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Contents

Transport of Membranes and Vesicle Contents During Exocytosis

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Introduction

In many electronmicroscopical studies, it has been observed that secretory vesicles release their contents into the extracellular fluid during exocytosis and their limiting membranes become inserted into the cell membrane. From these observations it has been deduced that secretory vesicle membranes may act as precursors of cell membrane components and thus may be involved in the biogenesis of the cell membrane.

There is abundant evidence that many membrane proteins, including those destined for the cell membrane, are synthesized by membrane-bound ribosomes at the endoplasmic reticulum (see Kreibich, this vol.). It has been suggested that cell membrane components then join the secretory pathway and are transferred via the Golgi apparatus and secretory vesicles to the cell membrane. However, considerable transport of membranes also exists from the cell membrane back to the cytoplasm (endocytosis) which seems to be coupled with the exocytotic pathway. Endocytotic vesicles end up in lysosomes or are reused in the Golgi apparatus to reform secretory vesicles (membrane recycling) (see Herzog, this vol.).

Little is known concerning the biosynthesis of individual cell membrane proteins within the cell, the kinetics of their intracellular transport to the cell membrane (is it coupled to exocytosis?), their removal from the cell membrane (endocytosis) and, if not degraded within lysosomes, their reuse in secretory vesicles. All of these mechanisms are involved in the biogenesis of the cell membrane and in the turnover of its components.

Since secretory vesicles are everted during exocytosis, those membrane proteins localized at the extracellular side of the cell membrane should also be found at the intracisternal side of secretory vesicles. The first part of this contribution will discuss whether this postulate is fulfilled. The second part will deal with the insertion mechanism itself, the initiation of membrane fusion and the respective roles of membrane lipids and proteins.

Presence of Cell Membrane Components on the Intracisternal Side of Secretory Vesicles

5'-nucleotidase is considered to be a cell membrane marker and is widely used as such. However, 5'-nucleotidase is not restricted in its dis-

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tribution to the cell membrane, as is usually assumed, but has also been found to be present in the endoplasmic reticulum (Widnell 1972) where it is synthesized (Bergeron et al. 1975), and in secretory vesicles from rat liver (Farquhar et al. 1974; Little and Widnell 1975).

5'-nucleotidase activity in isolated secretory vesicle fractions is latent. Thus, the enzyme was inaccessible to both antibody and concanavalin A and an increase of activity was found when detergent was included in the assay, suggesting an intravesicular location of this enzyme. This was confirmed by cytochemical procedures with which the reaction product of 5'-nucleotidases was localized on the inside of secretory vesicles (Farquhar et al. 1974; Little and Widnell 1975). With cell membranes, however, reaction product is localized on the extracellular side of the membrane both in isolated cell fractions and in situ (cf. ref. Little and Widnell 1975). These studies are in agreement with the postulated exocytotic insertion of the secretory vesicle membrane 5'-nucleotidase into the cell membrane, since the inner aspect of the secretory vesicle membranes becomes the outer aspect of the cell membrane when secretory vesicles are everted during exocytosis.

Although the primary biological action of insulin is probably exerted at the cell surface, receptors for this hormone have also been found within several intracellular membranes including secretory vesicles (Bergeron et al. 1973). Freeze-thawing markedly augmented the binding of insulin as well as that of growth hormone in secretory vesicle fractions (Bergeron et al. 1978) This behavior is compatible with the location of the hormone-binding sites on the cisternal face of secretory vesicles.

In chromaffin cells, acetylcholinesterase has been histochemically demonstrated on the cell membrane as well as in the cisternae of endoplasmic reticulum. The Golgi apparatus, where the secretory vesicles are formed, very rarely showed acetylcholinesterase activity. In secretory vesicles themselves, however, no reaction product has been found (Somogyi et al. 1975). Despite this, release of acetylcholinesterase and catecholamines into the perfusate has been observed when chromaffin cells were stimulated to secrete with depolarizing concentrations of K^+ or carbachol. The fact that the presence of Ca^{2+} in the external medium is necessary before acetylcholinesterase is released provided evidence that the release is by the processes of exocytosis (Chubb and Smith 1975).

