Synexin and GTP increase surfactant secretion in permeabilized alveolar type II cells

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Synexin and GTP increase surfactant secretion in permeabilized alveolar type II cells. Am J Physiol Lung Cell Mol Physiol 280: L991–L998, 2001.—We have previously suggested that synexin (annexin VII), a Ca<sup>2+</sup>-dependent phospholipid binding protein, may have a role in surfactant secretion, since it promotes membrane fusion between isolated lamellar bodies (the surfactant-containing organelles) and plasma membranes. In this study, we investigated whether exogenous synexin can augment surfactant phosphatidylcholine (PC) secretion in synexin-deficient lung epithelial type II cells. Isolated rat type II cells were cultured for 20–22 h with [3H]choline to label cellular PC. The cells were then treated with β-escin, which forms pores in the cell membrane and releases cytoplasmic proteins including synexin. These cells, however, retained lamellar bodies. The permeabilized type II cells were evaluated for PC secretion during a 30-min incubation. Compared with PC secretion under basal conditions, the presence of Ca<sup>2+</sup> (up to 10 μM) did not increase PC secretion. In the presence of 1 μM Ca<sup>2+</sup>, synexin increased PC secretion in a concentration-dependent manner, which reached a maximum at ~5 μg/ml synexin. The secretagogue effect of synexin was abolished when synexin was inactivated by heat treatment (30 min at 65°C) or by treatment with synexin antibodies. GTP or its nonhydrolyzable analog GTPγS augmented PC secretion in permeabilized type II cells. The PC secretion was further increased in an additive manner when a maximally effective concentration of synexin was added in the presence of 1 mM GTP, suggesting that GTP acts by a synexin-independent mechanism to increase membrane fusion. Thus our results support a direct role for synexin in surfactant secretion. Our study also suggests that membrane fusion during surfactant secretion may be mediated by two independent mechanisms.

The lung surfactant is a phospholipid-rich lipoprotein-like substance that is essential for lowering surface tension during end expiration to prevent alveolar collapse (reviewed in Ref. 2). The major component of lung surfactant is phosphatidylcholine (PC). The lung epithelial type II cells synthesize and store PC and other surfactant components in lamellar bodies, which are membrane-bound distinct surfactant storage organelles that are unique to type II cells. The secretion of lung surfactant occurs after membrane fusion between lamellar bodies and plasma membrane and release of lamellar body contents. Several agents modulate lung surfactant secretion by generating specific intracellular messengers (reviewed in Refs. 11 and 32). Although significant advances have been made in understanding the signal transduction processes leading to generation of second messengers, the mechanism(s) of membrane fusion and factors that regulate such membrane fusion during exocytosis of lamellar body contents have remained poorly investigated.

We have previously proposed that synexin (annexin VII), a member of the annexin family of proteins, is involved in membrane fusion between lamellar bodies and plasma membrane (14). Annexins are Ca<sup>2+</sup>-dependent phospholipid-binding proteins that share significant sequence homology in the carboxy-terminal domains but contain a unique amino-terminal domain with variable chain length (15, 19). These proteins have been invoked in Ca<sup>2+</sup> homeostasis and in membrane fusion during exocytosis, endocytosis, and other intracellular membrane fusion events that may occur during vesicular trafficking. Our previous studies indicated that synexin can facilitate in vitro fusion of isolated lamellar bodies with plasma membrane (14). Our observations that certain stilbene disulfonic acids demonstrate similar inhibition potency and inhibition constants toward membrane fusion (23) and toward basal or stimulated PC secretion in type II cells (12) suggest that stilbene compounds may inhibit synexin-dependent membrane fusion during surfactant secretion. Our recent studies have shown that synexin can bind to specific proteins in the plasma membrane and the lamellar body fractions of lung (37). Together, these studies suggest a role for synexin in membrane fusion during surfactant secretion.

