Molecular Mechanisms of Membrane Fusion

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STRATEGIES FOR THE INVESTIGATION OF EXOCYTOTIC MEMBRANE FUSION

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INTRODUCTION

The transfer of hormones from the cell into the extracellular space during secretion by exocytosis is accomplished by the fusion of the secretory vesicle membrane with the plasma membrane. As opposed to the study of artificial membranes or isolated natural membranes as models, suitable modified secretory cells have been introduced in the past few years to investigate exocytotic membrane fusion. The groups working in the latter field are attempting to learn as much as possible from nature with the aim of characterizing the properties of membrane fusion as it occurs in the intact cell.

The inherent problem in such investigations is that the interacting membranes are not accessible from outside the cells. Manipulation of the cytoplasm in a rather limited way can be carried out using ionophores. Direct access to the cytoplasm and its complete control requires the breakdown of the plasma membrane as a permeability barrier. With single cells this can be done using cells attached to pipettes (c.f. Fernandez et al., 1984). Cell suspensions have been permeabilized with different techniques without impairing the process of exocytosis. With high-voltage discharges and detergents, it has been possible to permanently permeabilize cells for secretion studies. In both ways, if carefully controlled, the cells seem not to be altered greatly in so far as the release process is concerned (c.f. Knight and Baker, 1982; Wilson and Kirshner, 1983; Dunn and Holz, 1983). The lesions evoked in the cell membrane with these methods differ in size. With detergents, cellular macromolecules (like cytoplasmic lactate dehydrogenase) are lost, while with high voltage discharges few such molecules escape. In contrast to the techniques mentioned above, defined pores can be inserted into the cell membrane of secretory cells using staphylococcal alpha-toxin. This allows small solutes to be exchanged and keeps macromolecules necessary for secretion within the cells. The application of alpha-toxin for the analysis of exocytotic membrane fusion (Ahnert-Hilger et al., 1985a,b, 1987;

Bader et al., 1986; Lind et al., 1987; Ahnert-Hilger and Gratzl, 1987) will be described in this chapter. The characteristics of hormone release from permeabilized cells will be compared with secretion by intact cells and fusion of isolated secretory vesicle membranes and of liposomes.

PERMEABILIZATION OF SECRETORY CELLS WITH ALPHA-TOXIN

Alpha-toxin is a water soluble protein produced by most strains of Staphylococcus aureus. The mechanism of the toxins attack of mammalian cells has been established in a series of investigations (Füssle et al., 1981; Bhakdi et al., 1981, 1984; for review see Bhakdi and Tranum-Jensen, 1987). First the toxin (molecular weight 34 KD) is bound to the cells, then it hexamerizes in the plasma membrane and forms stable transmembrane pores, which do not permit the passage of macromolecules with a molecular weight of 4 KD and larger but allows for the release of small molecules from erythrocytes.

Secretory cells can also be permeabilized by alpha-toxin. Bovine chromaffin cells, rat pheochromocytoma or insulinoma cells in culture, upon addition of alpha-toxin, release small solutes like monovalent cations or ATP and take up trypan blue while large proteins such as cytoplasmic lactate dehydrogenase are retained by the cells (Ahnert-Hilger et al., 1985; Bader et al., 1986; Lind et al., 1987). In other words the size of the alpha-toxin pore allows for the removal of all small molecules from the cytoplasm and their replacement by other ones. Therefore this technique can serve to add buffer substances for the control of pH and divalent cations. In addition, drugs known to interfere with defined intracellular regulator sites, but ineffective in intact cells due to limited access, can easily be introduced into secretory cells. Finally, the remarkable insensitivity of secretory vesicle fusion with the plasma membrane to the incorporation of alpha-toxin allows sufficient time for the experimental manipulation of the permeabilized cells.

As a consequence of the small size of the alpha-toxin pore, the toxin monomer itself is excluded from the cytoplasm. Thus the toxin's attack is restricted to the plasma membrane which is an advantage when compared to the action of detergents used for cell permeabilization. Also detergents like digitonin harbor the hazards of modifying the composition of the interacting membrane and thus distorting the data obtained.