Recently, we have isolated highly purified secretory vesicles from bovine adrenal medulla by differential and density gradient centrifugation on iso-osmolal gradients using Percoll™ (Gratzl et al. 1980). These vesicles gradually released their content (e.g., adrenalin) when incubated in media of osmolalities <400 mosm/kg (Fig. 1). Under these conditions acetylcholinesterase activities increased to values comparable to values found in assays with Triton X 100 (0.12% final) included (Fig. 2). The marked increase in enzyme activity (10-15 times), resulting from the osmotic lysis or the permeability changes when detergent was added seems to establish that acetylcholinesterase is localized on the inside of adrenal medullary secretory vesicles. The origin of secretory vesicle acetylcholinesterase is difficult to evaluate. The presence of this enzyme in the endoplasmic reticulum of chromaffin cells suggests that the enzyme in secretory vesicles may be, at least in part, newly synthesized by these cells. On the other hand, in adrenal medulla, fusion of small coated vesicles with prosecretory vesicles has been observed (Bendeczky and Smith 1972). Coated vesicles in this cell have also been detected along exocytotic profiles at the cell membrane (cf. ref. Winkler 1977). Therefore, acetylcholinesterase synthesized and

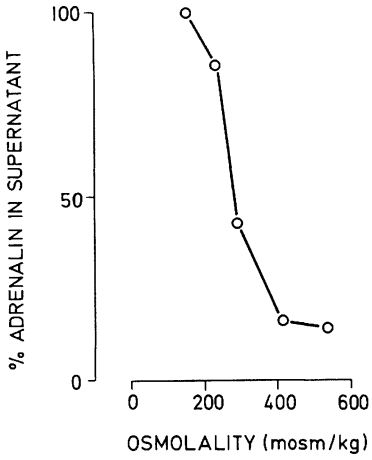

Fig. 1. Stability of secretory vesicles isolated from bovine adrenal medulla in media of different osmolality. Vesicles were incubated for 30 min at 37°C in 20 mM MOPS (pH 7.0), 5 mM EGTA and sucrose to obtain the osmolality indicated at the abscissa. Vesicles were separated from the media by centrifugation (12,000 *g* for 10 min) and the adrenalin released into the supernatant was determined (Gratzl et al., unpubl.)

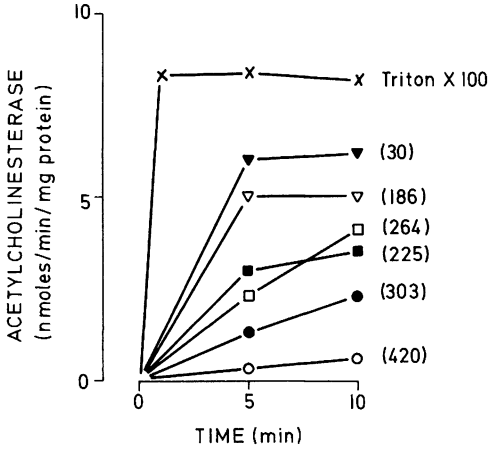

Fig. 2. Latency of acetylcholinesterase of adrenal medullary secretory vesicles. Isolated secretory vesicles were lyzed at 20°C in media of different osmolality as described in Fig. 1 or with Triton X 100 (0.12% final concentration) and the specific activity of this enzyme was determined (Ellman et al. 1961) at the times indicated at the abscissa. The values given in *brackets* represent the osmolalities of the incubation media (Gratzl et al., unpubl.)

released by the adrenal medullary cells or other cells in this tissue might have been taken up by coated vesicles during exocytosis and then transferred to secretory vesicles.

The cell membrane components found on the intracisternal side of secretory vesicles, as well as the biochemical evidence for this localization, are summarized in Table 1.