Several investigators have used permeabilized cells to evaluate the effects of membrane-impermeable agents on intracellular processes like protein trafficking and secretion (1, 3, 21, 24, 27, 29, 31). The permeabilized cells appear to support these physiological...
functions under specific conditions. Several agents (ATP4\(^-\), bacterial toxins, and detergents) can be used to form pores in the cell membrane. The choice of agent depends on the specific pore size required for passage of substances across the cell membrane. Previous studies have used ATP4\(^-\) to form membrane pores for passage of low-molecular-mass substances (~1 kDa) into mast cells (3). However, this agent could not be used for our studies because type II cells could not be permeabilized with ATP4\(^-\) (up to 5 \(\mu\)M ATP in divalent-free medium) and because we wished to introduce large-molecular-mass substances (synexin and its antibodies) into type II cells. In different types of cells, a bacterial toxin (streptolysin O) and a saponin (\(\beta\)-escin) have been used to evaluate the role of Ca\(^{2+}\) (21, 31), GTP (21, 31), or annexin II (24) in exocytic secretion. Both streptolysin O and saponin can bind and remove cholesterol in the cell membrane and render the cells permeable to large molecules (5, 10a). Saponin treatment has been shown to form small or large pores in the cell membrane depending on its concentration (21). Liu and associates (24) have previously used \(\beta\)-escin to introduce large molecules like annexin II into type II cells. In this study, we used \(\beta\)-escin-permeabilized type II cells to demonstrate that purified bovine synexin or GTP can independently increase surfactant PC secretion. Thus our study shows a direct role of synexin in lung surfactant secretion. Our results also show the presence of two separate mechanisms for membrane fusion during surfactant secretion in type II cells.

**MATERIALS AND METHODS**

GTP, \(\beta\-\gamma\)-imidoguanosine-5'-triphosphate (NHP\(g\)Gpp), \(\beta\)-escin, fatty acid-poor bovine serum albumin, antibiotics, and other standard chemicals were from Sigma Chemical (St. Louis, MO). SuperSignal reagent for chemiluminescence analysis of Western blots was purchased from Pierce (Rockford, IL). All plasticwares were obtained from Fisher Scientific (Pittsburgh, PA). All cell culture supplies were from Gibco BRL (Gaithersburg, MD). [\(methyl-\^{\text{3}}\)H]choline and \([^{14}\text{C}]\)dipalmitoyl PC (\([^{14}\text{C}]\)DPPC) were obtained from American (Arlington Heights, IL). The antibody to recombinant synexin peptide was a kind gift from Dr. A. Noegel (36).

**Isolation of type II cells.** Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 180–200 g were used for isolation of alveolar type II cells after elastase digestion of lungs (17) as described previously (12). The cells were plated in tissue culture plastic dishes for 20–22 h in minimum essential medium (MEM) containing 10% fetal calf serum at a cell density of 3 \(\times\) 10⁶ cells/\(ml\). At the end of this culture period, the adherent cells were >95% type II cells as determined by phosphine fluorescence, and >97% of these cells excluded the vital dye erythrosin B.

**Permeabilization of type II cells.** The adherent type II cells were washed with buffer A (Krebs-Ringer bicarbonate buffer containing 10 mM glucose, 30 mM HEPES, pH 7.4, and 1 mM EGTA). Buffer A was equilibrated for >30 min with 5% CO₂ in air before use. The cells were incubated for 10 min at 37°C in 1 ml of permeabilization buffer (buffer A containing indicated concentrations of \(\beta\)-escin). At the end of this incubation, the permeabilization buffer was removed, and the cells were washed (3 times) with buffer A. In some experiments, the attached cells were scraped into buffer A, and the scraped cells and the permeabilization buffer were stored at ~80°C until further analysis. The cells on the dishes were also examined for uptake of trypan blue (0.1%). At least 100 cells in four random fields were counted, and the percentage of trypan blue-stained (nonviable) cells was calculated.