As evidence for exocytosis in alpha-toxin permeabilized cell preparations, the same criteria can be used which were applied to intact cells: E. g. in order to exclude simple leakage of hormones from secretory vesicles the parallel release of small and large molecular weight secretory products present within the vesicles can be measured. This co-release, in conjunction with the observation that large molecules residing in the cytoplasm remain within the cells clearly indicates that hormone release occurs via exocytosis (Ahnert-Hilger et al., 1985b; Bader et al., 1986). Another approach to demonstrate exocytosis makes use of the compartmentation of enzymes metabolizing catecholamines (Ahnert-Hilger et al., 1987). Catecholamines are released

unchanged from intact pheochromocytoma cells because the cytoplasm containing the oxidizing enzymes is circumvented if secretion occurs by exocytosis. When transferred into the cytoplasm (by reversing the driving force for vesicular uptake of catecholamines) oxidized catecholamines are formed. Since the same situation is seen in alpha-toxin permeabilized cells, it is safe to conclude that the secretory vesicle contents are directly transported by exocytosis into the extracellular space.

Recently, in addition to the other documented procedures (c.f. references Bhakdi and Tranum-Jensen, 1987) a rapid and effective purification procedure for alpha-toxin from the culture medium of Staphylococcus aureus (strain Wood 46) has been described (Lind et al., 1987). This technique facilitates again the study of membrane fusion in permeabilized secretory cells.

CHARACTERIZATION OF EXOCYTOSIS IN ALPHA-TOXIN PERMEABILIZED CELLS

In the first series of experiments the minimal (cytoplasmic) requirements for secretory vesicle/plasma membrane fusion have been established in alpha-toxin permeabilized PC12 cells, a rat pheochromocytoma cell line. It was demonstrated that the release of dopamine from these cells upon addition of alpha-toxin was reduced from "extracellular" (mM) to intracellular (μM) calcium concentrations (Ahnert-Hilger et al., 1985a). In media containing defined free concentrations of calcium (adjusted with chelators and controlled with a calcium-specific electrode) a characteristic biphasic response as a function of the free calcium concentration could be observed (Fig. 1 and Ahnert-Hilger et al., 1985a,b; Ahnert-Hilger and Gratzl, 1987). One phase already activated below 1 μM free calcium, plateaued at about 1-5 μM free calcium and a second occurred in the presence of larger amounts of free calcium (10-100 μM). Calcium-induced dopamine release could be observed at pH 6.6 as well as at pH 7.2, it was insensitive to changes in the nature of monovalent cations or anions. Magnesium was unable to replace calcium. However, when present simultaneously with calcium in 1-5 μM concentrations it augmented hormone release (Fig. 2 and Ahnert-Hilger and Gratzl, 1987). It is interesting to note that the effect of magnesium was only seen at the low calcium concentrations which are likely to occur in stimulated cells (see next chapter). Further attempts to characterize or identify the targets (receptors) of calcium within the pheochromocytoma cells using group-specific reagents or drugs interfering with calmodulin (trifluoperazine) have not yet been successful.

One reason for that may be that additional modulatory reactions involved in the regulation of exocytosis are working in alpha-toxin permeabilized cells. From the inhibitory action of GTP-gamma-S on calcium-induced exocytosis, a modulatory role of GTP binding proteins has been inferred as for the activation by TPA an involvement of the proteinkinase C cascade (Ahnert-Hilger et al., 1987). The latter effect requires the presence of ATP while the calcium-induced exocytosis in the pheochromocytoma cells used is entirely

independent on this nucleotide (Ahnert-Hilger et al., 1985a,b; Ahnert-Hilger and Gratzl, 1987).

Interestingly, chromaffin cells in primary culture also in the absence of TPA require additional ATP for calcium-induced exocytosis (Bader et al., 1986). Other nucleotides could not replace ATP suggesting a very specific role in these cells. The ATP requirement of secretion in intact cells will be discussed in detail in the next section of this article.

COMPARISON OF EXOCYTOSIS IN ALPHA-TOXIN PERMEABILIZED CELLS WITH MEMBRANE FUSION OF BIOLOGICAL MEMBRANES AND OF LIPOSOMES

The importance of extracellular calcium in stimulus-secretion coupling was realized more than 25 years ago (Douglas and Rubin, 1961). Injection studies with mast cells and the giant synapse indicated that intracellular calcium may trigger secretion (Kanno et al., 1973; Miledi, 1973). After it was observed that transmitter release is actually paralleled by an increase in intracellular free calcium (Llinàs and Nicholson) this issue was further investigated in a variety of secretory cells. These studies soon became experimentally easier to be carried out because new techniques became available. Resting pheochromocytoma cells or adrenal medullary chromaffin cells in primary culture, i. e. the cells used up to now for permeabilization with alpha-toxin, contain about 0.1 µM free calcium.