Recently, another "cell membrane enzyme" has been found in subfractionation studies in secretory vesicles. The activity of this enzyme, adenylate cyclase, in secretory vesicle membranes was even higher than in cell membranes (Cheng and Farquhar 1976a). But, however, its sidedness was exactly opposite to that of 5'-nucleotidase, hormone receptors or acetylcholinesterase. In both secretory vesicles and cell membranes it faces the cytoplasmic side; a fact which is compatible with the possibility that adenylate cyclase is also inserted exocytotically into the cell membrane (Cheng and Farquhar 1976b).

Table 1. Cell membrane components on the intercisternal side of secretory vesicles

Component	Evidences
5'-Nucleotidase	inaccessible to antibody and Con A, activation of enzyme activity by detergent, cytochemical localization
Hormone receptors (Insulin, Growth Hormone)	enhancement of hormone binding by freeze thawing
Acetylcholinesterase	activation of enzyme activity by detergent, activation of enzyme activity by hypotonic treatment

In stimulated cells, secretory vesicles fuse with the cell membrane as well as with each other, a process termed "compound exocytosis" which has been observed in many secretory cells (Fig. 3, cf. ref. Dahl et al. 1979; Gratzl et al. 1980). It is reasonable to assume that the membrane components responsible for the specific attachment and fusion of the membranes are localized on the interacting surfaces of the membranes; namely on the cytoplasmic surfaces of both secretory vesicle membranes and cell membranes and are, therefore, similarly arranged as adenylate cyclase.

The elucidation of the molecular mechanism of membrane fusion during exocytosis has been hampered by the lack of suitable systems for studying this process. This is mainly due to the difficulties involved in the isolation of both interacting membranes in a reasonable state of purity and, especially cell membranes, in an appropriate orientation. The fact that secretory vesicles fuse together in stimulated cells indicates that the components required for membrane fusion are present in secretory vesicle membranes. This points to the possibility of studying this process in vitro using isolated secretory vesicles. In such experiments substances, suggested to trigger exocytosis in stimulated cells, can be tested for their ability to induce membrane fusion. Furthermore, questions concerning the role of membrane lipids and proteins in this process can probably be answered.

Fusion of Secretory Vesicles in Vitro

Secretory vesicles isolated from liver (Gratzl and Dahl 1976, 1978), pancreatic islets (Dahl and Gratzl 1976; Gratzl et al. 1980), neurohypophyses (Gratzl et al. 1977) and adrenal medulla (Dahl et al. 1979;

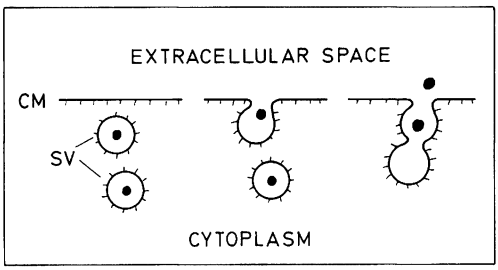

Fig. 3. Schematic representation of secretory vesicles (*SV*) close to the cell membrane (*CM*) (*left*). The cytoplasmic surfaces of the membranes are marked with *strokes*. During exocytosis (*middle*) secretory vesicles fuse with the cell membrane and discharge their content into the extracellular space. The inner surface of the secretory vesicle membrane becomes the outer surface of the cell membrane. Compound exocytosis (*right*) is characterized by fusion of secretory vesicles with each other and the cell membrane

Gratzl et al. 1980) have been subject of fusion studies on the subcellular level.