**Studies on PC secretion.** In experiments designed to measure PC secretion, the cells were incubated for 20–22 h in MEM containing 10% fetal bovine serum and \([^{3}\text{H}\)-methyl-\(^{\text{3}}\)H]choline (0.3 \(\mu\)Ci/ml MEM). The cells were then permeabilized as described above. After the last wash, the cells were incubated for 5 min in 1 ml buffer A. In some experiments, EGTA in buffer A was replaced by 1,2-bis-(aminohexyl)-N,N,N',N'-tetraacetic acid, with essentially similar results. In experiments evaluating the effect of Ca\(^{2+}\), appropriate amounts of CaCl₂ were added to the medium to provide 0.5–10 \(\mu\)M Ca\(^{2+}\) buffered with 1 mM EGTA and accounted for the presence of other ions including Mg\(^{2+}\) (4). In each set of experiments, the medium and cells were collected from one plate after the 5-min incubation. This comprised the "zero-time" sample. For other plates, indicated agents or vehicle (<1% by vol) were added at the end of 5 min, and the incubations were continued for the next 30 min. Thereafter, the media were removed to terminate incubations. The medium samples were centrifuged to remove free cells, if any, which were pooled with the cells on plates. The media and the cells on plates were extracted for lipids (6) after addition of egg PC (0.4 mg each) and \([^{14}\text{C}]\)DPPC as described previously (12). Individual lipid samples were analyzed for the \(^{3}\)H radioactivity, which was corrected for the recovery of \([^{14}\text{C}]\)-DPPC. The PC secretion was calculated from the corrected \(^{3}\)H radioactivity in the medium and cell samples [\%PC secretion = (100 \(\times\) \(^{3}\)H in the medium/molH in the medium plus cells)]. The changes in PC secretion are also expressed relative to the control secretion, which was expressed as 100%.

**Purification of bovine synexin.** Synexin was isolated from the soluble fraction (100,000 g for 60 min supernatant) of bovine lung homogenates as described (37). The purified protein migrated as a single band of ~47 kDa molecular mass on 10% SDS-PAGE. The purified protein was lyophilized and stored at ~80°C. It was reconstituted in water at 0.1–1 mg/ml.

**Other methods.** Protein samples were separated by 10% SDS-PAGE under reducing conditions and stained with Coomassie blue to visualize proteins. For Western analysis, the protein samples were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. These membranes were probed with monoclonal antibody to recombinant synexin peptide (36) and visualized by chemiluminescence using SuperSignal reagent. For pretreatment of synexin with the antibody, aliquots containing indicated amounts of synexin were incubated for 20 h at 4°C with the synexin antibody (10 \(\mu\)l). An equal volume of the antibody without synexin or synexin without the antibody was incubated in parallel. These were then evaluated for effects on surfactant PC secretion in permeabilized cells. For heat inactivation of synexin, appropriate amounts of synexin were incubated for 30 min at 65°C, cooled to room temperature, and evaluated for secretagogue effects in permeabilized type II cells. Lactate dehydrogenase (LDH) activity was assayed by monitoring the reduction of NAD at 340 nm (22). Proteins were measured by reaction with the protein-binding dye reagent (Bio-Rad Laboratories, Richmond, CA) using bovine \(\gamma\)-globulin as a standard (7).

**Statistical analysis.** Results were analyzed for statistical significance by Student’s t-test for experiments paired with respect to cell preparations. For comparison of results from
multiple groups, the results were analyzed by ANOVA followed by Newman-Keuls post hoc analysis. All differences were considered significant at $P < 0.05$.

RESULTS

Cell permeabilization. The permeabilization of type II cells was evaluated by measuring cell uptake of the vital dye trypan blue and by following the release of LDH, cellular proteins, and synexin from type II cells. After a 10-min incubation with 10 $\mu$g/ml $\beta$-escin, a significantly higher proportion of cells was stained with trypan blue, while there was only a slight increase in the LDH release when compared with the controls (Fig. 1). These findings suggested that low concentrations of $\beta$-escin formed small pores in type II cell membranes as previously shown for mast cells (20). When incubated with increasingly higher concentrations of $\beta$-escin, the LDH release and trypan blue uptake increased in a concentration-dependent manner. Cells treated with 40 $\mu$g/ml $\beta$-escin released $\sim$70% of cellular LDH, and $\sim$90% of these cells could be stained with trypan blue (Fig. 1). In parallel with the release of LDH, other cellular proteins (Fig. 2A) and synexin were also released (Fig. 2B). The release of these cell constituents also increased with $\beta$-escin concentration. Type II cells treated with 40 $\mu$g/ml $\beta$-escin, however, retained lamellar bodies as shown by phosphine staining of lamellar bodies (Fig. 3). Because phosphine binds to lipids in the lamellar bodies, these findings suggest that the lamellar bodies in these cells were intact. In light of previous reports that digitonin-permeabilized type II cells do not show regulated secretion (24, 27), we elected to use only $\beta$-escin for cell permeabilization.

