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CONTENTS

MODELS FOR EXOCYTOTIC MEMBRANE FUSION

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Introduction

Based on morphological studies, the concept of exocytotic secretion was developed about twenty years ago (De Robertis & Vaz Ferreira, 1957; Palade, 1959). At nearly the same time, the essential role of calcium in secretory processes was recognized and the concept of calcium mediated stimulus–secretion coupling was proposed by Douglas & Rubin (1961). Since then, data have accumulated which strongly support the validity of both concepts for the release of hormones, enzymes and neurotransmitters. The electron-microscopical observation of exocytotic figures has been extended to a variety of secretory cells and the frequency of such figures is found to be correlated with the secretory activity of the cells. Further the quantal nature of transmitter release is most compatible with an exocytotic mechanism. Finally, exocytosis is biochemically evidenced by the observation of a stoichiometric release of the various substances stored in secretory vesicles. These substances differ considerably in size, e.g. ATP, catecholamines and the protein chomogranin from the adrenal medulla. While the involvement of calcium in secretory processes was first recognized by its requirement in the extracellular fluid, flux measurements and, more directly, the use of calcium-sensitive dyes, have revealed that a rise of the intracellular free Ca^{2+} concentration parallels secretion. The ability of Ca^{2+} even to initiate secretion has been demonstrated by injection of the ions into cells. For further details and references, see the reviews by Douglas (1974) and Winkler *et al.* (1974). Whereas the involvement of Ca^{2+} in exocytotic secretion has been well documented, its precise role remains unclear. Consequently, several alternative actions have been attributed to this ion: gel to sol change of the cytoplasm, interaction with the microtubular–microfilamen-

tous system, change of surface charge of membranes and initiation of membrane fusion (cf. Rubin, 1974; Baker, 1977).

The present report is concerned with recent progress made with cell-free systems which may help to clarify some molecular events of exocytotic membrane fusion and the role of Ca^{2+} in this process.

Model systems

Because of the complexity of events during exocytotic secretion and the inaccessibility of the interacting membranes to test substances, the necessity of model systems is quite obvious. Hence, many model systems have been proposed to clarify the fusion process, such as cell–cell fusion mediated by chemicals, viruses or liposomes, or liposome–cell fusion (Harris, 1970; Papahadjopoulos, Poste & Schaeffer, 1973; Ahkong *et al.*, 1975; Poste, Papahadjopoulos & Vail, 1976). Despite their merits in enabling the handling of many biological problems, such as hybridization of cells, most of these model systems suffer from their own complexity, which even may exceed that of the secreting cell. Furthermore, in some cases the substances and experimental conditions required for these fusion processes are more exotic than exocytotic.

Two types of experimental approach, which promise to lead towards an understanding of the mechanisms of exocytotic membrane fusion have been developed in the last few years. These are the classical methods of natural sciences, which complement each other.

(1) The inductive method

As a drastic simplification, the basic components of biological membranes can be separated and the individual components tested with respect to the function of interest. Subsequently, a stepwise recombination of the various components may reflect the biological process. Liposomes (artificial membranes) prepared from phospholipids which are well defined in chemical and physical terms are used in several laboratories for this purpose (Blioch, Glagoleva, Liberman & Nenashev, 1968; Maeda & Ohnishi, 1974; Papahadjopoulos, Poste, Schaeffer & Vail, 1974; Prestegard & Fellmeth, 1974; Breisblatt & Ohki, 1975; Van der Bosch & McConnell, 1975; Miller & Racker, 1976; Dunham *et al.*, 1977).

(2) The deductive method

Starting with the complex system, it can be simplified step by step. Tissue-fractionation techniques can be applied to isolate membranes

involved in physiological fusion processes and their fusion can be followed *in vitro*. The contribution of the various membrane components to the fusion reaction can be investigated, for example, by modification of the membranes – by enzymatic treatment or by extraction procedures. Interactions of isolated secretory vesicles with cell membrane fractions have been reported by Davis & Lazarus (1976) (see also Lazarus, this volume) and by Milutinović, Argent, Schulz & Sachs (1977). Unfortunately, in both studies it is not clear to what extent membrane fusion actually occurred. Furthermore, at present it seems to be difficult to obtain adequate cell membrane fractions. After disruption, most membranes exhibit the tendency to form closed vesicles which predominantly are orientated right side out (Altendorf & Staehelin, 1974; Losa, 1976; Dahl, Schudt & Gratzl, 1978). Thus, in a cell-free system, secretory vesicles will only have a reduced chance to gain access to the previous cytoplasmic surface of the isolated cell membranes.

