Calcium-dependence of synexin binding may determine aggregation and fusion of lamellar bodies

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Synexin (annexin VII) is a member of the annexin family of calcium and phospholipid binding proteins that promote calcium-dependent aggregation and fusion of lipid vesicles or secretory granules. We have previously suggested that synexin may be involved in membrane fusion processes during exocytosis of lung surfactant since it promotes fusion *in vitro* of lamellar bodies with plasma membranes. In this study, we characterized calcium-dependency of synexin binding to lamellar bodies and plasma membranes, since such binding is the initial, and, therefore, may be the rate-limiting step in membrane aggregation and fusion. The binding of biotinylated synexin to lamellar bodies and plasma membranes increased in a calcium-dependent manner reaching a maximum at approx. 200 μM Ca^{2+}. Binding to lamellar bodies was completely inhibited by unlabelled synexin. Gel-overlay analysis showed that synexin bound to an approx. 76 kDa protein in the lamellar body and plasma membrane fractions. The calcium kinetics were noticeably similar for synexin binding to lamellar bodies and plasma membranes, aggregation of lamellar bodies, and fusion of lamellar bodies with lipid vesicles. At low calcium concentrations, aggregation of lamellar bodies could be increased with increasing synexin concentration, and arachidonic acid increased all three parameters (binding, aggregation, and fusion) in a similar manner. The effects of calcium and arachidonic acid on these three parameters suggest that synexin binding to lamellar bodies may be a rate-determining step for fusion during surfactant secretion. Furthermore, at near physiological calcium levels, the membrane fusion may be enhanced by elevated concentrations of synexin and poly-unsaturated fatty acids.

INTRODUCTION

Lung lamellar bodies are the secretory organelles for lung surfactant. During surfactant secretion, the limiting membrane of these organelles must fuse with the plasma membrane to release their contents into the alveolar lumen. Our previous studies *in vitro* have shown that synexin (annexin VII) promotes fusion of lamellar bodies with plasma membranes [1]. Furthermore, our previous studies showing inhibition of lung surfactant secretion in alveolar type II cells and of synexin-dependent membrane fusion by stilbene disulphonic acids have suggested a physiological role for synexin in surfactant secretion [2,3].

Synexin shares the common characteristic of calcium-dependent binding to acidic phospholipids and biological membranes with the other twelve members of annexin family [4]. Annexins are characterized by the presence of 4 or 8 repeats of conserved 70 amino acid sequence in the C-terminus region which form the core of the protein and are hypothesized to bind multiple calcium ions [5]. The existence of such multiple putative calcium/phospholipid binding domains has been suggested to underlie the ability of these proteins to aggregate phospholipid vesicles and biological membranes [5].

Although precise physiological roles for annexins have yet to be defined [6,7], a number of studies have suggested that synexin may be involved in membrane fusion during exocytosis [1,2,3,6]. Further evidence is derived from the ability of synexin to insert in membranes and form voltage-dependent calcium channels *in vitro* [8]. The postulated scheme of synexin mediated membrane fusion involves: (1) self-association of synexin, and (2) binding to the membranes, that is followed by (3) aggregation, and (4) fusion of membranes. Previous investigators have suggested aggregation to be a rate limiting step in the membrane fusion process [9]. Since self-association of membrane-bound synexin may differ from that of the soluble synexin [10], we postulated that synexin binding, rather than membrane aggregation, may be the primary and rate limiting step for membrane fusion. In this study we compared the calcium-dependence of synexin binding to lamellar bodies with the synexin-mediated aggregation of lamellar bodies and synexin-dependent fusion of lamellar bodies with lipid vesicles. Calcium kinetics for all three parameters suggest that synexin binding to biological membranes may be the initial calcium-dependent rate limiting step in the process of membrane fusion. Parts of these studies have been presented in abstract form [11].

MATERIALS AND METHODS

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylcholine (NBD-PC) and *N*-(lissamine rhodamine B sulphonyl)phosphatidylethanolamine (Rh-PE) were obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). ELISA plates (Falcon Pro Bind) were from Fisher Scientific (Philadelphia, PA, U.S.A.). Avidin D–horseradish peroxidase (avidin–-HRP; 1.5 mol HRP/mol of avidin) was from Vector Laboratories (Burlingame, CA, U.S.A.). Fluoreporter Mini-Biotin-XX protein labelling kit (F-6347) was from Molecular Probes (Eugene, OR, U.S.A.). Gel electrophoresis reagents and the tetramethylbenzidine peroxidase immunoassay substrate kit were from Bio-Rad Laboratories (Richmond, VA, U.S.A.). Biotinylated-BSA and all other reagents were from Sigma

Abbreviations used: HRP, horseradish peroxidase; NBD-PC, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Rh-PE, *N*-(lissamine rhodamine B sulphonyl)phosphatidylethanolamine.

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Chemical Company (St. Louis, MO, U.S.A.). Sephacryl S-200 was from Pharmacia (Piscataway, NJ, U.S.A.).

Purification and assay of synexin

Synexin was purified from bovine lungs as described previously [1] with slight modifications. Briefly, lungs were powdered and suspended in 300 mM sucrose for 60 min. The suspension was centrifuged for 30 min at 20000 ***g*** and then for 60 min at 100000 ***g***. The supernatant proteins were precipitated twice with 22 % and 20 % (w/v) $(NH_4)_2SO_4$ respectively, and resuspended in 300 mM sucrose/40 mM histidine/2 mM EGTA buffer, pH 6.0, containing 0.02 % (w/v) sodium azide. Synexin-enriched (> 90 % pure) fractions were obtained after three passes through a Sephacryl S-200 column. Synexin activity was assayed by following the aggregation of PC/PS (3:1, w/w) liposomes at 540 nm as described previously [1].

