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MINI-REVIEW

Evaluation of the Annexins as Potential Mediators of Membrane Fusion in Exocytosis

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Abstract

Membrane fusion is a central event in the process of exocytosis. It occurs between secretory vesicle membranes and the plasma membrane and also among secretory vesicle membranes themselves during compound exocytosis. In many cells the fusion event is regulated by calcium. Since the relevant membranes do not undergo fusion in vitro when highly purified, much attention has been paid to possible protein mediators of these calcium-dependent fusion events. The annexins comprise a group of calcium-dependent membrane-aggregating proteins, of which synexin is the prototype, which can initiate contacts between secretory vesicle membranes which will then fuse if the membranes are further perturbed by the addition of exogenous free fatty acids. This review discusses the secretory pathway and the evidence obtained from *in vitro* studies that suggests the annexins may be mediators or regulators of membrane fusion in exocytosis.

Key Words: Calcium-binding proteins; membrane-binding proteins; chromaffin granules; synexin; calpactin; lipocortin; secretion.

Introduction

Eukaryotic cells contain a number of distinct compartments bounded by membranes. During the life of the cell, communication between these intracellular compartments is frequently necessary, including the transfer of macromolecules. Since the membrane bilayer is for practical purposes normally an impenetrable barrier to proteins, special mechanisms have developed to permit such substrates to cross membrane barriers. During the synthesis

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of secretory proteins, linear polypeptide chains are slipped through membranes by specialized machinery coincident with their translation from messenger RNA. Preformed macromolecules have greater difficulty crossing membranes and frequently rely on the fusion of membranes of separate compartments to enable them to pass from compartment to compartment or to the external environment. Nowhere is this more obvious than in the secretory pathway of eukaryotic cells. Since the fusion events in this pathway appear temporally regulated and spatially directed, it has been natural to propose that protein molecules must provide the orchestration of these fusion events. However, only a limited number of proteins have been identified that may be involved in these events. Particularly recent discoveries include the GTP-binding proteins (Salminen and Novick, 1987; Segev *et al.*, 1988) and the NEM-sensitive protein (Wilson *et al.*, 1989) that genetic analyses in yeast have demonstrated play essential roles in membrane fusion. However, the exact functions of these recently described proteins in the fusion process are quite unclear. On the other hand, proteins of the annexin family, as first described over ten years ago with the discovery of synexin (Creutz *et al.*, 1978), possess the ability to promote membrane fusion events *in vitro* that appear similar to the membrane fusion events occurring in the secretory pathway. Since the annexins are calcium-dependent and interact with secretory vesicle and plasma membranes, and some annexins, such as calpactin, are localized just below the plasma membrane (Burgoyne and Cheek, 1987), they are excellent candidates for mediators of membrane fusion in exocytosis. However, the annexins are distinct from viral fusion proteins in that they are not fusogenic themselves but merely promote the close association of membranes which will then fuse if further perturbed by free fatty acids (Creutz, 1981; Drust and Creutz, 1988). This review will begin with an overview of secretion so that it can be appreciated where the annexins may act intracellularly. Then we will discuss the nature of the physical interaction of these proteins with membrane lipids. Finally we will consider possible mechanisms underlying the actual fusion event mediated by these proteins.

Overview of the Secretory Process

Exocytosis is the process of secretion in which materials within intracellular vesicles (secretory granules) are released from the cell by the fusion of the granule membrane with the plasma membrane, followed by discharge of the granule contents into the extracellular space. This process plays a vital role in membrane biogenesis and is the basis for such important phenomena as neurotransmission and hormone and enzyme secretion (Campbell, 1983). The pathway of exocytosis has been well defined (Butcher, 1978). It begins

with the synthesis of secretory proteins on ribosomes attached to the rough endoplasmic reticulum (ER). These proteins enter the lumen of the ER and are thought to be transported to the Golgi complex by ER-derived transport vesicles (Kelly, 1985). In the Golgi complex the proteins are modified, sorted, concentrated, and packaged into vesicles which bud from the Golgi apparatus. Small molecules, which are the active secretory components in many cell types, appear to be actively transported into secretory vesicles that have already formed (Pollard *et al.*, 1985). After synthesis and assembly in the Golgi, the granules may be considered to proceed through a series of distinct stages involving (1) movement through the cytoskeletal matrix, (2) attachment to, and subsequent fusion with, the plasma membranes, and (3) recovery by endocytosis to either be recycled through the Golgi or discarded through the lysosomes.

Constitutive and Regulated Secretion

Although the basic pathway of exocytotic secretion is similar in most cells, there is some variability between cell types in certain individual steps. In constitutive secretory cells such as myocytes, fibroblasts, and hepatocytes, secretory products such as extracellular matrix components reach the cell surface minutes after leaving the Golgi. This process occurs independently of extracellular stimuli and proceeds at a steady rate. The transport vesicles have a short half-life, and when viewed in electron micrographs, lack the electron-dense core found in electron micrographs of conventional secretory vesicles. Regulated secretory cells such as endocrine and exocrine cells, however, are specialized to release, for a brief period, large amounts of protein at a rapid rate. These cells store newly synthesized secretory product in vesicles with a half-life of days. These vesicles have characteristic electron-dense cores and accumulate in the cytoplasm of the cell, fusing with the cell membrane only when the level of some intracellular messenger is altered.

Much accumulated evidence has led to the suggestion that an increase in the intracellular concentration of free Ca^{2+} is the intracellular signal for exocytosis in a wide variety of secretory cells (Rubin, 1982). The source of this Ca^{2+}, however, may vary for different cell types. In many cells secretion is dependent on the presence of extracellular calcium. Calcium may enter these cells through voltage-dependent Ca^{2+} channels in the plasma membrane if excitation is associated with depolarization of the cell, or through receptor-operated Ca^{2+} channels activated through binding of excitatory hormones to their receptors. In other cells, secretion is independent of extracellular calcium, and the source of this cation appears to be the endoplasmic reticulum (Berridge, 1984). The magnitude, rate of rise, and