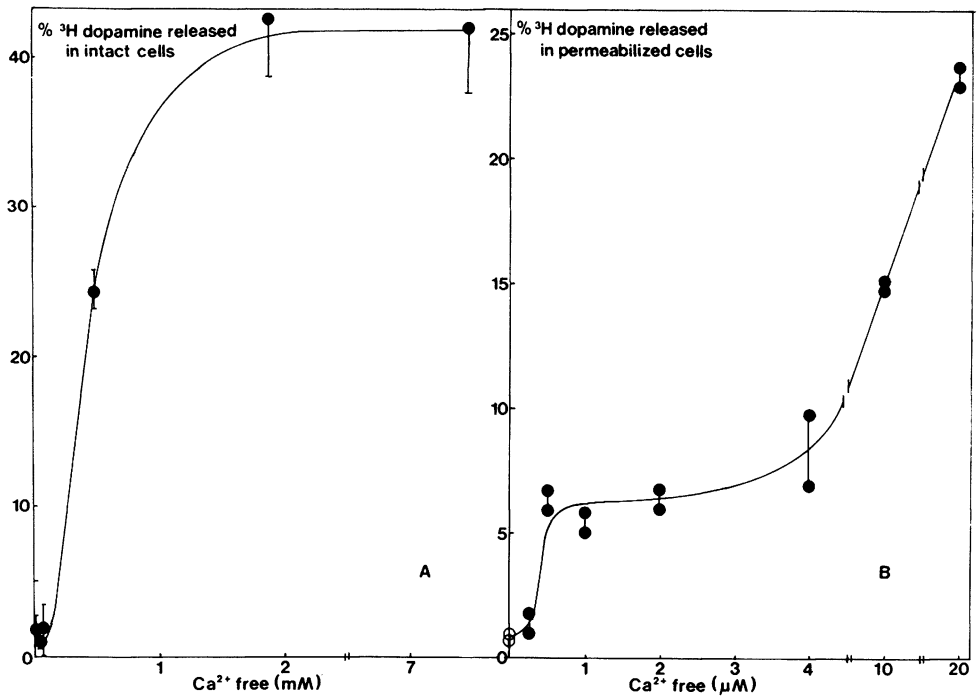

Fig. 1. Calcium requirement of dopamine release by intact and by alpha-toxin permeabilized pheochromocytoma cells (from Ahnert-Hilger et al., 1985a, by permission).

When stimulated, the free calcium increases to about 0.5 μM (Knight and Kesteven, 1983; Meldolesi et al., 1984). It is worth mentioning, however, that an average intracellular free calcium concentration has been measured in these studies and the true concentrations just beneath the plasma membrane may be higher as a consequence of the calcium influx during stimulation. In another study (Kao and Schneider, 1986) values of about 10 μM free calcium have been obtained in chromaffin cells stimulated with nicotine.

In any case, the calcium dependence of catecholamine secretion by intact cells is in accordance with the situation seen in alpha-toxin permeabilized pheochromocytoma cells and adrenal medullary chromaffin cells in primary culture (see previous chapter). Interestingly enough, also the dependence on ATP in alpha-toxin permeabilized chromaffin cells (Bader et al., 1986) is paralleled by a similar observation in intact cells (Rubin, 1969; Kirshner and Smith, 1965). Intact pheochromocytoma cells, on the other hand, do not depend on cytoplasmic ATP during secretion (Reynolds et al., 1982), a fact which is supported by the insensitivity of calcium-induced dopamine release to ATP in the same cells permeabilized with alpha-toxin (Ahnert-Hilger et al., 1985a,b; Ahnert-Hilger and Gratzl, 1987).