Isolation of subcellular fractions

Lamellar bodies and lamellar-body membranes were isolated from rat lung as described previously [12,13]. Plasma membranes were isolated from rat lung homogenate in 10 mM phosphate buffer containing 0.32 M sucrose, 1 mM $MgCl_2$, 30 mM NaCl, 5 μM PMSF, 0.02 % NaN_3 and 10 μg/ml DNase according to Belsham et al. [14] as described previously [1]. Briefly, the homogenate was layered over a discontinuous gradient consisting of 10 ml of 1.2 M and 5 ml each of 0.9 M, 0.7 M and 0.5 M sucrose. After centrifugation for 1 h at 95000 ***g***, the plasma-membrane enriched interface between 1.2 M and 0.9 M was diluted to 0.2 M sucrose and centrifuged for 30 min at 95000 ***g***. The pellet was resuspended in 0.3 M sucrose and mixed in a ratio of 1:4 (v/v) with a mixture of 7 ml of Percoll, 1 ml of 2 M sucrose in 8 mM EDTA/80 mM Tris/HCl, pH 7.4 and 32 ml of 0.25 M sucrose/2 mM EDTA/10 mM Tris/HCl, pH 7.4. The mixture was centrifuged for 15 min at 10000 ***g*** to achieve a self-forming gradient and the plasma membrane fraction was collected from just below the top of the gradient, diluted 5-fold and centrifuged for 15 min at 100000 ***g***. The pellet was washed three times with the buffer. Alkaline phosphatase activity was used as a marker for the plasma membrane [15]. The plasma membrane fraction showed 9-fold enrichment in alkaline phosphatase when compared with lung homogenate (plasma membrane, 8.24 ± 0.53; lung homogenate, 0.94 ± 0.07 μmol P_i liberated/h per mg of protein, mean $\pm$ S.E.M., $n = 4$) and undetectable levels of succinate dehydrogenase, a mitochondrial marker [16], but showed slight contamination with the microsomal marker, NADPH:cytochrome *c* reductase [17]. The microsomal and the cytosol fractions from lung homogenate were isolated according to a method published previously [18]. The NADPH:cytochrome *c* reductase activities in plasma membrane and the microsomal fractions were 0.45 ± 0.04 and 22.4 ± 1.7 nmol of cytochrome *c* reduced/min per mg of protein, respectively ($n = 3$). Thus microsomal contamination of the plasma membrane fraction was approx. 2 %.

Preparation of liposomes

Mixtures of indicated lipid composition in chloroform solution were dried under a stream of N_2, and resuspended in an appropriate volume of buffer (buffer composition was chosen according to the experimental protocol). The lipid suspension was sonicated for 6 min at 50 ° C in a Cup-Horn Sonicator (Branson sonifier 250, Thomas Scientific, Swedesboro, NJ, U.S.A.) at 70 % of maximal output, and the process was repeated three times. The suspension was then allowed to cool to room temperature, stored overnight at 4 °C, and centrifuged at 1000 ***g*** for 15 min to sediment large aggregates. The supernatant fraction has previously been shown to contain mostly small unilamellar liposomes [19]. Approximately 44 % of the total phospholipids were recovered in the supernatant fraction, which was then used for aggregation and fusion studies.

Synexin binding assay

A nonisotopic method of receptor-binding based assay [20] was adapted to measure synexin binding to various subcellular fractions. In this competitive-inhibition assay, a biotinylated ligand competes with biotinylated BSA for reaction with avidin–HRP, whereby ligand binding to a receptor (or subcellular fraction) would inhibit such competition. Briefly, 100 μl of 0.001 % biotinylated BSA was pipetted into the wells of a microtitre plate for adsorption during overnight incubation at 4 °C. The wells were rinsed (× 3) with PBS (10 mM sodium phosphate/0.1 M NaCl, pH 7.4) to remove unadsorbed biotinylated BSA, and incubated for 1 h with 250 μl of blocking solution (Pierce Chemical Co., Rockford, IL, U.S.A.) to block non-specific binding sites. The plates were then rinsed (× 3) with PBS and used for the binding assay.

Purified bovine lung synexin was biotinylated using the biotin derivative according to the manufacturer's protocol (Molecular Probes). Subcellular fractions of interest (50 μg/ml protein each) were incubated for 2 h in ELISA plates with biotinylated synexin in the absence or presence of the indicated calcium concentrations, buffered with 1 mM EGTA. The incubation buffer (total volume 150 μl) contained 50 mM Tris/HCl, pH 7.4/120 mM NaCl/30 mM KCl [21]. All incubations were carried out, with shaking, at room temperature. For competitive binding, lamellar-body suspension was incubated for 30 min with unlabelled synexin (4.7–900 nM). Biotinylated synexin was then added and incubations were continued for the next 2 h. Thereafter, 50 μl of avidin–HRP was added (final concentration, 0.2 nM) and incubations were continued for another hour. Initial studies showed that maximum binding of avidin–HRP to biotinylated wells was achieved during a 1 h incubation. The plates were washed (× 5) with PBS, the enzyme reaction was started by adding 100 μl of tetramethylbenzidine peroxidase immunoassay substrate solution and the incubation was continued for another 20 min. The reaction was terminated by addition of 0.5 M H_2SO_4 (100 μl) and the absorbance was measured at 490 nm in an ELISA reader. Enzyme activity varied somewhat from day to day even with the same amount of avidin–HRP bound to the well. Therefore, to standardize the data, results were expressed as a percentage of maximum activity (100 %) that was obtained in each experiment by incubating 0.2 nM avidin–HRP without any other additions.