distribution of intracellular Ca^{2+} necessary for secretion is currently a matter of debate (Pollard *et al.*, 1985). Several investigators have attempted to measure or calculate the magnitude of the intracellular rise in calcium concentration achieved during cell stimulation. Using the fluorescent probe quin-2, it was found that the Ca^{2+} concentration in resting chromaffin cells was approximately 0.1 μM (Baker and Knight, 1984; Knight and Kestenen, 1983). In response to a secretory stimulus the Ca^{2+} concentration rose to near 1.0 μM following Ca^{2+} influx from the extracellular space (Baker and Knight, 1984). A major drawback of these studies, however, is that quin-2 gives only an estimate of the average Ca^{2+} concentration throughout the cytoplasm, and provides no information on Ca^{2+} transients much greater than 1–2 μM. Creutz (1984), using the Ca^{2+} flux data of Llinas *et al.* (1976) from the presynaptic terminal of the squid giant synapse and a few simplifying assumptions, calculated the free Ca^{2+} concentrations achieved at various depths in the cytoplasm as a function of time. These calculations demonstrated that Ca^{2+} concentrations as high as 70 μM may be achieved transiently at the inner surface of the plasma membrane. This estimated value would be even higher if the calcium entered only at discrete regions of the membrane such as the sites of the Ca^{2+} channels, and the internal calcium were not uniformly distributed, but preferentially associated with negatively charged membrane surfaces. Because of these unknown parameters, Kelly *et al.* (1979) reasoned that the internal Ca^{2+} concentrations in the region of the exocytotic site may reach between 1 μM and 1 mM during cell stimulation.

Recently, several investigators have used chromaffin cells rendered permeable by electric discharge, detergents, or channel-forming bacterial toxins to investigate directly the Ca^{2+} concentration dependence of exocytosis (Baker and Knight, 1981; Dunn and Holz, 1983; Wilson and Kirshner, 1983; Bader *et al.*, 1986). In most studies, one phase of secretion titrated roughly between 0.1 and 10 μM free Ca^{2+}. However, in some studies, additional phases of secretion were observed to titrate between 1 and 100 μM, and between 100 μM and 1 mM free Ca^{2+}. It is unclear which phase more closely mimics the naturally occurring process of Ca^{2+}-dependent exocytosis. Indeed, it is possible that each phase may represent activation of a different contributory step in the overall pathway.

Although the requirement for Ca^{2+} in regulated secretion has been well recognized, the molecular mechanisms by which Ca^{2+} acts remain largely unknown. In view of the common occurrence of membrane transport and fusion in all cell types, the study of Ca^{2+}-triggered exocytosis must focus on factors inhibiting membrane fusion in the absence of Ca^{2+} and/or factors promoting fusion in the presence of Ca^{2+}. Given the wide range of Ca^{2+} concentrations potentially important for exocytosis, this must include a study of both higher-affinity Ca^{2+}-binding proteins such

as calmodulin, and proteins with lower affinity sites that may be at least partially activated and contribute to the regulation and/or mechanism of exocytosis.

The Role(s) of Increased Intracellular Ca^{2+}

In theory, the involvement of Ca^{2+} at several possible sites in the exocytotic pathway is possible. However, kinetic data from studies by Llinas *et al.* (1976) on the squid stellate ganglion show that calcium enters the nerve terminal and elicits secretion over a time interval that is too short to allow calcium to diffuse more than a short distance from the plasma membrane. This indicates that wherever Ca^{2+} acts it must be close to the site where secretory vesicles and plasma membrane interact. One approach that has been taken to identify potential sites of Ca^{2+} regulation has been to isolate components of secretory cells and recombine them in model systems that recreate one or more steps in the exocytotic pathway (Creutz and Pollard, 1983). A majority of studies have utilized catecholamine-secreting adrenal medullary chromaffin cells and their constituent organelles and proteins since exocytosis in this system has been especially well characterized and large quantities of secretory vesicles (chromaffin granules) are easily obtained (Pollard *et al.*, 1985).

One potential site of Ca^{2+} regulation is at the level of translocation of the secretory granule to the plasma membrane. Chromaffin granules have been demonstrated by electron microscopy to be embedded within a three-dimensional filamentous lattice in the cortical cytoplasm (Kondo *et al.*, 1982). Dissolution of this cytoplasmic matrix may be required for organelle movement to the plasma membrane during exocytosis. There is some experimental support for this idea in neutrophils and adrenal chromaffin cells. Boyles and Bainton (1981) devised a technique for examining filaments attached to the inner surface of the plasma membrane of neutrophils. In contrast to the more or less extensive network in control cells, in secreting cells the filaments appeared fewer in number and aggregated in such a way as to leave areas of plasma membrane uncovered. In addition, Perrin and Aunis (1985) observed that fodrin, a peripheral membrane protein, is redistributed into discrete patches in the subplasmalemmal region of stimulated chromaffin cells. Fowler and Pollard (1982) developed, as a model of the cortical cytoplasm in unstimulated cells, a high-viscosity gel-like structure formed from chromaffin granules and F-actin. Micromolar levels of Ca^{2+} were found to inhibit the formation of this gel, consistent with a model in which Ca^{2+} influx following cell stimulation results in release of chromaffin granules from the cytoskeletal network permitting movement to sites of exocytosis. Unfortunately, the site of Ca^{2+} action in this system has not been determined.

A second potential site of Ca^{2+} action is at the level of intermembrane contact. Electron microscopic studies, using quick freezing (Schmidt *et al.*, 1983), as well as conventional fixation methods (Palade and Bruns, 1968), reveal that prior to the actual release event secretory vesicles form contact regions with the plasma membrane, and during compound exocytosis, with the membranes of granules already fused with the plasma membrane. This observation of granule–granule contact has led to the study of the interaction of isolated chromaffin granules as a model for events occurring during compound exocytosis (Morris *et al.*, 1983). These studies revealed that unphysiologically high Ca^{2+} levels were required to promote membrane aggregation and fusion in this system, and lead to the proposal that the chromaffin granules in the stimulated cell may become associated with cytosolic and/or cytoskeletal components which mediate interactions between the granules and other subcellular structures. Indeed, Creutz *et al.* (1978) found that the soluble fraction of an adrenal medullary homogenate promoted Ca^{2+}-dependent contacts between chromaffin granules, as indicated by an increase in turbidity of a granule suspension (26). Using this Ca^{2+}-dependent turbidity increase as an assay, it was possible to isolate a single protein from the cytosol that was responsible for inducing granule aggregation. The protein was named synexin from the Greek "synexis" meaning "meeting," because of the "meetings" it initiated between granule membranes. Further study revealed that synexin is a 47-kDa Ca^{2+}-binding protein, widely distributed in nature, which binds to both biological (Creutz and Sterner, 1983; Creutz *et al.*, 1983) and acidic phospholipid (Creutz *et al.*, 1983; Hong *et al.*, 1982) membranes at low levels of Ca^{2+} ($K_d = 4\,\mu M$ at pH 7.2) and self-associates (Creutz *et al.*, 1979; Sterner *et al.*, 1985) and promotes intermembrane contacts (Creutz *et al.*, 1978) at higher Ca^{2+} concentrations ($K_d = 150$–$200\,\mu M$). The membrane contacts formed by synexin between chromaffin granules are relatively stable (Creutz, 1981), and when viewed in thin section by electron microscopy, appear as pentalaminar structures similar to those observed in electron microscopic studies of living cells undergoing exocytosis (Creutz *et al.*, 1978). This observation, together with the fact that the formation of these contacts is inhibited by phenothiazine drugs, with the same relative potency as inhibition of catecholamine secretion from cultured cells, suggested a possible role for synexin activity *in vivo* (Pollard *et al.*, 1983).