As seen in freeze-fractured suspensions, secretory vesicles in buffered sucrose media containing EGTA are dispersed. Upon addition of divalent cations (10^{-4}M final concentration) to the medium, vesicles become attached to each other. If the media were supplemented with Ca^{2+}, in addition, fused vesicles could be detected. Fused rat liver secretory vesicles are shown in Fig. 4 in freeze-fracture electronmicrographs. The continuity of the membranes of "twinned vesicles" is indicated by the continuous cleavage plane in both membrane faces exposed by freeze-fracturing. Interaction of vesicle contents after exposure of rat liver secretory vesicles to Ca^{2+} was demonstrated by the mixing of vesicles containing labeled proalbumin but inactivated converting enzyme with unlabeled, active vesicles. Addition of 10^{-4}M Ca^{2+} increased the conversion of proalbumin into albumin within the vesicles and provides quite strong evidence for the induction of fusion between the two types of vesicles (Quinn and Judah 1978).

The number of fused secretory vesicles increased with the Ca^{2+} concentration in the medium. If the percentage of fused vesicles is plotted as a function of the free Ca^{2+}-concentration the curve shown in Fig. 5 is obtained. The percentage of fused vesicles increases sigmoidally between 10^{-7}M and 10^{-4}M Ca^{2+} and reaches a plateau. None of the other divalent cations tested was able to induce fusion of secretory vesicles in concentrations lower than 1 mM. Simultaneous addition of Mg^{2+} and Ca^{2+} to secretory vesicles resulted in lower percentages of fused vesicles than was observed in the presence of 10^{-4}M Ca^{2+} alone (Table 2). Pretreatment of rat liver secretory vesicles with proteolytic en-

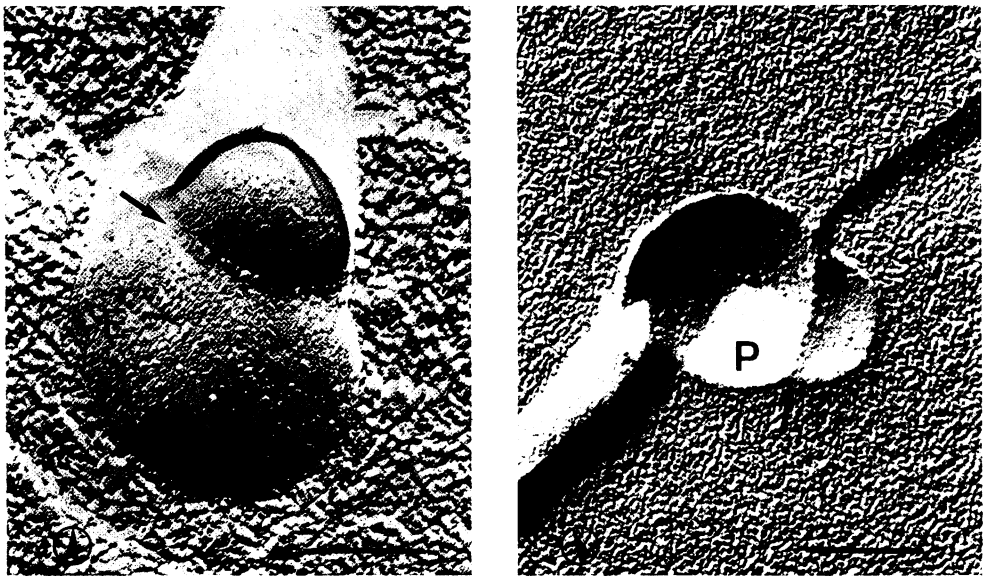

Fig. 4. Secretory vesicles isolated from rat liver in media containing 2×10^{-5}M Ca^{2+}. Twinned vesicles with a continuous cleavage plane in the membrane EF-face (*left*) as well as the membrane PF-face (*right*). *Encircled arrowhead* indicates direction of shadowing. Scale: 0.2 µm. Fracture faces are denoted according to the nomenclature introduced (Branton et al. 1975). (Gratzl and Dahl 1976)