In an effort to circumvent these difficulties, we have omitted a plasma membrane fraction and followed the fusion of isolated secretory vesicles with each other (Dahl & Gratzl, 1976; Gratzl & Dahl, 1976; Dahl, Gratzl & Ekerdt, 1976; Gratzl, Dahl, Russell & Thorn, 1977). To take intervesicular fusion as a model system for exocytosis is justified by its occurrence in a wide spectrum of secreting cells (Ekholm, Zelander & Edlund, 1962; De Virgilis, Meldolesi & Clementi, 1968; Amsterdam, Ohad & Schramm, 1969; Normann, 1970; Farquhar, 1971; Röhlich, Anderson & Uvnäs, 1971; Berger, Dahl & Meissner, 1975) where, by 'compound exocytosis' (Douglas, 1974), the contents of several vesicles are ejected through one orifice at the cell membrane. The site of secretion seems to be determined by membrane factors (Meldolesi, this symposium). It could be argued that for compound exocytosis such cell membrane factors will diffuse into the vesicle membrane after establishment of membrane continuity with the cell membrane and thus permit subsequent fusion of a further vesicle to this site as shown in Fig. 1. But this becomes unlikely if we take into account the fact that the serial fusion of vesicles is a very fast process which seems to occur within milliseconds. In mast cells, where the dynamics of compound exocytosis has been impressively shown in a microkinematographic study by Douglas (1974), secretory vesicles are of a diameter of 0.5 μm and the circumference is 1.5×10^{-4} cm. The upper limits of the diffusion coefficients for membrane proteins and lipids are in the order of 10^{-10} and 10^{-8} $cm^2 s^{-1}$ respectively (Lee, 1975). From