Since the initial discovery of synexin in 1978, a number of related proteins have been discovered. One of these, calelectrin, was isolated from the cholinergically innervated electric organ of *Torpedo marmorata* as a 34-kDa protein which selectively binds to membranes at low Ca^{2+} concentrations (Walker, 1982). This protein was later demonstrated to self-associate

and promote chromaffin granule aggregation in a Ca^{2+}-dependent manner similar to that of synexin (Sudhoff *et al.*, 1982). Sudhof *et al.* (1984) subsequently reported the isolation, from bovine liver and adrenal medulla, of two Ca^{2+}-binding proteins which bound to chromaffin granule membranes at low Ca^{2+} concentrations ($K_d = 5.5\,\mu M$) and potentiated the Ca^{2+}-induced aggregation of these membranes at higher levels of Ca^{2+} ($Ca^{2+} > 10\,\mu M$). These proteins, which have masses of 32 kDa and 67 kDa, are similar in amino acid composition to the *Torpedo* calelectrin, and were named "mammalian calelectrins." Another protein, which very likely is identical to the 67-kDa calelectrin, was isolated by Pollard and Scott (1982) as a Ca^{2+}-insoluble, EGTA-soluble component of a bovine liver membrane fraction. This protein was found to inhibit the Ca^{2+}-dependent chromaffin granule aggregating activity of synexin and was hence named "synhibin." The possibility that synexin, synhibin, and the calelectrins may function as co-regulators of membrane contact events in exocytosis is suggested by the observations of Geisow and Burgoyne (1982) and Creutz *et al.* (1983) that these proteins copurify during isolation by Ca^{2+}-dependent association with chromaffin granule membranes.

A third possible site of Ca^{2+} action is at the level of membrane fusion. Several model systems exist in which membrane fusion events involving chromaffin granules appear to have been reconstituted. In general, two types of fusion have been seen (Duzgunes, 1985). "Type I" fusion occurs at low Ca^{2+} concentration (10^{-7}–10^{-4} M), proceeds to only a limited extent, and is sensitive to enzymatic treatment of the membrane. "Type II" fusion requires millimolar levels of Ca^{2+} or other divalent cations, is extensive, and is insensitive to membrane perturbation. In some studies, Ca^{2+}-independent fusion has also been seen (Lelkes *et al.*, 1980), raising the possibility that the commonly observed Ca^{2+} requirement is not at the level of the fusion event itself, but at an earlier stage such as attachment of granules to one another and to the cellular plasma membrane. This hypothesis is consistent with the observation of Creutz *et al.* (1978) that synexin facilitates chromaffin granule aggregation but not fusion. However, Ca^{2+} may be important in fusion to the extent that it leads to fatty acid production, since these lipids cause chromaffin granules aggregated by synexin to fuse into vacuolar structures morphologically similar to those seen in extensively stimulated chromaffin cells (Creutz, 1981). Free fatty acids may be liberated by the action of a Ca^{2+}-dependent phospholipase A_2, or through the concerted action of a Ca^{2+}-dependent phospholipase C and diglyceride lipase (Rubin, 1982). Some support for this idea comes from the studies of Frye and Holz (1984), which showed that phospholipase A_2 inhibitors can block catecholamine secretion from cultured chromaffin cells.

The Annexin Family of Proteins

It is now appreciated that 32-kDa and 67-kDa calelectrin and synexin are but three members of a larger family of proteins that bind Ca^{2+} and phospholipid, and in many if not all cases, aggregate membranes in a Ca^{2+}-dependent manner (Geisow and Walker, 1986). The other members of this family include calpactin, lipocortin, endonexin II, and lipocortin III. These proteins have been referred to as "annexins" by Geisow (1986). Table I provides a summary of the nomenclature that has been used for these proteins. The members of this family are highly similar: Amino acid sequences show 40 to 60% identity, cross reactions are seen with some antisera, and they share multiple copies of a common 17 amino acid sequence, the endonexin fold (Geisow *et al.*, 1986). Each member of the annexin family of 30–50 kDa consists of four repeating 70 amino acid units, separated into two domains by a short (10–17 amino acid) connecting peptide. Within each 70 amino acid unit there are two regions which are homologous with the corresponding regions of other units. One of these, near the N-terminus of each repeat, is the 17 amino acid endonexin fold. The other, near the C-terminus, is a 12 amino acid sequence containing a conserved six-residue hydrophobic sequence. 32-kDa and 67-kDa calelectrin appear to have four

Table I. Nomenclature of the Annexins

Annexin	Name in this review	Common synonyms
Annexin I	Lipocortin	Lipocortin I, P35, calpactin II, chromobindin 9
Annexin II	Calpactin	Calpactin I, p36, lipocortin II, protein I, chromobindin 8
Annexin III	Lipocortin III	
Annexin IV	32-kDa Calelectrin	Endonexin, protein II, lipocortin 4, chromobindin 4
Annexin V	Endonexin II	Lipocortin V, pap, chromobindin 5/7
Annexin VI	67-kDa Calelectrin	p68, protein III, 67-kDa calcimedin, lipocortin VI, synhibin, chromobindin 20
Annexin VII	Synexin	Chromobindin 11

and eight of these 70 amino acid repeating units, respectively. These proteins lack "EF hand" Ca^{2+}-binding sequences and hence do not belong to the calmodulin-like family of proteins.