Gel overlay assay

After slight modifications of previously described protocol [22], the gel overlay assay was performed using biotinylated synexin as the ligand. Briefly, proteins (approx. 100 μg) of each fraction were separated by SDS/10 % (w/v) PAGE under reducing conditions [23], and transferred onto nitrocellulose paper. The nitrocellulose paper was rinsed with 0.2 % Tween-20 in 0.05 M borate buffer, pH 9.3, blocked by a 45 min incubation with 5 % (w/v) vitamin-free casein in TTBS [0.02M Tris/HCl/0.5 M NaCl, pH 7.5, containing 0.2 % (v/v) Tween-20] and incubated for 1 h with 200 nM biotinylated synexin. In parallel, another nitrocellulose paper with transferred proteins was incubated for 2 h with unlabelled synexin (1 μM) before incubation with biotinylated synexin. The membranes were incubated for 1 h

with avidin–HRP solution (5 μg/ml), washed with multiple changes of buffer (0.5 M NaCl/0.02 M Tris/HCl, pH 7.5), and then reacted with 4-chloro-1-naphthol to identify bound biotinylated synexin.

Aggregation and fusion of lamellar bodies

Aggregation of lamellar bodies (10 μg) was assayed at room temperature in a buffer (300 mM sucrose/40 mM histidine, pH 6.0) containing different concentrations of calcium buffered with 1 mM EGTA. After establishing baseline absorbance at 540 nm, 10 μg/ml synexin (215 nM using 47 kDa as the molecular mass for synexin) was added and the increase in absorbance was measured for the next 7 min. The percentage increase in absorbance (relative to initial absorbance) was expressed as aggregation.

Fusion was measured by following the transfer of resonance energy between two fluorescent lipid probes, donor NBD-PC and acceptor Rh-PE [24]. A typical assay was carried out at 37 °C in a total volume of 1.0 ml containing 200 mM sucrose/30 mM KCl/25 mM histidine, pH 6.0, containing 2 μM labelled liposome (PC/PS/PE/NBD-PC/Rh-PE; 65:25:10:0.75:0.75, by weight) and lamellar bodies (20 μg of protein). After equilibration for 5 min, the indicated concentrations of calcium were added and the fluorescence (excitation, 450 nm; emission, 530 nm) was followed for another 10 min. Synexin (215 nM) was then added to initiate fusion, which was monitored by following the increase in fluorescence. The fusion rate (fluorescence increase per min) was calculated from the initial, linear part of the trace.

Protein was determined with protein-binding dye reagent (Bio-Rad) using bovine γ-globulin as standard [25]. Phospholipid phosphorous was measured according to Marinetti [26] as described previously [27]. Alkaline phosphatase activity was measured by following the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at pH 8.5 [15]. The activity of succinate dehydrogenase was measured using cytochrome *c* as electron acceptor [16]. NADPH:cytochrome *c* reductase was assayed spectrophotometrically by following reduction of cytochrome *c* [17].

RESULTS

Synexin binding

Biotinylated synexin appeared as a single approx. 47 kDa protein, when determined by electrophoresis on SDS/PAGE, transfer to nitrocellulose paper and reaction with avidin–HRP (Figure 1A). Compared with native protein, biotinylated synexin retained approx. 95% of biological activity as determined by protein-induced liposomal aggregation in the presence of 1 mM calcium.

The percentage enzyme activity of 0.2 nM avidin–HRP decreased with increasing concentrations of biotinylated synexin, reaching almost 20% of initial activity at 15 nM synexin (Figure 1B). Based on the enzyme-activity curve, 15 nM biotinylated synexin was used for all subsequent binding assays. The free biotinylated synexin concentration was then calculated from the slope of the curve by plotting $1-(1/\text{relative activity})$ against $1/[\text{biotinylated synexin}]$, where relative activity = enzyme activity/maximum enzyme activity (Figure 1B, inset), as described for the receptor binding assay [20].

Synexin binding to lamellar bodies increased with increasing concentration of lamellar-body proteins. Binding was observed at as low as 0.5 μg and reached a maximum at 7.5 μg of protein/well (Figure 2). At higher lamellar-body concentrations, a plateau was observed due to binding of all of the biotinylated synexin in the well. Therefore in subsequent experiments, 7.5 μg of lamellar-body protein was used for the binding assay. Binding of biotinylated synexin to lamellar bodies could be inhibited with unlabelled synexin in a concentration-dependent manner reaching a maximum (97%) at 470 nM (Figure 3).