PC secretion. Because permeabilized type II cells retained lamellar bodies, we evaluated them for PC secretion. During the 30-min incubation in $\beta$-escin-free medium, these cells released $\sim$1% of the cellular PC into the medium (Table 1). The control secretion, however, showed variation between different cell preparations. Some of this variation is inherent to the cell preparation. In our previous studies with nonpermeabilized cells, we have observed that the control secretion can vary by $\sim$100% (13). The permeabilization of type II cells with a detergent ($\beta$-escin) and the variation in the number of cells rendered permeable to large molecules, as indicated by the LDH release (Fig. 1), also likely contribute to the variations in control secretion and in synexin-dependent secretion (see below). Nevertheless, the PC secretion under the indicated experimental conditions showed a consistent pattern of changes in different cell preparations. To minimize the effect of such variation, the PC secretion under all experimental conditions of a group was measured in...
the same cell preparations whenever possible. In our initial studies, we evaluated the effects of varying free 
Ca$_{2+}$ concentrations on PC secretion. The PC secretion was unchanged when low concentrations of Ca$_{2+}$ (0.5–10 µM) buffered with EGTA (4) were present in the incubation buffer (Fig. 4). These studies suggested that some of the factors, possibly proteins, had leaked out of the cells and, therefore, Ca$_{2+}$ was unable to increase PC secretion in these cells. Next, we evaluated the effects of purified synexin on PC secretion in the absence or presence of 1 µM Ca$_{2+}$. The addition of syn-

Table 1. Effect of Ca$_{2+}$ and synexin in permeabilized type II cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>%[^3]H]PC Secretion in 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−Ca$_{2+}$</td>
</tr>
<tr>
<td>Control</td>
<td>1.16 ± 0.26</td>
</tr>
<tr>
<td>0.71 µg/ml Synexin</td>
<td>1.25 ± 0.13</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5 separate experiments. Isolated type II cells were incubated for 20–22 h in MEM containing 10% fetal bovine serum and [methyl-[^3]H]choline. At the end of incubation, the adherent cells were washed and permeablized for 10 min with β-escin (40 µg/ml). The cells were washed again and studied for phosphatidylicholine (PC) secretion in the absence (control; −) and presence (+) of indicated agents. *P < 0.05 by Student’s t-test for observations paired with respect to cell preparation when compared with PC secretion in the presence of 1 µM Ca$_{2+}$.

Fig. 3. Permeabilized type II cells retain lamellar bodies. Isolated and adherent type II cells were permeabilized for 10 min with 40 µg/ml β-escin, washed with MEM, treated with 0.01% phosphine, and viewed with a fluorescence microscope. Fluorescence micrographs of the control (A) and β-escin-treated (B) cells show bright fluorescence in vesicles (lamellar bodies). Magnification, ×600.

Fig. 4. Effect of Ca$_{2+}$ on phosphatidylcholine (PC) secretion in permeabilized type II cells. Isolated type II cells were incubated for 20–22 h with [[^3]H]choline, and the adherent cells were permeabilized for 10 min with 40 µg/ml β-escin and evaluated for PC secretion during a 30-min incubation in the absence and presence of indicated concentrations of Ca$_{2+}$ buffered with 1 mM EGTA. Results are expressed as percentage of secretion in the absence of Ca$_{2+}$ and are means ± SE of experiments in 3–5 cell preparations. Ca$_{2+}$ did not increase PC secretion at any of the indicated concentrations.
exin increased the secretion of PC in the presence but not in the absence of 1 μM Ca\(^{2+}\) (Table 1). The Ca\(^{2+}\) requirement for synexin effect on PC secretion is consistent with the previously described Ca\(^{2+}\) requirement for synexin-dependent membrane aggregation and fusion activity (37). This increase in PC secretion was dependent on synexin concentration (Fig. 5). The maximal increase in secretion was observed at ~5 μg/ml synexin.