Criteria for Ca^{2+} Receptor Status

In addition to promoting calcium-dependent membrane aggregation *in vitro*, in order for the annexins to play a general role in Ca^{2+}-stimulated exocytosis they must meet certain other criteria. One such criterion is that they must exist in all tissues secreting via Ca^{2+}-stimulated exocytosis. Data from a large number of laboratories have shown that the annexins appear to satisfy this condition. Synexin, although routinely isolated from liver, also appears to be present in the adrenal medulla, brain, spleen, parotid, and peripheral blood leucocytes (Creutz, 1984; Meers *et al.*, 1986). The 32-kDa and 67-kDa calelectrins also appear to have a wide tissue distribution. They have been isolated from brain, liver, heart, skeletal muscle, testis, pancreas, mammary epithelial cells, adrenal, intestinal mucosa, gastric smooth muscle, neutrophils, lymphocytes, and syncytiotrophoblast (Geisow and Walker, 1986; Moore and Dedman, 1982; Meers *et al.*, 1986; Booth *et al.*, 1986). When the location of these proteins is examined at the light or electron microscopic level using immunological probes, their subcellular distribution appears to differ depending on the cell type. Synexin shows a diffuse cytoplasmic staining pattern in adrenal chromaffin cells, the only cell type examined for synexin localization (Pollard *et al.*, 1981). 67-kDa calelectrin appears diffusely distributed along the inner membrane in fibroblasts and lymphocytes (Geisow and Walker, 1986), but concentrated to the brush border of syncytiotrophoblasts (Booth *et al.*, 1986), and the cap and tail region of bovine spermatozoa (Sudhof, 1984). 32-kDa calelectrin is localized to endoplasmic reticulum-like elements in the fibroblast (Geisow and Walker, 1986), and exclusively the cap region in spermatozoa (Sudhoff, 1984). The distribution of several annexins in the adrenal medulla has been examined by Western blot analysis of sucrose gradient fractions (Drust and Creutz, 1989). Each protein seems to have a unique distribution. For example, 68-kDa calelectrin is predominantly bound in an EGTA-insensitive manner to the plasma membrane, whereas endonexin and lipocortin are predominantly cytosolic. Interestingly, calpactin was found in several locations: Soluble, plasma membrane-associated, and chromaffin-granule associated.

Immunocytochemical studies have demonstrated that much of the chromaffin cell calpactin is located on or immediately below the plasma membrane in the chromaffin cell (Burgoyne and Cheek, 1987). The significance of these distributions regarding the proteins' putative role(s) in

Ca^{2+}-triggered exocytosis is unclear. It is also unknown whether any of these proteins redistribute to morphologically identifiable sites of exocytosis, or secretory granules, in cells triggered to undergo Ca^{2+}-dependent exocytosis.

A second criterion for a protein having a general role in exocytosis is the capability of interacting with the secretory granules and plasma membranes from a wide variety of cell types. The annexins appear to satisfy this criterion as well. Synexin binds to both chromaffin granules (Creutz and Sterner, 1983) as well as chromaffin cell plasma membranes (Scott *et al.*, 1985), and the calelectrins appear to bind to the membranes of each tissue from which they are isolated. This apparent lack of specificity may be explained by their ability to bind the phospholipid components of membranes. However, this interaction is not totally nonspecific. Morris showed that the liposome-aggregating effect of synexin was sensitive to the ratio of PS to PC in SUV; no aggregation was observed at $>40\%$ PC (Morris *et al.*, 1982). Hong *et al.* (1982) observed that the ability of synexin to lower the Ca^{2+} requirement for liposome fusion was dependent on the lipid composition. Recently, Geisow *et al.* (1986) studied the phospholipid binding properties of 32-kDa calelectrin. The protein bound in a Ca^{2+}-dependent manner to liposomes formed from equimolar amounts of PC and PI, PE, or PA, but not PS, sphingomyelin, or cholesterol, or PC alone. In general, these studies of lipid specificity reveal preferential binding to acidic phospholipids, a property apparently shared by most if not all extrinsic membrane proteins (Devaux and Seigneuret, 1985). The role of membrane proteins in the binding of the annexins has been a matter of debate. Some (Dabrow *et al.*, 1980), but not all (Shadle and Weber, 1985; Geisow and Burgoyne, 1982; Morris *et al.*, 1982), investigators have found that binding is sensitive to protease treatment of the membranes. We investigated the nature of the chromaffin granule membrane receptor for these proteins by examining the effect of organic solvent extraction of chromaffin granule membranes on annexin binding (Zaks, 1987; Zaks and Creutz, 1989a). We observed that all binding at less than $100\,\mu$M Ca was abolished by this procedure, although 0–80% of control binding at greater than $100\,\mu$M Ca was still seen. Hence it appears that the annexins bind solely to lipid at less than $100\,\mu$M Ca and are capable of binding to membrane proteins to varying degrees at greater than $100\,\mu$M Ca. However, since trypsin or pronase treatment of the intact membrane had no effect on annexin binding at greater than $100\,\mu$M Ca, it appears that these proteins are inaccessible in the intact membrane. It is conceivable that the binding sites on the delipidated proteins are masked by lipid in the intact membrane.

A third requirement for a putative intracellular Ca^{2+}-receptor is functional activation at Ca^{2+} levels found in stimulated cells. We have studied the Ca^{2+} requirements for activation of synexin, 32-kDa and 68-kDa calelectrin, and calpactin in detail. For 32-kDa and 67-kDa calelectrin, chromaffin

granule membrane binding was found to occur at lower Ca^{2+} concentrations (K_d = 10 μM) than did either granule aggregation (K_d = 126 μM for 32 kDa and 22 μM for 67 kDa) or fusion (K_d = 100 μM). A similar phenomenon has been reported for synexin (Creutz and Sterner, 1983). However, in a recent study of synexin (Zaks and Creutz, 1989a,b) binding, aggregation and fusion occurred at similar Ca^{2+} levels (K_d = 30–100 μM). Of particular importance is the recent observation of the high calcium sensitivity of the calpactin tetramer which was found to aggregate chromaffin granules at 1 μM Ca^{2+}, a level of calcium that initiates exocytosis in permeabilized chromaffin cell models (Drust and Creutz, 1988).