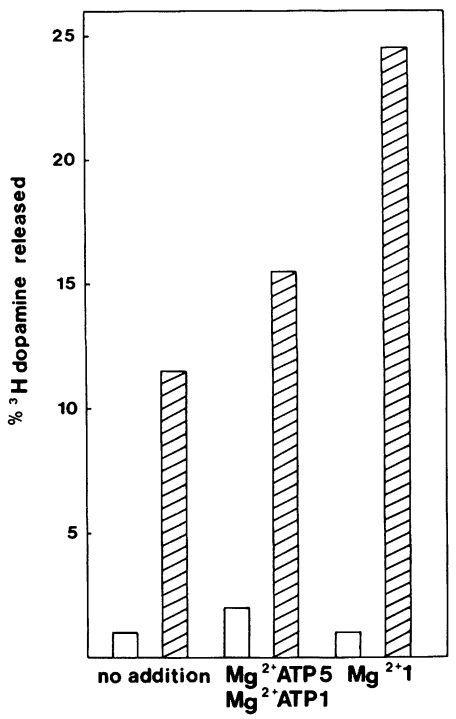

Fig. 2. Effect of magnesium with or without ATP on calcium-induced dopamine release by permeabilized pheochromocytoma cells. The open bars represent controls, the hatched bars alpha-toxin treated cells. The medium contained 10 μM free calcium and the indicated amounts of free magnesium (from Ahnert-Hilger and Gratzl, 1987, by permission).

The exact reason for the necessity of ATP in permeabilized chromaffin cells in primary culture is not known. One possibility is that regulatory sequences (like the proteinkinase C system) have all components activated but one key protein is dephosphorylated and requires ATP for rephosphorylation. In order to identify such sites phosphorylation studies have been carried out using intact cells, permeabilized cells, or subcellular fractions (Schulman and Greengard, 1978; Amy and Kirshner, 1981; Treiman et al., 1983; Niggli et al., 1984). However, also the topography of chromaffin vesicle distribution in the different cells investigated must be considered. In cultured pheochromocytoma cells secretory vesicles are concentrated near the plasma membrane (Watanabe et al., 1983) and thus are close to the site of exocytosis. On the other hand within adrenal medullary chromaffin cells secretory vesicles are more randomly distributed (c.f. Bader et al., 1981) and ATP dependent vesicle movement (Allen et al., 1985; Vale et al., 1985) could be necessary.

The step requiring ATP is not the process of membrane fusion itself but a preceding one, since isolated secretory vesicles isolated from chromaffin and other secretory cells fuse without ATP added. These biological membranes fuse half maximal with about 1-10 μM free calcium, i. e. the same concentration observed in stimulated intact cells or the amount of calcium effective in alpha-toxin permeabilized cells (Dahl and Gratzl, 1976; Ekerdt et al., 1981; Gratzl and Dahl, 1976, 1978; Gratzl et al., 1977, 1980). When the membranes investigated were further simplified by the preparation of liposomes from the membrane lipids of chromaffin vesicles, characteristic properties like the susceptibility to low concentrations of calcium for the fusion process were lost (Ekerdt et al., 1981). In conjunction with the observation that treatment with chemicals or enzymes of the secretory vesicle membranes led to the same changes in membrane fusion properties, it was concluded that membrane proteins are responsible for these properties (Ekerdt et al., 1981). Actually liposomes containing acidic phospholipids exhibit very similar fusion properties as do the liposomes prepared from the membrane lipids of isolated secretory vesicles (Ekerdt et al., 1981; c.f. for ref. Düzgünes, 1985). In attempts to reduce the calcium requirements of these membranes from mM to more physiological (μM) concentrations the effect of synexin, a cytoplasmic protein present in a variety of secretory cells has been investigated (Hong et al., 1982a,b). It turned out that with this protein, in the presence of mM magnesium, the threshold of the liposomes to undergo fusion could be reduced to about 10 μM calcium. This indicates that fusion of liposomes can be modulated in a way to approach the milieu existing in resting or stimulated cells.

CONCLUSION

Permeabilization of secretory cells by staphylococcal alpha-toxin indeed is a valuable technique to investigate membrane fusion during exocytosis because it allows the permanent control of the composition of the cytoplasm with respect to small molecules (MW 1 KD) without perturbing the fusion process itself. The secretion observed in such

permeabilized cells is compatible with secretion by intact cells and closely resembles membrane fusion studied with isolated secretory vesicle membranes.

After extensively studying the effects of small solutes, inhibitors or activators, the concept of using natural occurring pore forming substances for cell permeabilization opens further possibilities. Permanent pores which are big enough to release even large molecular weight substances from the cells or to introduce antibodies directed against membrane components still residing in permeabilized cells can be used to identify the relevant membrane proteins involved in exocytotic membrane fusion. Then they can be isolated and incorporated into liposomes with the aim of modifying their properties in a way that the fusion process in this simplified system is compatible with the situation seen in intact secretory cells. This would then elucidate the molecular mechanism of membrane fusion during exocytosis.