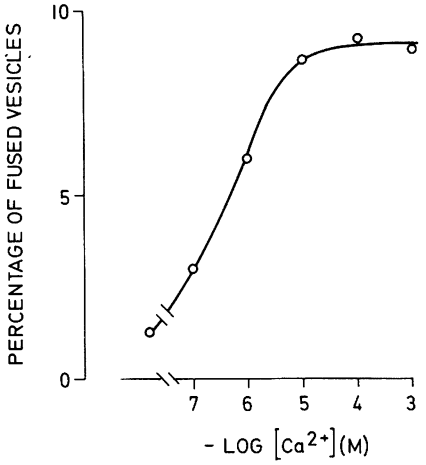

Fig. 5. Percentage of fused vesicles as function of the Ca^{2+}-concentration. The experiments were evaluated by counting 500 vesicles for each Ca^{2+}-concentration (Gratzl and Dahl 1976, 1978)

Table 2. Cation specificity of the fusion of rat liver secretory vesicles

Cations	Percentage of fused vesicles
-	1.3
10^{-4}M Ca^{2+}	10.2
10^{-4}M Mg^{2+}	1.5
10^{-4}M Sr^{2+}	1.8
10^{-4}M Ba^{2+}	1.7
10^{-4}M La^{3+}	1.4
10^{-4}M Mn^{2+}	2.6
10^{-4}M Ca^{2+} + 10^{-4}M Mg^{2+}	7.0
10^{-4}M Ca^{2+} + 10^{-3}M Mg^{2+}	5.0

The experiments were evaluated by counting 400 vesicles for each incubation (Gratzl and Dahl 1976, 1978).

zymes, neuraminidase, or glutaraldehyde abolished fusion induced by Ca^{2+} <1 mM (Gratzl and Dahl 1978).

Fusion of isolated secretory vesicles in vitro, triggered by low [μM] concentrations of Ca^{2+}, the ineffectiveness of other divalent cations in replacing Ca^{2+}, and the inhibition of Ca^{2+}-induced fusion by Mg^{2+} are all properties common to the fusion of secretory vesicles isolated from different tissues (Gratzl and Dahl 1976, 1978; Dahl and Gratzl 1976; Gratzl et al. 1977, 1980; Gratzl et al. 1977; Dahl et al. 1979; Gratzl et al. 1980). The different efficacy of the alkaline earths and the Ca^{2+}/Mg^{2+} antagonism may well provide a clue to the biochemical nature of the Ca^{2+}-sensitive apparatus in secretory vesicle membranes.

Ca^{2+}-specificity for fusion (albeit at higher concentrations) and sensitivity to proteolytic attack was also observed with isolated cell membranes from myoblasts (Schudt et al. 1976; Dahl et al. 1978). Ca^{2+}-specific fusion of these membranes was not only abolished by enzymatic pretreatment or prefixation with glutaraldehyde but was also reduced when cell membranes were isolated from cultures supplemented with cycloheximide (Dahl et al. 1978). All of these experiments support the suggestion that membrane proteins participate in the Ca^{2+}-specific fusion of biological membranes.

Trypsinized secretory vesicles from adrenal medulla, which are unable to fuse with μM Ca^{2+}, could only be fused when exposed to very high concentrations of Ca^{2+} (>2.5 mM). However, at these concentrations Mg^{2+} and other divalent cations were equally effective (Dahl et al. 1979; Gratzl et al. 1980). Apparently, upon enzymatic attack of the membrane proteins, secretory vesicles not only lose their ability to fuse with low [Ca^{2+}], but also their specificity for this cation.

To establish further the role of membrane proteins or lipids in the fusion of isolated secretory vesicles, liposomes have been prepared from the extracted lipids of adrenal medullary secretory vesicle membranes. These liposomes, as trypsinized secretory vesicles, fuse with Ca^{2+} or other divalent cations in concentrations higher than 2.5 mM. Moreover, if Ca^{2+} and Mg^{2+} are added to the liposomes, the effect of both ions is additive (Dahl et al. 1979; Gratzl et al. 1980). Thus, the ionic requirements of liposome fusion are similar for liposomes prepared from either the membrane lipids of secretory vesicles or pure phospholipids (cf. ref. Papahadjopoulos 1978).