An interesting feature of the Ca^{2+} dependence for activation of the annexins is that it appears to vary with the nature or composition of the membrane present. For example, 32-kDa calelectrin binds to PS/PC (1 : 1w/w) multilammellar vesicles over a broad range of Ca^{2+} concentrations centered at pCa 5.2, but binds in a highly Ca^{2+}-cooperative manner to chromaffin granule membranes with EC_{50} of 10 μM, and in a less cooperative fashion to mitochondrial membranes with an EC_{50} of 28 μM (Zaks, 1987; Zaks and Creutz, 1989a). Since we observed that only 15% of 32-kDa calelectrin binds to PS/PC vesicles at pCa 5.0, it is understandable that, using a less sensitive assay, Geisow *et al.* (1986) reported the failure of this lipid mixture to support binding. It is likely that the differences in annexin binding to the different biological membranes reflect dissimilarities in membrane lipid composition. In a related study, Glenney (1986) observed that binding of calpactin to phospholipid increased the affinity of the protein for Ca^{2+}, and that the effectiveness of the phospholipid varied with PS being more effective than PI, and PC being inactive. Two possible mechanisms may be postulated to explain the effect of phospholipid on Ca^{2+} binding to protein. One, originally put forward to explain the lower Ca^{2+} requirements for binding of protein kinase C in the presence of certain acidic phospholipids, fatty acids, and diacylglycerols, is the formation of a ternary chelation complex of Ca^{2+}, protein, and negatively charged lipid (Ganong *et al.*, 1986). This mechanism implies that high-affinity Ca^{2+} binding observed in the presence of phospholipid membranes does not exist on the isolated protein. However, both highly purified 32-kDa and 67-kDa calelectrin have been reported to bind Ca^{2+} with high affinity in the absence of membranes (Sudhof *et al.*, 1984; Shadle *et al.*, 1985; Moore, 1986; Mathew *et al.*, 1986). The other mechanism follows from thermodynamic considerations; since Ca^{2+} increases the affinity of the protein for lipid, the presence of lipid will increase the apparent affinity of the protein for calcium. A physical interaction between Ca^{2+} and phospholipid need not exist. This relationship, between the affinity for Ca^{2+} in the presence and absence of effector, has been shown for calmodulin–enzyme interaction to lead to

differences in affinity greater than an order of magnitude (Olwin and Storm, 1985).

The ability of different phospholipids to regulate annexin Ca^{2+} affinity may play an important role in differential cellular localization and function of these proteins. It is possible that only those cellular membranes or regions of membranes having the appropriate lipid composition bind the annexins at physiological Ca^{2+} concentration. Furthermore, different sites of protein localization and exocytosis might occur at different Ca^{2+} levels. It may be no coincidence that 32-kDa calelectrin needs less Ca^{2+} to bind chromaffin granule membranes than mitochondrial membranes if the protein is designed to interact with granule membranes selectively in the cell (Zaks, 1987; Zaks and Creutz, 1989a). Despite this selectivity, the Ca^{2+} concentrations required for activating synexin and 32-kDa and 67-kDa calelectrin (especially the granule aggregation and fusion processes) are higher than generally believed to exist in stimulated cells. However, free fatty acids could significantly decrease these Ca^{2+} requirements; in the presence of 5% by weight oleic acid, annexin binding and chromaffin granule aggregation and fusion occurred at micromolar Ca^{2+} levels (Zaks and Creutz, 1988, 1989a,b). This phenomenon may be of potential physiological importance. The liberation of free fatty acids by phospholipase A_2 activation during secretion (Frye and Holz, 1984) may be a mechanism by which the cell can change its membrane composition so as to favor annexin binding and activation. Furthermore, fatty acids may play a general role in cells as modulators of Ca^{2+}-dependent proteins, since they have been shown to alter the Ca^{2+} affinity of phospholipase C (Takenawa and Yoshitaka, 1981) and protein kinase C (McPhail *et al.*, 1984) as well. However, there may be a greater specificity for cis-unsaturated fatty acids in affecting the latter proteins than the annexins. Saturated fatty acids are about 50% as effective as cis-unsaturated fatty acids as modulators of the annexins (Zaks, 1987), but appear to be ineffective as modulators of phospholipase C (Takenawa and Yoshitaka, 1981) and C kinase (McPhail *et al.*, 1984). The mechanism by which fatty acids affect the annexins as well as other proteins is unknown. It is possible that since the proteins appear to interact better with negatively charged lipids, the fatty acids are acting solely by increasing the surface charge of the membrane. However, if this were the case, one might not expect to observe a difference between saturated and unsaturated fatty acids. These two classes of fatty acids have also been postulated to interact differently with gel and fluid lipid domains in membranes (Karnovsky *et al.*, 1982). Since cis-unsaturated free fatty acids preferentially interact with fluid lipid domains, preferential binding of protein to these regions might provide a mechanism for selective interaction with this class of fatty acid. A third possibility is that the fatty acids may interact directly with the proteins, independent of the membrane. Evidence that such an interaction occurs and

is significant comes from the observations of Murakami *et al.* (1986) and Sterner *et al.* (1985). Murakami *et al.* (1986) observed that free fatty acids alone, below their critical micelle concentration, could activate protein kinase C. Sterner *et al.* (1985) showed that substoichiometric amounts of cis-unsaturated fatty acids increase the magnitude and decrease the Ca^{2+} requirements for the Ca^{2+}-dependent light scattering increase associated with synexin polymerization. The possibility exists of directly testing this hypothesis by examining the effects of free fatty acids on the Tb^{3+} binding proteins of these proteins, both in the absence and presence of phospholipid vesicles. Attempts to study the association of the fluorescent free fatty acid, cis-parinaric acid, with synexin were unsuccessful, however, due to lack of evidence for energy transfer between them (Zaks and Creutz, unpublished).

A fourth criterion for Ca^{2+}-receptor status in exocytosis is activation by Ba^{2+} and Sr^{2+}, since the chemistry of these ions is similar enough to Ca^{2+} to replace it in exocytosis (Rubin, 1982). We observed that synexin and 32-kDa and 68-kDa calelectrin were all activated by Sr^{2+} and Ba^{2+}, albeit with less affinity than by Ca^{2+} (Zaks, 1987; Zaks and Creutz, 1989a), an observation consistent with secretion data from permeabilized cells (Wilson and Kirshner, 1983).