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REFERENCE LIST

Ahnert-Hilger, G., and Gratzl, M., 1987, Further characterization of dopamine release by permeabilized PC12 cells, J. Neurochem., in press.

Ahnert-Hilger, G., Bhakdi, S., and Gratzl, M., 1985a, Alpha-toxin permeabilized cells - A new approach to investigate stimulus secretion coupling, Neurosci. Lett., 58:107.

Ahnert-Hilger, G., Bhakdi, S., and Gratzl, M., 1985b, Minimal requirements for exocytosis - a study using PC 12 cells permeabilized with staphylococcal alpha-toxin, J. Biol. Chem., 260:12730.

Ahnert-Hilger, G., Bräutigam, M., and Gratzl, M., 1987, Calcium-stimulated catecholamine release from alpha-toxin permeabilized PC12 cells: Biochemical evidence for exocytosis and its modulation by TPA and GTP-gamma-S, submitted

Allen, R. D., Weiss, D. G., Hayden, J. H., Brown, D. T., Fujiwake, H., and Simpson, M., 1985, Gliding movement of and bidirectional transport along single native microtubules from squid axoplasm: Evidence for an active role of microtubules in cytoplasmic transport, J. Cell Biol., 100:1736.

Amy, C. M., and Kirshner, N., 1981, Phosphorylation of adrenal medulla cell proteins in conjunction with stimulation of catecholamine secretion, J. Neurochemistry, 36:847.

Bader, M. F., Ciesielski-Treska, J., Thiersé, D., Hesketh, J. E., and Aunis, D., 1981, Immunocytochemical study of microtubules in chromaffin cells in culture and evidence that tubulin is not an integral protein of the chromaffin granule membrane, J. Neurochem., 37:917.

Bader, M. F., Thiersé, D., Aunis, D., Ahnert-Hilger, G., and Gratzl, M., 1986, Characterization of hormone and protein release from alpha-toxin permeabilized chromaffin cells in primary culture, J. Biol. Chem., 261:5777.

Bhakdi, S., Muhly, M., and Füssle, R., 1984, Correlation between toxin binding and hemolytic activity in membrane damage by staphylococcal alpha-toxin, Infect. Immun., 46:318.

Bhakdi, S., Füssle, R., and Tranum-Jensen, J., 1981, Staphylococcal alpha-toxin: Oligomerization of hydrophilic monomers to form amphiphilic hexamers induced through contact with deoxycholate detergent micelles, Proc. Natl. Acad. Sci. USA, 78:5475.

Bhakdi, S., and Tranum-Jensen, J., 1987, Damage to mammalian cells by proteins that form transmembrane pores, Rev. Physiol. Biochem. Pharmacol., 107:147.

Dahl, G., and Gratzl, M., 1976, Calcium-induced fusion of isolated secretory vesicles from the islet of Langerhans, Cytobiologie, 12:344.

Douglas, W. W., and Rubin, R. P., 1961, The role of calcium in the secretory response of the adrenal medulla to acetylcholine, J. Physiol. (London), 159:40

Dunn, L. A., and Holz, R. W., 1983, Catecholamine secretion from digitonin-treated adrenal medullary chromaffin cells, J. Biol. Chem., 258:4989.

Düzgünes, N., 1985, Chapter 5: Membrane Fusion, in: "Subcellular Biochemistry," Vol. 11, D. B. Roodyn, ed., Plenum Press, New York.

Ekerdt, R., Dahl, G., and Gratzl, M., 1981, Membrane fusion of secretory vesicles and liposomes: two different types of fusion, Biochim. Biophys. Acta, 646:10.

Fernandez, J. M., Neher, E., and Gomperts, B. D., 1984, Capacitance measurements reveal stepwise fusion events in degranulating mast cells, Nature, 312:453.

Füssle, R., Bhakdi, S., Sziegoleit, A., Tranum-Jensen, J., Kranz, T., and Wellensiek, H.J., 1981, On the mechanism of membrane damage by Staphylococcus aureus alpha-toxin, J. Cell Biol., 91:83.