Comparison of Secretory Vesicle Fusion in Vitro and Secretion by Exocytosis of Intact Cells

This chapter will discuss whether fusion of secretory vesicles in vitro matches the properties of exocytosis by intact cells.

The intracellular concentration of Ca^{2+} in resting cells is low ($\leqslant 10^{-7}$M) (Baker et al. 1976; Di Polo et al. 1976) but increases during stimulation. Release of transmitter is directly correlated with a rise in the intracellular Ca^{2+}-concentration (Llinás and Nicholson 1975). This was shown in the giant synapse, where the intracellular concentration of Ca^{2+} was directly monitored with injected Ca^{2+} indicators. Similarly, the intracellular Ca^{2+}-concentration of sea urchin eggs, activated to release cortical vesicle contents by exocytosis, increased to a mean value of 2.5-4.5 μM (Steinhardt et al. 1977) and in the medaka egg in the space beneath the cell membrane to 30 μM (Gilkey et al. 1978).

To find out whether the observed increase in intracellular [Ca^{2+}] parallels exocytosis or controls exocytosis directly, intracellular concentration of Ca^{2+} was increased by microinjection. Actually Ca^{2+}, injected into the presynaptic nerve terminal of the giant synapse induced transmitter release, while Mg^{2+} and Mn^{2+} were ineffective. Mg^{2+} and Mn^{2+} led to a slight reduction in the amount of transmitter released by Ca^{2+} (Miledi 1973). Also Ca^{2+}, but not Mg^{2+} injected into mast cells, elicited extrusion of secretory granules or resulted in exocytosis of cortical vesicles of amphibian oocytes (Kanno et al. 1973; Hollinger and Schütz 1976).

The concentration of intracellular Ca^{2+} can also be modified in cells rendered permeable to small molecular weight substances by high voltage

discharges (Baker and Knight 1978). Such "leaky" adrenal medullary cells release less than 1% of total intracellular catecholamine when EGTA is present in the incubation medium. Addition of μM Ca^{2+} induces release of catecholamines, but not lactate dehydrogenase from the cytoplasm. Raising the Mg^{2+}-concentration to 2-50 mM reduces the amount of catecholamines released by Ca^{2+} (Baker and Knight 1978).

By comparing exocytosis by intact cells and fusion of secretory vesicles in vitro, it is obvious that both processes occur under similar conditions. Secretory vesicle fusion is low at the Ca^{2+}-concentration found in resting cells but increases with $[Ca^{2+}]$ found in stimulated cells. Exocytosis by intact cells can be triggered by increasing experimentally the intracellular Ca^{2+}-concentrations. Mg^{2+} and other divalent cations are ineffective in inducing both exocytosis and secretory vesicle fusion. Moreover, Mg^{2+} inhibits exocytosis as well as the secretory vesicle fusion activated by Ca^{2+}. Thus, it appears that Ca^{2+} is able to act specifically as the final trigger of exocytosis by initiating membrane fusion.

The fusion of liposomes prepared from the membrane lipids of secretory vesicles and trypsinized secretory vesicles does not retain the characteristic ionic requirements of the fusion of intact secretory vesicles. Fusion of the former requires >mM concentration of Ca^{2+}, whilst Mg^{2+} and other divalent cations can replace and supplement the Ca^{2+}-effect. Therefore, it can be concluded that membrane proteins of secretory vesicles account for the characteristic properties of secretory vesicle fusion. The precise role of membrane proteins in secretory vesicle fusion and exocytosis by intact cells is not known yet. They may bind Ca^{2+} specifically, act as recognition sites between the interacting membranes and/or be involved in the membrane rearrangements taking place during membrane fusion. It has recently been demonstrated that binding of Ca^{2+} to high affinity sites on secretory vesicle membranes parallels Ca^{2+}-induced fusion (Dahl et al. 1979). Further characterization and identification of the membrane components with which Ca^{2+} interacts promises progress towards the molecular mechanism of exocytotic membrane fusion.

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