From the previous discussion we can conclude that there are few differences that have been recognized among the annexins in terms of their sites of action, requirements for activation, and susceptibility to inhibition. One feature that did distinguish synexin and 32-kDa and 67-kDa calelectrin when they were carefully examined in parallel studies is their relative efficacy in chromaffin granule aggregation (Zaks and Creutz, 1989b). At physiological Ca^{2+} concentrations, loosely defined as < 1 mM, synexin appeared to be approximately twofold more efficacious than 32-kDa calelectrin, which in turn is approximately sevenfold more efficacious than 67-kDa calelectrin as a granule aggregator. The importance of such efficacy differences in the cell is unclear, however, since the relative amounts of each protein at the sites of exocytosis are unknown.

An interesting consequence of these efficacy differences is seen when combinations of proteins are tested (Creutz *et al.*, 1987; Zaks and Creutz, 1989b). 67-kDa calelectrin was observed to inhibit both 32-kDa- and synexin-induced granule aggregation at pCa 3.0, at concentrations which do not inhibit 32-kDa binding to granule membranes. 32-kDa calelectrin was also observed to inhibit synexin-induced granule aggregation at high concentrations of synexin. At low synexin and 32-kDa concentrations, the two proteins appeared to interact synergistically, while at intermediate concentrations, the combined effect was usually less than additive. These interactions may be of physiological importance if one or more of these proteins is localized to similar regions of the cell. Indeed, immunofluorescence

microscopy of bovine spermatozoa revealed that both 32-kDa and 67-kDa calelectrin are present in the cap region, which is the site of fusion with the egg membrane (Sudhof, 1984).

In summary, it appears that several of the annexins are effective at aggregating chromaffin granules at physiological Ca^{2+} levels. In addition, these proteins may interact to enhance their overall effect. 67-kDa calelectrin, on the other hand, is only a weak aggregator on its own, but functions as an effective inhibitor of 32-kDa calelectrin and synexin. A principal role for 67-kDa may therefore be as an inhibitory modulator of the other proteins (i.e., as a "synhibin," Pollard and Scott, 1982). Why does a cell need several proteins with a common function, such as the ability to aggregate and fuse membranes? Perhaps differential localization would provide a mechanism for regulating exocytosis from different regions of the cell or perhaps enable secretion to occur from distinct pools of secretory granules, such as the specific and azurophil granules of neutrophils. It is also possible that a cell's secretory activity may be regulated by its complement of granule-aggregating proteins. This complement if subject to change may provide a mechanism for chronic changes in secretory function.

Membrane Fusion Mediated by the Annexins

Several model systems exist in which to study membrane fusion, and these are described briefly in a review by Duzgunes (1985). In general, two types of Ca^{2+}-dependent fusion are seen in these models. One type of fusion event occurs at low Ca^{2+} concentrations ($< 10\ \mu M$), is restricted to biological membranes, and is generally of limited extent. This type of fusion in many cases is poorly documented to be actual fusion, and exhibits requirements which vary from system to system and are of unclear physiological significance. The other type of fusion event occurs at unphysiologically high Ca^{2+} concentrations (> 1 mM), is associated with extensive membrane aggregation, is often insensitive to perturbation of membrane proteins, and is also observed with acidic phospholiposomes. Neither of these types of fusion model provide the required Ca^{2+} sensitivity and generality expected for a widespread and highly conserved process such as exocytosis. A model which lacks these problems is the Ca^{2+}-annexin-dependent fusion of secretory granules in the presence of cis-unsaturated free fatty acids. In our recent study (Zaks and Creutz, 1988) two sensitive fluorescence assays were applied to study fusion in this model system and a detailed analysis of the characteristics has been obtained.

It was observed that chromaffin granule fusion requires an intact native chromaffin granule (Zaks and Creutz, 1988). Granules lysed by hypoosmotic

lysis fail to fuse. Although the mechanism for this selectivity is unclear, it was observed that fusion is also inhibited by increased osmolarity. It is believed that the mechanism for osmotic sensitivity of membrane fusion in some systems is inhibition of osmotically induced vesicle swelling (Lucy and Ahkong, 1986). This swelling event has been shown to facilitate fusion of vesicles to planar phospholipid bilayers (Finkelstein *et al.*, 1986). Since chromaffin granules have potentially hyperosmotic core contents (Sudhof, 1982), these organelles are potentially capable of applying an osmotic force to their membranes. If the above theory of osmotic pressure sensitivity applies to this system, it would suggest that an osmotic force on the granule membrane due to the granule core contents plays a role in chromaffin granule fusion. We may speculate that membrane fusion has two requirements. First the membranes must be brought into close apposition by the adhesive forces of the granule-aggregating proteins. Second, the apposed membranes must be physically forced together with sufficient energy to bring about membrane reorganization. For the latter process to occur, both membranes must be resistant to movement away from the opposing membrane. The internal osmotic pressure provides this resistive force. If this idea is correct, it suggests that exocytosis is localized to sites of plasma membrane stress, and would fail to occur at sites of easily deformable membrane. Changes in membrane tension could potentially be locally regulated in the cell by gel–sol transformations as well as by interactions with cytoskeletal elements.

Most models for membrane fusion require some perturbation of the membrane phospholipids for fusion to occur (Lucy and Ahkong, 1986). This perturbation may take the form of an increase in tension due to pressure imbalance as postulated above, or it may result from physiochemical interactions. The observed ability of cis-unsaturated free fatty acids to promote fusion may be the result of the second mechanism. These fatty acids decrease the polarization of diphenylhexatriene in biological membranes consistent with a "disordering" of the bilayer (Karnovsky *et al.*, 1982). This increase in fluidity would presumably increase the probability of lipid interactions between apposed membranes.