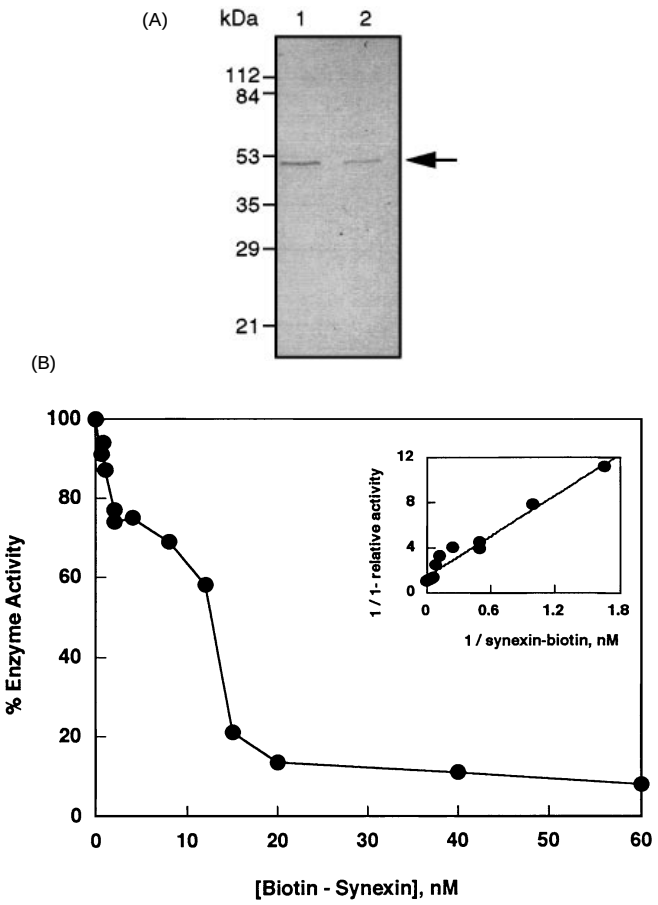

Figure 1 Characterization of biotinylated synexin

(**A**) Western blot of biotinylated synexin (indicated by arrow) under reducing conditions. Biotinylated synexin was resolved by SDS/(10%) PAGE, transferred to nitrocellulose membranes and reacted with avidin–HRP. Molecular-mass standards are indicated in the left margin. Lane 1, 1 μg of protein; lane 2, 300 ng of protein. (**B**) Percentage enzyme activity plotted against concentration of biotinylated synexin [biotin-synexin]. Biotin-synexin decreases HRP-reaction product in a concentration-dependent manner. The results shown are from a single experiment. Inset, double reciprocal plot. The concentration of free biotinylated synexin was determined from the slope of the regression line ($y = 6.0x + 1.4$).

Next, we determined the calcium kinetics for synexin binding to biological membranes (Figure 4). Synexin binding to lamellar bodies and plasma membranes increased progressively with increasing calcium concentrations reaching a plateau at approx. 200 μM calcium. Slight binding of synexin was observed in the absence of calcium, which was subtracted from binding in the presence of calcium. Synexin binding to each of these fractions was seen at calcium concentrations as low as 2.5 μM. The apparent EC_{50} for synexin binding to lamellar bodies and plasma membranes was 30 and 50 μM calcium respectively. Binding of biotinylated synexin to lamellar bodies, plasma membrane and cytosol was 220 ± 14, 34 ± 1, and 26 ± 10 pmol/μg of protein respectively (means $\pm$ S.E.M, $n = 4$ for each). Since lamellar bodies are phospholipid-rich organelles, we evaluated also synexin binding to lamellar-body membranes that contain relatively low levels of phospholipids when compared with lamellar

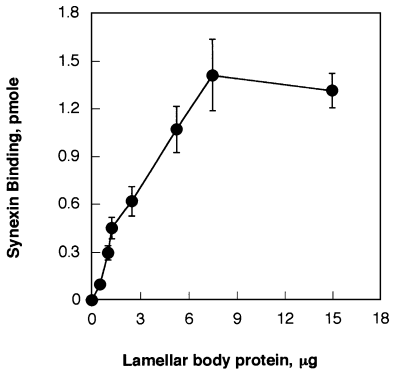

Figure 2 Synexin binding to lamellar bodies

Biotinylated synexin (15 nM) and 0.5–15 μg lamellar-body protein/well were incubated with 200 μM calcium. The results are the means $\pm$ S.E. of three experiments.

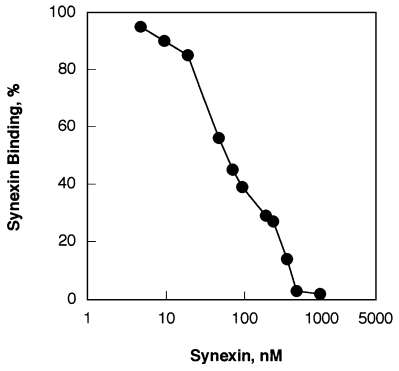

Figure 3 Inhibition of synexin binding to lamellar bodies

The binding assay was carried out after incubation of biotinylated synexin (15 nM) and various amounts of unlabelled synexin (5–940 nM) with lamellar bodies (7.5 μg of protein/well), and in the presence of 200 μM calcium. The results are from one experiment and are expressed as a percentage of protein binding in the absence of added unlabelled synexin.

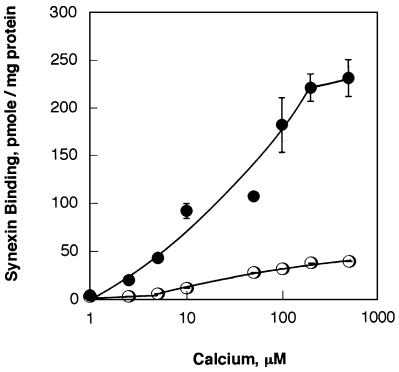

Figure 4 Synexin binding to lamellar bodies and plasma membranes as a function of calcium concentration

Binding of biotinylated synexin to lamellar bodies and plasma membranes (7.5 μg of protein each) were determined in the presence of calcium/EGTA buffer to provide the stated calcium concentrations. Lamellar bodies, (●). The results are the means $\pm$ S.E.M. of four different experiments. Plasma membranes, (○). The results for binding to plasma membranes are from one experiment.