**Specificity of synexin effect on PC secretion.** Next, we investigated the specificity of synexin effects on surfactant secretion. Addition of bovine serum albumin at concentrations comparable with those of synexin did not increase PC secretion (Table 2). The effect of synexin could be abolished with heat inactivation of synexin (30 min at 65°C). These results suggest that the synexin effect on PC secretion is not nonspecific. In another set of experiments, we evaluated whether antibody treatment of synexin would abolish the synexin effect on PC secretion. For these studies, indicated amounts of synexin were incubated for 18–20 h at 4°C with the synexin antibody. The antibody-synexin mixture was then used to evaluate its effect on PC secretion. An equivalent amount of synexin antibody was similarly treated in parallel but without synexin and evaluated for its effect on PC secretion. The antibody or the antibody-synexin mixture did not increase PC secretion (Table 3). These observations suggest that the effect of synexin on PC secretion in type II cells was specific. Furthermore, because synexin antibody alone did not decrease PC secretion when compared with control secretion, we suggest that the residual (possibly cell membrane-associated) synexin does not mediate the control (basal) secretion (Fig. 2B). However, we cannot exclude the possibility that synexin antibody may not be able to react with membrane-associated synexin and, therefore, is unable to inhibit its activity.

**Synexin and GTP.** In several cell types, GTP is known to promote exocytic secretion by mechanisms involving members of heterotrimeric or low-molecular-weight G proteins. Other investigators have postulated a role for G proteins in membrane fusion during exocytosis of lung surfactant. Previous studies on GTP binding to lamellar bodies revealed the presence of several low-molecular-mass G proteins, some of which could be related to the Ras family of proteins (34). By Western blot analysis, these authors reported the presence of Ras p21 and Rho proteins in the lamellar bodies. It is likely that some of these low-molecular-mass G proteins regulate lung surfactant secretion. Synexin has also been postulated to act as a Ca\(^{2+}\) sensor in the presence of GTP, since it could bind and hydrolyze GTP (10). Therefore, we investigated the effects of GTP and its nonhydrolyzable analog N\(_7\)HppGpp on PC secretion in permeabilized type II cells. The effects of GTP were investigated in the presence of 1 μM Ca\(^{2+}\). The PC secretion in the presence of 1 mM GTP was elevated in the presence of Ca\(^{2+}\) when compared

### Table 3. Effect of synexin antibody on synexin-stimulated PC secretion

<table>
<thead>
<tr>
<th>% [\text{H}2\text{PC} \text{Secretion in 30 min} ]</th>
<th>% Control</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.51 ± 0.55</td>
</tr>
<tr>
<td>+1 μM Ca(^{2+})</td>
<td>1.29 ± 0.47</td>
</tr>
<tr>
<td>+1 μM Ca(^{2+}) + 0.71 μg synexin + synexin Ab</td>
<td>3.12 ± 0.64</td>
</tr>
<tr>
<td>+1 μM Ca(^{2+}) + 0.71 μg synexin + synexin Ab</td>
<td>1.94 ± 0.99</td>
</tr>
<tr>
<td>+1 μM Ca(^{2+}) + synexin Ab</td>
<td>1.55 ± 0.85</td>
</tr>
</tbody>
</table>

Values are means ± SE of experiments in 3 preparations of type II cells. Isolated type II cells were permeabilized and evaluated for PC secretion during the 30-min incubation in the absence and presence of indicated agent. Synexin with and without synexin antibodies (Ab) were incubated for 20 h at 4°C before addition. Results are expressed as percentage of control values, which were analyzed for statistical significance by ANOVA followed by Newman-Keuls post hoc test. *P < 0.05 when compared with secretion in all other groups including secretion in the presence of 1 μM Ca\(^{2+}\).
Table 4. Synexin effects on GTP-stimulated secretion in permeabilized type II cells