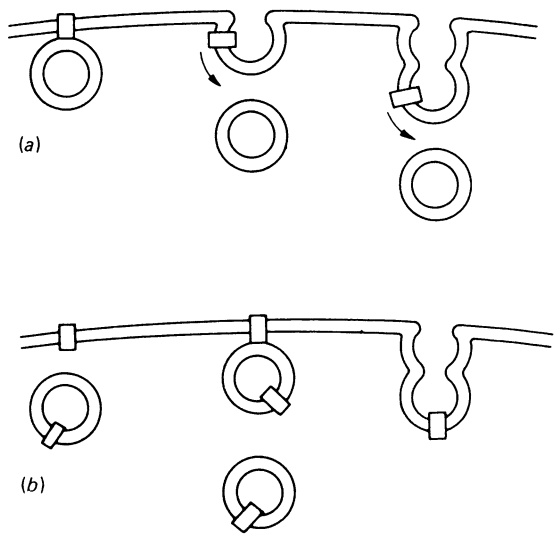


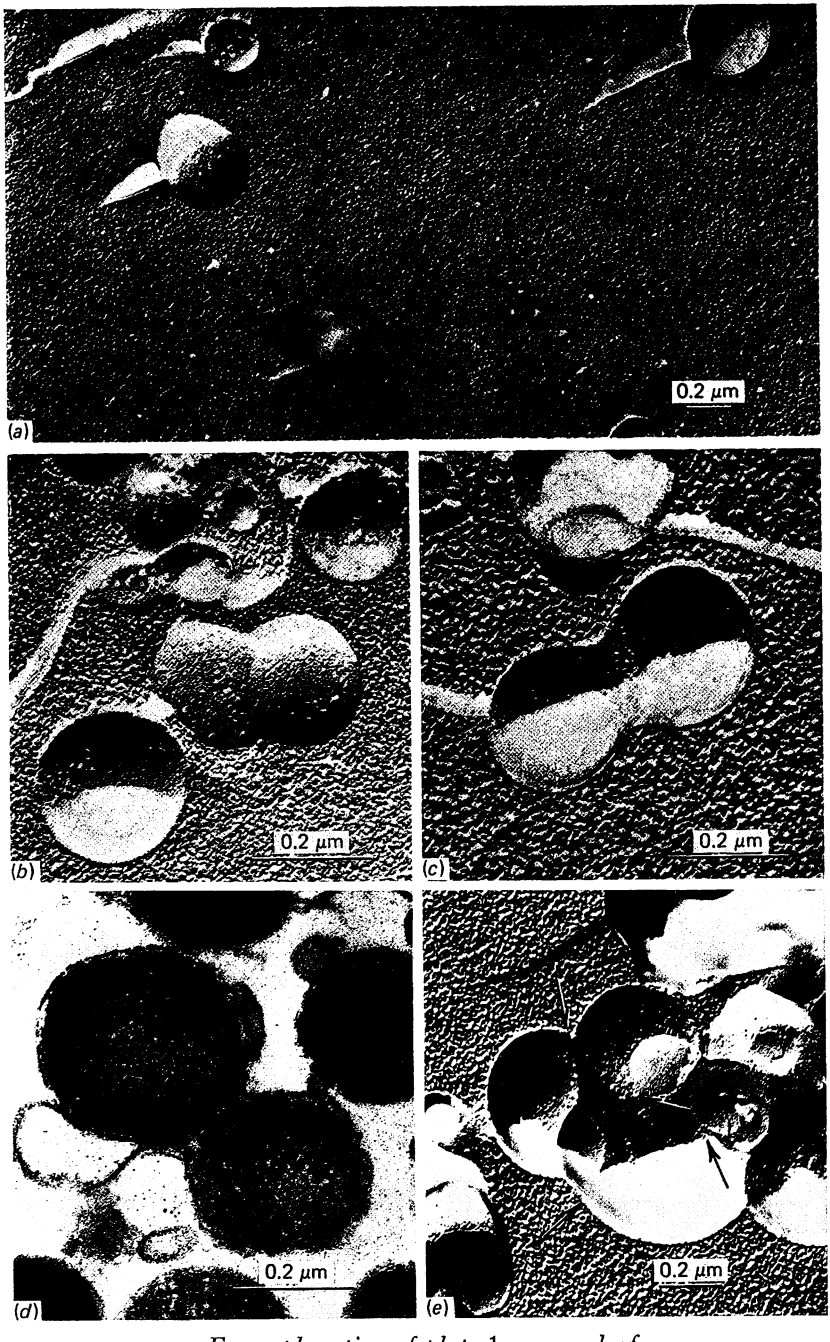
Fig. 1. Schematic representation of hypothetical membrane factors responsible for exocytotic membrane fusion. If such factors were present in the cell membrane exclusively, they would have to diffuse into the membrane of secretory vesicles after establishment of continuity with the cell membrane for compound exocytosis to occur (*a*). The coexistence of such factors in the cell membrane and in secretory vesicle membranes would allow compound exocytosis to proceed without a delay in time (*b*).

$T = X^2/2D$, where T is the time required for a membrane component to move by self-diffusion along the distance X and D is the diffusion coefficient in cm^2s^{-1}, it would take about 100 s for a proteinaceous factor or about 1 s for a lipid factor to diffuse over half of the circumference of the vesicle. From this estimate, the argument presented above is not valid. Therefore we have to conclude that the secretory vesicles already possess fusion sites themselves which account for intervesicular fusion.

Liposome fusion

Of the different studies on liposome–liposome fusion, those dealing with Ca^{2+}-induced fusion seem to be the most attractive to serve as model system for exocytosis, and we shall therefore be exclusively concerned with them in the following. Fusion of liposomes composed of single or mixed phospholipids has, to a great extent, been characterized (Papahadjopoulos *et al.*, 1976, 1977) and the major findings can be summarized as follows. For cationic-induced fusion,