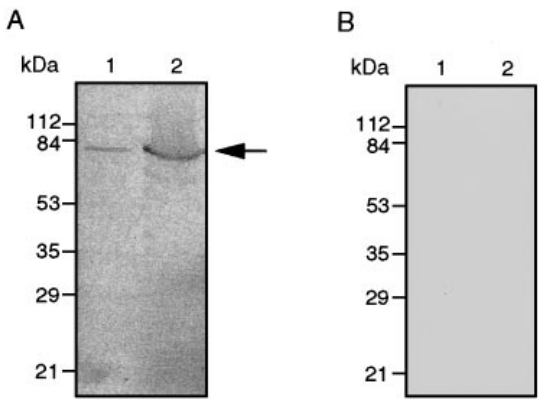

Figure 5 Synexin binding protein in lamellar bodies and plasma membranes

Membrane proteins (100 μg each) were resolved on SDS/(10%) PAGE under reducing conditions and transferred to a nitrocellulose paper that was then treated with biotinylated synexin in the absence (**A**) or presence (**B**) of unlabelled synexin. Bound biotinylated synexin was visualized by reaction with avidin–HRP. In both lanes in (**A**), similar protein (approx. 76 kDa, marked with an arrow) is seen to bind biotinylated synexin. Molecular-mass standards are indicated in the left margin. Lanes 1, lamellar bodies; lanes 2, plasma membranes.

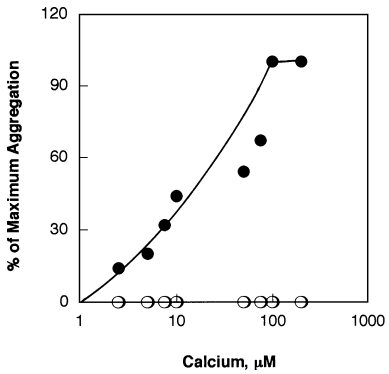

Figure 6 Calcium-dependence of synexin mediated membrane aggregation

Aggregation of lamellar bodies (10 μg of protein/ml) with 215 nM synexin was monitored by following the absorbance changes at 540 nm as a function of calcium concentration, using calcium/EGTA buffer. The aggregation is expressed as a percentage of the maximum, which was observed with 215 nM synexin plus 200 μM calcium. The results are from one experiment. Without calcium, (○); with calcium, (●).

bodies [13]. Synexin binding to lamellar-body membranes was 186 ± 24 pmol/μg of protein ($n = 4$). In a typical cytosol and a lamellar-body membrane preparation, the phospholipid/protein ratio (w/w) was 0.24 and 0.99 respectively. When expressed on a phospholipid basis, binding of synexin to lamellar-body membranes was higher when compared with that of the cytosol. Thus although synexin binding to phospholipids of lamellar bodies cannot be excluded, the higher binding to lamellar bodies suggests the presence of certain synexin binding proteins in lamellar bodies.

Gel overlays using biotinylated synexin as a ligand showed that synexin bound to an approx. 76 kDa protein in the lamellar-body and in the plasma-membrane fractions (Figure 5), but not in the cytosol fraction (results not shown). The associations of synexin with lamellar bodies and plasma membranes were specific, since preincubation with an excess quantity of unlabelled synexin abolished the binding of biotinylated synexin.

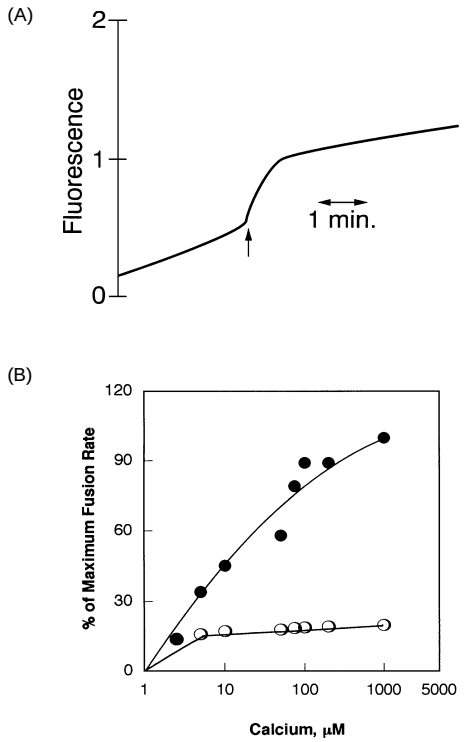

Figure 7 Calcium-dependence of synexin mediated membrane fusion

Labelled liposomes (2 μM) [PE/PS/PC/NBD-PC/Rh-PE (10:25:65:0.75:0.75, by weight)] and lamellar bodies (20 μg of protein/ml) were mixed with the concentrations of calcium indicated. (**A**) Representative trace of synexin-dependent fusion between lamellar bodies and liposomes at 1 mM calcium. Fusion was measured by following the increase in fluorescence (excitation, 450, emission, 530 nm). Fluorescence is given in arbitrary units. A small increase in fusion rate was observed with calcium. Addition of 215 nM synexin (indicated by an arrow) further increased the rate of fusion. (**B**) Results are expressed as a percentage of maximum fusion rate with 215 nM synexin plus 1 mM calcium. The results are from one experiment. Without synexin, (○); with synexin, (●).