Gratzl, M., and Dahl, G., 1976, Calcium-induced fusion of Golgi derived secretory vesicles isolated from rat liver, FEBS Letters, 62:142.

Gratzl, M., and Dahl, G., 1978, Fusion of secretory vesicles isolated from rat liver, J. Membrane Biology, 40:343.

Gratzl, M., Dahl, G., Russell, J. T., and Thorn, N. A., 1977, Fusion of neurohypophyseal membranes in vitro, Biochim. Biophys. Acta, 470:45.

Gratzl, M., Schudt, Ch., Ekerdt, R., and Dahl, G., 1980, Fusion of isolated biological membranes. A tool to investigate basic processes of exocytosis and cell-cell fusion. in: "Membrane structure and function," E. E. Bittar, ed., John Wiley, New York, p 59.

Hong, K., Düzgünes, N., Ekerdt, R., and Papahadjopoulos, D., 1982, Synexin facilitates fusion of specific

phospholipid vesicles at divalent cation concentrations found intracellularly, Proc. Natl. Acad. Sci. USA, 79:4942.

Hong, K., Düzgünes, N., and Papahadjopoulos, D., 1982, Modulation of membrane fusion by calcium-binding proteins, Biophys. J., 37:297.

Kanno, T., Chochrane, D. E., and Douglas, W. W., 1973, Exocytosis (secretory granule extrusion) induced by injection of calcium into mast cells, Can. J. Physiol. Pharmacol., 51:1001.

Kao, L. S., and Schneider, A. S., 1986, Calcium mobilization and catecholamine secretion in adrenal chromaffin cells, J. Biol. Chem. 261:4881.

Knight, D. E., and Baker, P. F., 1982, Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields, J. Membrane Biol., 68:107.

Knight, D. E., and Kesteven, N. T., 1983, Evoked transient intracellular free calcium changes and secretion in isolated adrenal medullary cells, Proc. R. Soc. Lond. B, 218:177.

Lind, I., Ahnert-Hilger, G., Fuchs, G., and Gratzl, M., 1987, Purification and application for cell permeabilization of alpha-toxin from staphylococcus aureus, Anal. Biochem., in press.

Llinás, R., and Nicholson, C., 1975, Calcium role in depolarization-secretion coupling: An aequorin study in squid giant synapse, Proc. Natl. Acad. Sci. USA, 72:187.

Meldolesi, J., Huttner, W. B., Tsien, R. Y., and Pozzan, T., 1984, Free cytoplasmic Calcium and neurotransmitter release: studies on PC12 cells and synaptosomes exposed to alpha-latrotoxin, Proc. Natl. Acad. Sci. USA, 81:620.

Miledi, R., 1973, Transmitter release induced by injection of calcium ions into nerve terminals, Proc. R. Soc. Ser. B, 183:421.

Niggli, V., Knight, D. E., Baker, P., F., Vigny, A., and Henry, J.-P., 1984, Tyrosine hydroxylase in "leaky" adrenal medullary cells: Evidence for in situ phosphorylation by separate calcium and cyclic AMP-dependent systems, J. Neurochem., 43:646.

Schulman, H., and Greengard, P., 1978, Calcium-dependent protein phosphorylation system in membranes from various tissues, and its activation by "calcium-dependent-regulator", Proc. Natl. Acad. Sci. USA, 75:5432.

Treiman, M., Weber, W., and Gratzl, M., 1983, 3', 5'-cyclic adenosine monophosphate- and Calcium-calmodulin-dependent endogenous protein phosphorylation activity in membranes of the bovine chromaffin secretory vesicles: Identification of two phosphorylated components as tyrosine hydroxylase and protein kinase regulatory subunit type II, J. Neurochem., 40:661.

Vale, R. D., Schnapp, B. J., Reese, T. S., and Sheetz, N. P., 1985, Organelle, bead, and mikrotubule translocations promoted by soluble factors from the squid giant axon, Cell, 40:559.

Watanabe, D., Torda, M., and Meldolesi, J., 1983, The effect of alpha-latrotoxin on the neurosecretory PC12 cell line: Electron microscopy and cytotoxicity studies, Neuroscience, 10:1011.

Wilson, S. P., and Kirshner, N., 1983, Calcium-evoked secretion from digitonin-permeabilized adrenal medullary chromaffin cells, J. Biol. Chem., 258:4994.