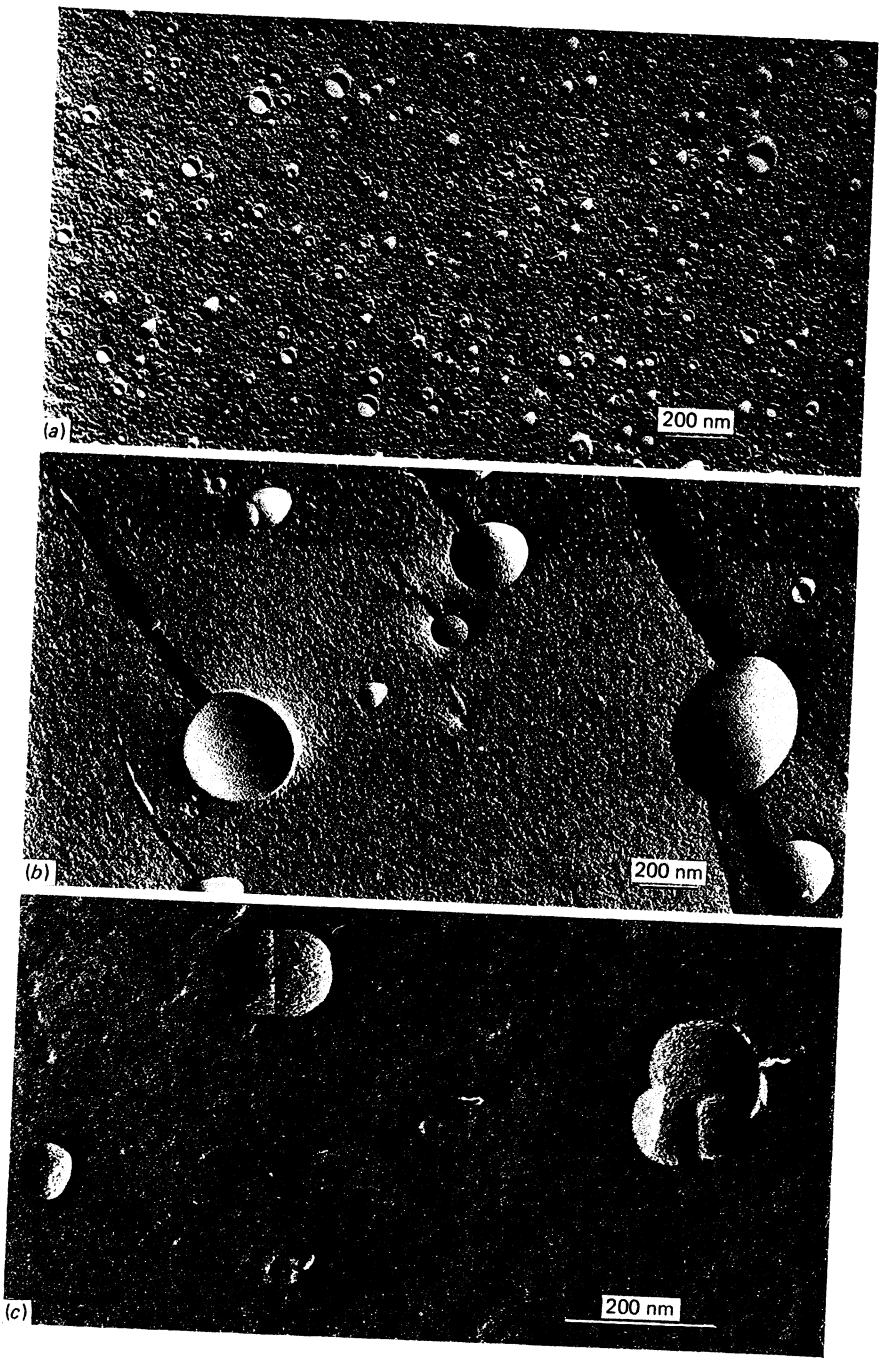
PLATE 1



For explanation of plate 1 *see overleaf*

(*Facing p.* 352)

PLATE 2



Explanation of plates

Plate 1. Electron micrographs of secretory vesicles isolated from the adrenal medulla. Vesicles are dispersed in a cacodylate-buffered sucrose medium containing 1 mM EGTA (*a*). Vesicles incubated with 10^{-4} M Ca^{2+} (5 min, 37 °C) are clustered, and twinned vesicles occur, indicating intervesicular fusion (*b*, *c*, *d*). Twinned vesicles exhibit continuous cleavage planes in the membrane E-face as well as in the P-face in freeze-fractures (*b*, *c*) and two electron-dense cores surrounded by a continuous membrane in thin sections (*d*). Vesicles incubated with 10^{-2} M Ca^{2+} are heavily aggregated and multiple fusions (arrows) result in a large structure, irregular in shape (*e*).