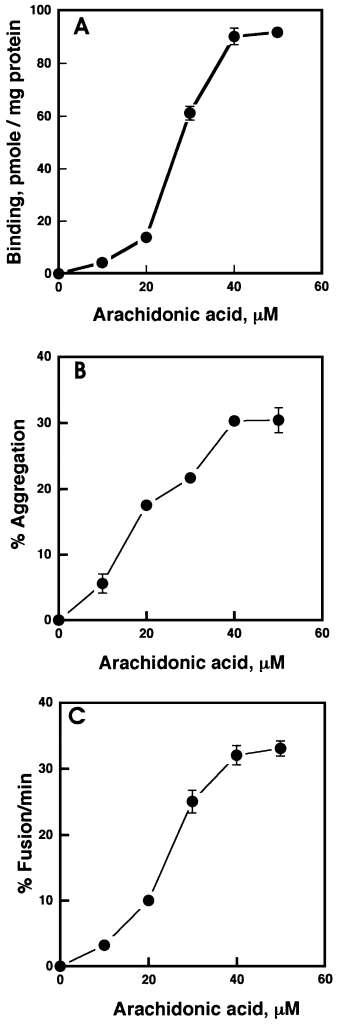

Figure 8 Effects of arachidonic acid on synexin binding, and on synexin-mediated aggregation and fusion of lamellar bodies

Lamellar bodies were preincubated for 5 min in 1 μM free calcium and the indicated concentration of arachidonic acid before the addition of synexin. The results are the means ± S.E.M. of three separate experiments. For some data points, the error bars are masked by the symbol. (**A**) Binding, (**B**) aggregation, and (**C**) rate of fusion.

Calcium-dependence of lamellar-body aggregation and fusion

Synexin also increased lamellar-body aggregation in a calcium-dependent manner, with an apparent EC_{50} of 20 μM. As with synexin binding, aggregation was seen at calcium concentrations as low as 2.5 μM and increased with increasing concentration reaching a maximum at approx. 100 μM calcium. The presence of calcium did not affect the lamellar-body aggregation in the absence of synexin (Figure 6). At low calcium (10 μM), aggregation was dependent on synexin concentrations. Aggregation (means ± S.E.M.) was 8.9 ± 2.0 %, 20.2 ± 3.9 % and 29.3 ± 4.3 % at 27 nM, 54 nM and 108 nM synexin respectively ($n = 3$ for each).

As with aggregation, synexin also increased the fusion rate of liposomes with lamellar bodies that was dependent on the calcium concentration (Figure 7). Addition of 1 mM calcium caused some fusion between lamellar bodies and liposomes (Figure 7A). The addition of 215 nM synexin further enhanced the rate of fusion. Fusion could be observed at 2.5 μM and reached a maximum at approx. 100 μM calcium, with an apparent EC_{50} of 30 μM Ca^{2+} (Figure 7B). In the absence of synexin, the fusion of lamellar bodies with liposomes also increased with increasing calcium concentrations. When the calcium concentration was increased from 2.5 μM to 1 mM the fusion rate increased from 13.5 % to 19.8 % of the rate observed with 215 nM synexin and 1 mM calcium (Figure 7B). Thus synexin significantly increased the calcium-dependent fusion of lamellar bodies. As shown previously with fusion between two populations of liposomes [1], increasing synexin concentrations will be likely to further increase the fusion of lamellar bodies and liposomes.

Synexin may promote fusion between membranes that are in close apposition by bringing them together in a calcium-dependent manner. Arachidonic acid has been shown previously to increase the calcium sensitivity of self-association of synexin in solution [28], and to increase synexin-dependent liposome fusion at high calcium concentrations [3]. Next, we evaluated the effect of arachidonic acid on synexin binding to lamellar bodies, and on aggregation and fusion of lamellar bodies at low calcium concentration (1 μM), which is close to that achieved in various stimulated secretory cells [29]. Arachidonic acid increased all three parameters, i.e. synexin binding to lamellar bodies, synexin-dependent aggregation of lamellar bodies, and synexin-dependent fusion of lamellar bodies with liposomes (Figure 8). The

arachidonic acid concentration kinetics were similar for all three parameters. Maximally effective concentrations of arachidonic acid were similar to those reported previously for liposome fusion by synexin [3].

DISCUSSION

Previous studies demonstrating calcium-dependence of synexin binding to chromaffin granules or oriented chromaffin cell plasma membranes have suggested a role for synexin in membrane fusion during exocytosis [30]. The present study describes the calcium-dependence of synexin interaction with lamellar bodies as reflected by synexin binding, and aggregation and fusion of lamellar bodies. We had postulated that calcium-dependent synexin binding to membranes, rather than aggregation of synexin as described for the hypothetical scheme of synexin-dependent membrane fusion [9,31], may be the primary step in membrane fusion. Our studies showing noticeably similar calcium kinetics for binding, aggregation, and fusion suggest that synexin binding to lamellar bodies may be an early determinant for membrane fusion and surfactant secretion. The calcium-dependence of synexin binding also suggests that an increase in calcium at the site of exocytosis [32,33] would facilitate synexin association with the plasma membrane and the secretory organelles. This interaction would then be followed by molecular reorganization and subsequent steps postulated for membrane-fusion [31].

A significant finding from our study is that, at low calcium concentrations, the binding and membrane-fusion processes may be modulated by certain factors. We have identified synexin and arachidonic acid concentrations among these factors. Our results with arachidonic acid (Figure 8) also suggest that the calcium-dependent synexin binding to membranes may be a rate-determining step. The physiological significance of binding, aggregation and fusion *in vitro* is often questioned on the basis of the rather high calcium levels used in these studies. However, studies have demonstrated that calcium may be compartmentalized in the cell and may exist at rather high concentrations localized close to the plasma membrane, in juxtaposition to calcium channels [32], or at the site of exocytosis [33]. Furthermore, various cofactors may modify the calcium requirement in intact cells [34,35]. It is likely that, besides synexin and arachidonic acid, other substances may also regulate synexin binding, and aggregation and fusion of secretory organelles.

