Or does it require both Mg^{2+} and Ca^{2+}?

PAPAHADJOPOULOS: We have some chemical evidence indicating that synexin can be spun down with the vesicles in the presence of Ca^{2+}. It is removed from the supernatant in the presence, but not in the absence, of Ca^{2+}.

DEBER: Sometimes the addition of protein to vesicles causes them actually to become more permeable to cations. This has been established by some NMR studies with paramagnetic ions. Does synexin itself make individual vesicles permeable? Could some of your results be due to possible mixing of the two *labels* of Tb^{3+} and the fluorescent component actually outside of the vesicles rather than inside?

PAPAHADJOPOULOS: We have cases of vesicles with added lecithin where the vesicles aggregate in the presence of synexin and Ca^{2+} but not fuse. Those vesicles are not leaky.

I think, in most cases, there is no indication that synexin induces an independent leakage mechanism. The fusion events that are induced by synexin are to a certain extent leaky but we have not seen leakiness independent of fusion.