Synexin and 32-kDa and 67-kDa calelectrin were observed to promote chromaffin granule fusion at $> 10\,\mu M\ Ca^{2+}$ in the absence of exogenous free fatty acids (Zaks and Creutz, 1988, 1989a,b). In the presence of high concentrations of fatty acids, significant fusion was observed between $1–10\,\mu M$ Ca^{2+}, well within the physiological range of Ca^{2+} concentration. Not only did free fatty acids change the calcium sensitivity of the fusion process, but they also increased the total extent of fusion, an observation in agreement with that seen in other membrane fusion models. Several experimental observations indicated that the proteins did not appear to promote fusion solely by increasing the extent of membrane aggregation. First, the relative

rates of fusion at different Ca^{2+} levels were often different from the relative aggregation rates. Second, the relative chromaffin granule-aggregating activities of the two calelectrins (32-kDa and 67-kDa) failed to agree with their relative granule fusing activities. Third, fusion was inhibited at high protein concentration, although aggregation was not. Interestingly, the amount of protein required for inhibition of fusion varied as a function of Ca^{2+}, and this did not appear to be explained by different amounts of protein bound to the chromaffin granule membrane. This suggests that some functional property of the protein, other than the one regulating binding, is responsible for inhibition of fusion. One possibility is self-association, such that protein polymers at the sites of membrane contact physically prevent close membrane contact necessary for fusion.

In summary, the fusion of chromaffin granules by Ca^{2+}, annexin, and cis-unsaturated free fatty acid appears to be an excellent model for Ca^{2+}-dependent exocytosis in general. The critical component in this system is the granule-aggregating protein. It is reasonable to assume that this model system can be applied to study secretion from nonchromaffin cells simply by substituting other secretory granules for the chromaffin granules, since the annexins have the ability to recognize a wide variety of biological membranes. Because of this property, they are ideal candidates for a role in generalized secretory processes. Further, fusion in this system shares two important features with exocytosis: (1) The effective Ca^{2+} concentration is in the micromolar range in the presence of free fatty acids. Interactions with regions of specific lipid composition may conceivably decrease this Ca^{2+} requirement further. (2) Fusion is inhibited by hypertonicity. This model also provides a possible molecular basis for various secretory phenomena: (1) Differential secretion from one or more distinct populations of granules may be a consequence of nonhomogeneous distributions of different annexins, or differences in the lipid composition of the different granule types. (2) The limited extent of exocytosis observed in secreting cells, even in the presence of elevated Ca^{2+} levels, may be a consequence of the "autoinhibition" due to the recruitment and binding of large amounts of annexin over a prolonged period of stimulation. The model also predicts that fusion may be regulated by local changes in the state of tension of the plasma membrane, perhaps regulated by membrane–cytoskeleton interactions or cortical sol–gel transformations.

Mechanism of Action of the Annexins

The mechanism by which the annexins bind, aggregate, and fuse membranes should be interpretable in light of their known structural features.

The annexins all share the ability to bind Ca^{2+} and phospholipid. These proteins may also be capable of binding to other hydrophobic matrices to varying degrees. For example, Dedman and coworkers have isolated these proteins on the basis of Ca^{2+}-dependent binding to phenothiazine-ring-coupled Affi-Gel, and phenyl- and octyl-Sepharose affinity resins (Moore and Dedman, 1982; Smith and Dedman, 1986; Mathew *et al.*, 1986). Unlike calmodulin, which also interacts with these hydrophobic ligands, these proteins do not enhance the quantum yield of fluorescent hydrophobic probes in a Ca^{2+}-dependent manner (Sudhof, 1984). Rather, only Ca^{2+}-independent binding of these probes is observed, implying the existence of a hydrophobic site(s) on the protein surface accessible to these molecules. However, spectroscopic studies of calpactin I (Gerke and Weber, 1985) and 32-kDa calelectrin (Shadle *et al.*, 1985) reveal UV difference changes upon Ca^{2+} binding consistent with exposure to aromatic residues to the aqueous environment. It is possible this change would lead to increased hydrophobicity of the protein surface and would explain the interaction of these proteins with phospholipid membranes and other hydrophobic matrices.

The number of Ca^{2+}/lipid binding sites on these proteins and their location in the amino acid sequence is a matter of debate. Geisow has postulated that each endonexin fold forms, at least in part, a complex binding site for both Ca^{2+} and phospholipid (Geisow *et al.*, 1986). He observed that the endonexin fold had a sequence which was homologous both with part of the Ca^{2+}-binding site of phospholipase A2 and with the phosphate interaction site of dinucleotide-binding proteins. A more detailed structural model was recently presented by Taylor and Geisow (1987) that was developed by comparison of the structure of lipocortin and a classical EF hand containing protein, the 28-kDa intestinal calcium-binding protein. If indeed the endonexin fold is involved in Ca^{2+} binding, it would suggest that 32-kDa and 67-kDa calelectrin might be capable of binding four and eight Ca^{2+} ions per molecule, respectively. Preliminary studies from several laboratories suggest that this may be the case (Sudhof, 1984; Mathew *et al.*, 1986).

The annexins, although apparently sharing the ability to bind phospholipid and other hydrophobic matrices, differ in their reported ability to bind proteins. Only calpactin I and II have been demonstrated to bind other proteins (F-actin and spectrin) in a Ca^{2+}-dependent manner (Brugge, 1986). However, it appears that most if not all annexins self-associate in the presence of Ca^{2+}. This has been measured by 90° light scattering for both synexin and *Torpedo* calelectrin (Creutz *et al.*, 1979; Sudhof *et al.*, 1982). Although it has been claimed that 32-kDa calelectrin and calpactin do not self-associate (Shadle *et al.*, 1985), this conclusion was based on gel filtration experiments, which have been shown to be unreliable as a measure of self-association of synexin (Creutz *et al.*, 1979). Indeed, we have directly demonstrated using

both a light-scattering assay and a fluorescence energy transfer assay that 32-kDa and 67-kDa calelectrin self-associate in solution and on membrane surfaces (Zaks and Creutz, 1989c). Creutz *et al.* (1979) argued for a hydrophobic interaction as the basis for self-association due to the stimulatory and inhibitory effect of KCl and DMSO, respectively.