Our finding that arachidonic acid can modulate synexin binding at calcium concentrations reported to exist in some stimulated cells [29] attests to the significance of cofactors for synexin-dependent membrane fusion. Arachidonic acid is liberated from membrane phospholipids by the action of phospholipase A_2 in a variety of stimulated cells, and has been postulated to function as a second messenger [36]. Arachidonic acid increases intracellular calcium and stimulates surfactant secretion in alveolar type II cells [37,38]. It is possible that arachidonic acid also enhances synexin binding (and its function) in synergy with calcium. The effective concentrations of arachidonic acid used in this study are similar to those reported for activation of protein kinase C [39], and may be similar to those present in certain areas of the cell membrane at or near its site of production [40].

In the presence of calcium, synexin can self-associate and bridge adjacent vesicles (aggregation) to facilitate close contact and induce some degree of fusion between membranes. Previous studies have shown that appropriate concentrations of calcium (1 mM) can induce self-association of soluble synexin [3,41], which has been postulated to be an initial step in synexin-dependent membrane fusion [31]. The self-association of membrane-bound synexin, however, occurs at lower calcium concentrations than that of soluble synexin [10]. Possibly because of self-association following membrane binding, synexin appears to promote fusion of phosphatidic acid/PE (1:3, by wt.) vesicles at low calcium levels, which do not facilitate self-association of soluble synexin [41]. In fact, the membrane fusion activity of self-associated synexin in solution is greatly reduced [42]. One possibility is that self-associated forms of membrane-bound and soluble synexin may differ in protein conformation. This is supported by our previous study demonstrating that 4,4′-diisothiocyanato-2,2′-disulphonic acid promotes self-association of soluble synexin but inhibits aggregation and fusion of liposomes by synexin [3]. Thus synexin binding to membranes may be an important step in the overall process of membrane fusion. The foregoing considerations together with the results of this study suggest that the membrane-binding step may be a primary determinant of synexin activity. Although aggregation of membranes is an important initial step in exocytotic membrane fusion [31], it may be only an intermediate step in membrane fusion and may not be the rate-limiting step in the light of our observations (Figure 8).

The significance of preferential binding of synexin to lamellar bodies probably lies in these organelles being exocytotic in nature and in that they must fuse with the plasma membrane during surfactant secretion. It is possible that synexin binds to both the lipid and protein components of lamellar bodies. Although the lamellar bodies demonstrate significantly higher binding when compared with plasma membranes (Figure 4), some of this binding could be due to the high phospholipid content of lamellar bodies. We cannot exclude synexin binding to lipids, even though lamellar-body membranes, containing almost 85% less phospholipid than isolated lamellar bodies, bound synexin to a comparable extent, and synexin did not aggregate (and hence bind to) liposomes prepared from PC [9,42], which is the major lamellar-body phospholipid [12]. Since we observed relatively similar synexin binding to an approx. 76 kDa protein in lamellar bodies and plasma membranes (Figure 5), we suggest that the lipids or other proteins (not detectable by gel-overlay protocol) of lamellar bodies may contribute to high synexin binding.

Compared with total synexin binding, the calcium-dependence of synexin binding is likely to be a characteristic of synexin itself and not of the proteins or lipids of lamellar bodies, since similar calcium-kinetics were observed in both plasma membranes and lamellar bodies (Figure 4). Although the mechanism of synexin-dependent fusion of lamellar bodies is not clear, based on the observation that synexin apparently binds to the same protein in both lamellar bodies and plasma membranes it is tempting to speculate that the approx.76 kDa protein may serve as a docking protein similar to that identified at the active zone in synaptic vesicles and the presynaptic membranes [43,44]. Subsequent steps in the membrane fusion process would then include apposition and bridging of lamellar bodies with plasma membrane. A detailed analysis of synexin interaction with the approx. 76 kDa protein, however, must first be carried out in order to understand its role in synexin-dependent membrane fusion.

The lung contains a large number of annexins [1,3,45,46] of which at least two, synexin and annexin II, have been implicated in lung surfactant secretion. Western blot analyses have shown the presence of annexins I–IV and VI in type II cells [46]. Our recent studies demonstrate that synexin mRNA and proteins are present in type II cells (S. Adeniyi-Jones, L. Shiwu, M. Ahmad, J. Khillan, A. Spitzer and A. Chander, unpublished work). Recently, annexin IV from type II cells was shown to bind to

isolated lung lamellar bodies in a surfactant protein A- and calcium-dependent manner [47]. The role of annexin IV in lung surfactant secretion, however, is unclear. The precise physiological relevance of the presence of various annexins in one cell type is obscure, but may be related to membrane-fusion processes at different intracellular sites [6]. Alternatively, interactions among various annexins may result in improved or diminished membrane aggregation (and fusion) activities as previously demonstrated during annexin binding and chromaffin granule aggregation studies using synexin, P32 (annexin IV) and P67 (annexin VI) [48].

In summary, our results suggest that the calcium kinetics for synexin-dependent aggregation and fusion of lamellar bodies is similar to that for synexin binding to lamellar bodies, suggesting that calcium-dependent synexin binding to membranes may be a rate limiting step in the membrane fusion process. At near physiological calcium concentrations, polyunsaturated fatty acids (and possibly other unidentified factors), as well as synexin concentration, may modify the binding and subsequent aggregation and fusion reaction, supporting the premise that synexin binding to interacting membranes may determine the membrane fusion process.

These studies were supported in part by grant HL 49959 from the National Heart Lung and Blood Institute of the National Institutes of Health. The excellent secretarial assistance of Maria Mokan is also acknowledged.