<table>
<thead>
<tr>
<th></th>
<th>%[3H]PC Secretion in 30 min</th>
<th>%Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.62 ± 0.11</td>
<td>100</td>
</tr>
<tr>
<td>+1 μM Ca(^{2+})</td>
<td>0.82 ± 0.17</td>
<td>118 ± 24</td>
</tr>
<tr>
<td>+1 μM Ca(^{2+}) + 1 mM GTP</td>
<td>1.71 ± 0.42</td>
<td>318 ± 73</td>
</tr>
<tr>
<td>Synexin</td>
<td>1.36 ± 0.30</td>
<td>231 ± 42</td>
</tr>
<tr>
<td>+1 μM Ca(^{2+}) + 1 mM GTP + 3.5 μg/ml synexin</td>
<td>3.18 ± 0.69*</td>
<td>560 ± 92*</td>
</tr>
</tbody>
</table>

Values are means ± SE of experiments in 6 separate preparations of type II cells. *P < 0.05 by ANOVA followed by Newman-Keuls post hoc test when compared with secretion in the presence of Ca\(^{2+}\), GTP, or synexin.

Previous studies with streptolysin O-permeabilized type II cells showed that GTP can both stimulate and inhibit surfactant secretion and that the GTP effect was dependent on Ca\(^{2+}\) concentration in the assay medium (27). Later studies showed that activation of heterotrimeric G proteins may stimulate adenyl cyclase (28), which increases the cAMP levels and is associated with the increased secretion of lung surfactant (8, 26). More recently, exocytosis in type II cells was studied by measuring membrane capacitance and by following changes in the fluorescence of a dye, FM1-43, on binding of lipids in the lamellar body (25). These studies showed that guanosine 5′-(3-thiotriphosphate), a nonhydrolyzable analog of GTP, increases the formation of exocytic fusion pores in type II cells, suggesting increased secretion of lung surfactant. Our results of increased secretion with GTP and NHpGpp also suggest the involvement of G proteins in lamellar body exocytosis. The GTP-dependent mechanisms do not seem to require synexin for augmentation of PC secretion, since the effects of GTP were additive with those of synexin and the GTP modulation of PC secretion was observed in synexin-deficient type II cells (Fig. 2B and Table 4).

Several members of the low-molecular-mass G proteins, Ras, Rho, ADP-ribosylation factor (ARF), Rab, and Ran, which are active in GTP-bound form, are invoked to play a major role in various cellular processes like growth, morphogenesis, vesicle trafficking, and exocytosis (35). The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex is postulated to form the basic machinery for vesicle docking and membrane fusion during vesicle trafficking and exocytosis (33, 38). GTP is suggested to promote exocytic secretion in several cell types by acting on the low-molecular-mass G proteins such as Rab and ARF. It is suggested that the GTP-bound form of Rab recognizes the target SNAREs and facilitates their pairing with vesicle SNAREs to promote vesicle docking and membrane fusion (35). The low-molecular-mass G proteins are also present in type II cells and at least two of these, Ras p21 and Rho, and the substrates for ARF are present in lamellar bodies (34). If the GTP effect on PC secretion in type II cells is mediated by facilitating the SNARE pathway, then our observation of apparently additive effects of synexin and GTP would suggest that synexin acts independently of the SNARE pathway.

A previous study reported that only annexins I, II, III, and VI were present in type II cells (24). Our study shows that synexin is also present in type II cells. Like our study showing augmentation of surfactant secretion by synexin, the study by Liu et al. (24) showed that annexin II tetramer also increased PC secretion in permeabilized type II cells. These authors suggested that annexin II was necessary for surfactant PC exocytosis. However, our studies show that synexin can also increase PC secretion in β-escin-permeabilized type II cells that were shown to be deficient in annexin II (24). In PC12 cells also, the expression of structurally modified annexin II, which caused its aggregation
and functional depletion, or overexpression of annexin II did not affect Ca\(^{2+}\)-dependent dopamine secretion (20). Thus it is unlikely that annexin II is necessary for membrane fusion during exocytic secretion. Our studies also suggest that type II cells may contain annexin-dependent and annexin-independent (some of which may be GTP-dependent) mechanisms for secretion. Because of similar characteristics of synexin and annexin II (Ca\(^{2+}\)-dependent phospholipid binding, augmentation of surfactant PC secretion), we suggest that synexin and annexin II can substitute for each other during annexin-dependent membrane fusion for PC secretion in type II cells.