The ability to self-associate may be a general feature of proteins in the annexin class and a function of the common "core" region. Secondary structure predictions and computer modelling have suggested that each 70 amino acid domain in this region may be structurally similar to a pair of "EF hands," which in the calmodulin family of proteins is known to possess a hydrophobic protein- and drug-binding site. The ability of a calpactin-like protein from mammary ascites tumor cells to bind four molecules of chlorpromazine is consistent with this model. It is possible that such a hydrophobic site generated by calcium and phospholipid headgroup binding is the site of annexin self-association and perhaps the binding to other ligands as well. Some annexins have been shown to behave under some conditions as integral membrane proteins (e.g., Drust and Creutz, 1989). Perhaps, under these conditions, the hydrophobic sites gain access to the membrane interior and interact with the hydrophobic phospholipid tails. Interestingly, a similar ability has previously been demonstrated for calmodulin, which binds both proteins and membrane lipids under certain conditions (Bader *et al.*, 1985).

It has previously been proposed that self-association may take place between annexin molecules bound to separate membranes, thus explaining the membrane aggregating activity of these proteins (Creutz *et al.*, 1979). Alternatively, it has been suggested that each protein may contain several membrane-binding sites and crosslink membranes as a monomer (Hong *et al.*, 1982). Using a fluorescence energy transfer assay, we were able to demonstrate self-association between synexin and 32-kDa calelectrin molecules bound to separate chromaffin granule membranes at calcium concentrations less than 100 μM, but not at higher calcium levels, suggesting that both mechanisms may be operative depending on prevailing calcium concentrations (Zaks and Creutz, 1989c).

A model for annexin binding to membranes and membrane aggregation at different calcium concentrations is shown in Fig. 1. In this model each 30- to 50-kDa annexin is postulated to contain four similar domains (depicted by circles) which bind calcium and phospholipid and expose hydrophobic areas capable of self-association. At low calcium concentrations the proteins bind membranes and undergo intra-membrane self-association. At higher calcium levels more extensive intra- and intermembrane self-association occurs leading to membrane aggregation. At still higher calcium levels the protein binds two membrane surfaces as a monomer and undergoes intramembrane self-association. Intermembrane self-association becomes geometrically inefficient,

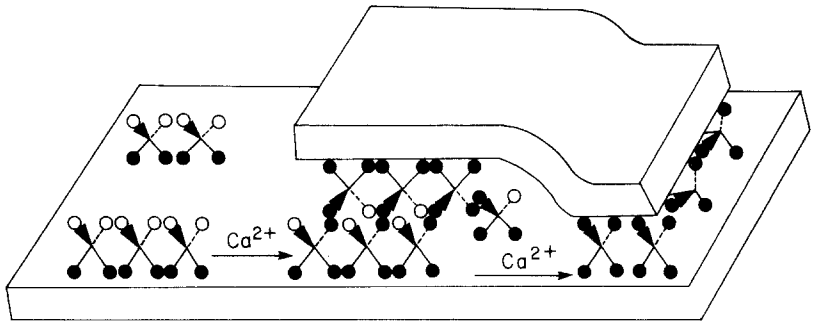

Fig. 1. Model of annexin action. Each 30- to 50-kDa annexin protein is envisioned as having its homologous 70 amino acid domains arranged in a tetrahedral orientation. (The wedge-shaped spokes project out from the paper, the dotted spokes project into the paper.) Blackened circles represent domains with bound calcium. At low calcium concentrations each protein is bound to the membrane via two calcium/phospholipid binding domains. These domains are also capable of self-associating to produce long annexin polymers. At higher calcium concentrations, annexin domains that are not bound to membranes bind calcium and then associate with similar domains on other annexin molecules bound to an apposing membrane, thus producing membrane–membrane attachment. At still higher calcium levels, calcium binds solely to membrane-associated annexin domains, enabling an annexin monomer to bridge two membrane surfaces. Side-to-side self-association of long annexin rods, initially bound to apposing membranes, is impossible because of the assumed tetrahedral arrangement of the interacting domains, and hence *inter*membrane self-association is inefficient as it is restricted to end-to-side associations. *Intra*membrane self-association under these conditions (not shown) would occur freely.

requiring right-angle interactions between annexin rodlike polymers because of the assumed tetrahedral arrangement of the associating domains. Such a model explains the decrease in intermembrane but not intramembrane annexin interactions seen to occur at high calcium levels (Zaks and Creutz, 1989c). With this model it is possible to envision extended structures, appearing as bundles of rods, forming between membrane surfaces, similar to those seen in negatively stained samples of isolated synexin in the presence of calcium (Creutz *et al.*, 1979). Furthermore, it is possible that these extended annexin aggregates, while promoting membrane adhesion, might actually inhibit membrane fusion by physically preventing intermembrane lipid interactions. Such a mechanism might explain the inhibition of membrane fusion seen at high annexin concentrations (Zaks and Creutz, 1988).

Future Directions

The annexins are members of a homologous family of Ca^{2+}-binding proteins with distinct but overlapping functional activities. The sequences of most of the annexins have now been determined. As has been the case for the

calmodulin family of proteins, detailed biophysical studies of these proteins and their proteolytic fragments (Forsen *et al.*, 1986), X-ray crystallographic analysis (Babu *et al.*, 1985), and site-directed mutagenesis will provide important information in the future on the structure of these proteins and their sites of interaction with other molecules. The greatest challenge remains the elucidation of the function of these proteins in the cell. Although these proteins function *in vitro* to closely mimic one or more steps in exocytosis, there is as yet no direct evidence for this role in the cell. Cell fusion or microinjection experiments utilizing intact annexins or their fragments, as well as sense and antisense mRNA, may yield some insight into the function of the proteins. In this regard, Ali *et al.* (1989) recently reported that exogenous calpactin could "reactivate" exocytosis in permeabilized chromaffin cells that had lost secretory activity coincident with the leakage of cellular proteins that might be components of the secretory machinery. In addition, this reactivation was blocked by a peptide with the sequence of an endonexin fold. These experiments strongly infer a role for calpactin in exocytosis. Genetic approaches to the study of the function of the annexins may also be important in the future, as has been recently applied to the function of clathrin (Payne and Schekman, 1985) and calmodulin (Davis *et al.*, 1986) in yeast. A group of calcium-dependent membrane binding proteins has recently been isolated from yeast extracts (Creutz *et al.*, 1989), although it is not yet clear if these proteins are true homologs of the annexins. Perhaps a better understanding of the function of these proteins will also come from an analysis of their role in other, nonsecretory systems, involving, for example, intestinal brush border mortility (Glenney and Glenney, 1985) and mammary epithelial cell spreading and differentiation (Braslau *et al.*, 1984).

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