REFERENCES

1 Chander, A. and Wu, R. D. (1991) Biochim. Biophys. Acta **1086**, 157–166
2 Chander, A. and Sen, N. (1993) Biochem. Pharmacol. **45**, 1905–1912
3 Liu, L. and Chander, A. (1995) Biochim. Biophys. Acta **1254**, 274–282
4 Crumpton, M. J. and Dedman, J. R. (1990) Nature (London) **345**, 212
5 Geisow, M. J. and Walker, J. H. (1986) Trends Biochem. Sci. **11**, 420–423
6 Creutz, C. E. (1992) Science **258**, 924–931
7 Raynal, P. and Pollard, H. B. (1994) Biochim. Biophys. Acta **1197**, 63–93
8 Pollard, H. B. and Rojas, E. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2974–2978
9 Meers, P., Bentz, J., Alford, D., Nir, S., Papahadjopoulos, D. and Hong, K. (1988) Biochemistry **27**, 4430–4439
10 Zaks, W. J. and Creutz, C. E. (1991) Biochemistry **30**, 9607–9615
11 Sen, N., Spitzer, A. R. and Chander, A. (1996) FASEB J. **10**, A357
12 Chander, A., Dodia, C. R., Gil, J. and Fisher, A. B. (1983) Biochim. Biophys. Acta **753**, 119–129
13 Chander, A. (1992) Biochim. Biophys. Acta **1123**, 198–206
14 Belsham, G. J., Denton, R. M. and Tanner, M. J. A. (1980) Biochem. J. **192**, 457–467
15 Loomis, W. F. (1969) J. Bacteriol. **100**, 417–422
16 Green, D. E., Mii, S. and Kohout, P. M. (1955) J. Biol. Chem. **217**, 551–567
17 Phillips, A. H. and Langdon, R. G. (1962) J. Biol. Chem. **237**, 2652–2660
18 Chander, A. and Fisher, A. B. (1988) Biochim. Biophys. Acta **958**, 343–351
19 Hamilton, R. L., Goerke, J., Guo, L. S. S., Williams, M. C. and Havel, R. J. (1980) J. Lipid Res. **21**, 981–992
20 Chen, L., Martin, G. B. and Rechnitz, G. A. (1992) Anal. Chem. **64**, 3018–3023
21 Bers, D. M. (1982) Am. J. Physiol. **242**, C404–C408
22 Glenny, J. R. and Weber, K. (1980) J. Biol. Chem. **255**, 10551–10554
23 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
24 Struck, D. K., Hoekstra, O. and Pagano, R. E. (1981) Biochemistry **20**, 4093–4099
25 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
26 Marinetti, G. V. (1961) J. Lipid Res. **3**, 1–20
27 Chander, A., Fisher, A. B. and Strauss, III., J. F. (1982) Biochem. J. **208**, 651–658
28 Sterner, D. C., Zaks, W. J. and Creutz, L. E. (1985) Biochim. Biophys. Acta **132**, 505–512
29 Kao, L.-S. and Schneider, A. S. (1986) J. Biol. Chem. **261**, 4881–4888
30 Scott, J. H., Creutz, C. E., Pollard, H. B. and Ornberg, R. (1985) FEBS Lett. **180**, 17–23
31 Plattner, H. (1989) Int. Rev. Cytol. **119**, 197–286
32 Simon, S. M. and Llinas, R. R. (1985) Biophys. J. **48**, 485–498
33 Sawyer, D. W., Sullivan, J. A. and Mandell, G. L. (1985) Science **230**, 663–666
34 Schlaepfer, D. D., Mehlman, T., Burgess, W. H. and Haigler, H. T. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6078–6082
35 Glenney, J. and Zokas, L. (1988) Biochemistry **27**, 2069–2076
36 Khan, W. A., Blobe, G. C. and Hannun, Y. A. (1995) Cell. Signalling **7**, 171–184
37 Peters-Golden, M., Coburn, K. and Chauncey, J. B. (1992) Exp. Lung Res. **18**, 535–551
38 Baybutt, R. C., Smith, J. E., Gillespie, M. N., Newcomb, T. G. and Yeh, Y. Y. (1994) Lipids **29**, 535–539
39 McPhail, L. C., Clayton, C. C. and Snyderman, R. (1984) Science **224**, 622–625
40 Ramanadham, S., Bohrer, A., Gross, R. W. and Turk, J. (1993) Biochemistry **32**, 13499–13509
41 Creutz, C. E., Pazoles, C. J. and Pollard, H. B. (1979) J. Biol. Chem. **254**, 553–558
42 Hong, K., Meers, P. R., Duzgunes, N. and Papahadjopoulos, D. (1991) in Membrane Fusion (Hoekstra, D. Wilschut, J., eds.), pp. 195–208, Marcel Dekker, New York
43 Landis, D. M. D., Hall, A. K., Weinstein, L. A. and Reese, T. S. (1988) Neuron **1**, 201–209
44 Hirokawa, N., Sobue, K., Kanda, K., Harada, A. and Yorifuji, H. (1989) J. Cell Biol. **108**, 111–126
45 Khanna, N. C., Helwig, E. D., Ikebuchi, N. W., Fitzpatrick, S., Bajwa, R. and Waisman, D. M. (1990) Biochemistry **29**, 4852–4862
46 Liu, L., Wang, M., Fisher, A. B. and Zimmerman, U.-J. P. (1996) Am. J. Physiol. **270**, L668–L676
47 Sohma, H., Matsushima, N., Watanabe, T., Hattori, A., Kuroki, Y. and Akino, T. (1995) Biochem. J. **312**, 175–181
48 Zaks, W. J. and Creutz, C. E. (1990) Biochim. Biophys. Acta **1029**, 149–160

Received 24 May 1996/25 September 1996; accepted 26 September 1996