The annexin-independent mechanism of membrane fusion may function during control (basal) secretion and during GTP-dependent secretion. Because of similar conditions for cell permeabilization (this study and Ref. 24), we assume that the β-escin-permeabilized type II cells were deficient in both synexin and annexin II. Thus the control secretion in these cells was through the annexin-independent mechanism. Because synexin antibodies did not inhibit PC secretion under control conditions (Table 3), we suggest that residual synexin in permeabilized type II cells did not mediate the control PC secretion. However, we cannot rule out that synexin antibody may not be able to inactivate cell-associated residual synexin. The GTP stimulation of PC secretion in synexin-deficient cells (Table 4) would also support the presence of an annexin-independent mechanism. It is likely that the proteins of the SNARE complex that are associated with the lamellar bodies and the plasma membrane bring about the control secretion, which is further facilitated with the addition of GTP because of its postulated role in the vesicle docking and membrane fusion process (35).

The synexin-dependent mechanism of membrane fusion and PC secretion requires Ca\(^{2+}\) (Table 1), which is consistent with previously described properties of synexin and other annexins (15, 19). In the studies reported here, we employed low concentrations of Ca\(^{2+}\) to evaluate synexin effects on surfactant PC secretion because of the low Ca\(^{2+}\) threshold for synexin binding to biological membranes (37). Although our earlier studies on membrane fusion between lamellar bodies and plasma membranes were carried out with high (1 mM) Ca\(^{2+}\) (14), our later studies suggest that synexin binding to biological membranes, which can occur at low Ca\(^{2+}\), may be the rate-limiting step in synexin-dependent membrane fusion (37). Therefore, we used low Ca\(^{2+}\) concentrations to evaluate PC secretion in permeabilized type II cells. Because synexin increased PC secretion at low Ca\(^{2+}\), we suggest that the threshold Ca\(^{2+}\) concentrations for synexin binding are sufficient to facilitate membrane fusion during surfactant secretion. The underlying reasons for different Ca\(^{2+}\) requirements for the in vitro fusion (14) and for PC secretion in permeabilized type II cells (this study) are not clear but may be related to the presence of membrane proteins, which may be lost during isolation of subcellular fractions.

The mechanism of synexin action during surfactant secretion is not clear. In vitro studies have shown that synexin can undergo Ca\(^{2+}\)-dependent self-association (16, 23). The self-associated form of synexin may bind to membranes and establish intermembrane contact, which is postulated as an essential step in the hydrophobic bridge hypothesis for membrane fusion (30). In analogy with the SNARE mechanism of membrane fusion, which involves several proteins, the synexin-dependent membrane fusion may also involve other proteins. It is noteworthy that some proteins can bind to synexin and thus may regulate its activity. We have previously shown that synexin binding to a specific protein (76 kDa) in the lung lamellar body and plasma membrane increases in a Ca\(^{2+}\)-dependent manner (37). Studies with recombinant synexin peptide showed that sorcin (soluble resistance-related Ca\(^{2+}\)-binding protein) can bind to the amino domain of synexin in a Ca\(^{2+}\)-dependent manner and inhibit synexin-mediated chromaffin granule aggregation (9). Thus the presence of these synexin-binding proteins suggests that synexin may not function alone in the membrane fusion process during surfactant secretion.

In summary, our studies show the presence of synexin in alveolar type II cells. Using permeabilized alveolar type II cells, we have provided direct evidence for involvement of synexin in surfactant secretion. Our studies also suggest that the membrane fusion for PC secretion in type II cells may be mediated by synexin (annexin)-dependent and -independent mechanisms. The presence of several independent mechanisms for membrane fusion during surfactant secretion possibly protects against defects in one pathway and underscores the importance of the secretion process. Although each mechanism can support secretion, the maximal secretion of surfactant may require that both mechanisms function properly.

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L998 SYNEXIN IN LUNG SURFACTANT SECRETION