Plate 2. Freeze-fracture electron micrographs of liposomes prepared from lipid extracts of adrenal chromaffin granule membranes. (*a*) After incubation in Ca^{2+}-free media the small unilamellar vesicles are rather homogeneous in size. (*b*) After incubation with 10^{-2} M Ca^{2+} (5 min, 37 °C) the number of vesicles is reduced while their size is increased considerably. The large vesicles are often spheres but can also be irregular in shape. (*c*) Liposomes incubated for 35 ms with 10^{-2} M Ca^{2+}. Structures composed of multiples of the initial vesicle size exhibiting continuous cleavage planes indicate that the large vesicles are generated by fusion of small vesicles rather than by molecular diffusion. Note the higher magnification of (*c*).

liposomes have to be composed of phospholipids bearing a net negative charge. In mixtures with neutral phospholipids, a threshold concentration of 40–60 % of charged lipids is required. The acidic phospholipids have to be in the liquid crystalline state; i.e. fusion does occur at temperatures above the phase transitions of the lipids. The minimal concentration of cations required to induce fusion depends on the type of phospholipid: e.g. 0.2 mM for phosphatidic acid (PA) and 10 mM for phosphatidylglycerol. In some cases, Mg^{2+} can fully replace Ca^{2+} (PA), and in others it is less effective.

From the correlation of phase separation, sharp increase in permeability and isothermal phase transition (crystallization) phenomena, which are all induced in comparable conditions by Ca^{2+}, Papahadjopoulos and co-workers (1977) have proposed a hypothesis for the mechanism of liposome fusion. According to their concept, fusion will occur at domain boundaries caused by divalent-cation-induced phase changes which lead to a transient destabilization of the lipid bilayer. Although this concept is very attractive, it seems to have an intrinsic weak point. Phase transitions are indicated to be complete within some milliseconds (U. Fischer, personal communication). The time course of liposome fusion, however, was reported to be of the order of some minutes. Thus, fusion would occur in liposomes whose lipids are already in the solid state and this is contradictory to the initial postulate.

Fusion of secretory vesicles

Following the deductive route, our group has studied, by electron microscopy, the fusion of secretory vesicles isolated from endocrine pancreas, liver, adrenal medulla and neurohypophysis (Dahl & Gratzl, 1976; Dahl *et al.*, 1976; Gratzl & Dahl, 1976; Gratzl *et al.*, 1977). Whereas isolated secretory vesicles are dispersed in a medium containing EGTA (Plate 1*a*), interactions of vesicle membranes are readily seen after incubation with Ca^{2+} (Plate 1*b*, *c*). Ca^{2+}-induced fusion of the vesicles is indicated by the appearance of twinned vesicles where the cleavage plane is continuous in both membrane faces exposed by freeze-fracturing. In thin sections, these structures contain two electron-dense cores surrounded by one continuous membrane (Plate 1*d*). In this common lumen, intermixing of the cores may be observed.

Biochemical evidence for intermixing of vesicle contents has recently been given by Quinn & Judah (1978) for secretory vesicles

of liver, following the same protocol as used in our studies. As known for many other proteinaceous secretory products (see Olsen & Berg, this symposium), a conversion of proalbumin to albumin precedes the secretion of albumin by hepatocytes. The inhibition of conversion by blocking the converting enzyme could be compensated when such vesicles had been co-incubated with untreated vesicles at low Ca^{2+} concentrations. The most plausible explanation for this observation is that the proalbumin of vesicles in which the converting enzyme has been inhibited, becomes the substrate of the enzyme of untreated vesicles by intervesicular fusion between both populations of vesicles.

In freeze-fractured suspensions, the number of twinned vesicles remains constant either if incubation is prolonged or if Ca^{2+} is withdrawn by an excess of EGTA. This shows that this process is not reversible. It also indicates that these structures are stable and do not tend to round up as would be expected by analogy to soap bubbles. This might be due to a preservation of the surface to volume ratio: a new sphere composed of the membranes of two vesicles would enclose a larger volume than is the sum of two vesicle volumes. These structures could also be stabilized by membrane components, as indicated by particles which are often found to form a ring-like aggregation at the waist. Interestingly, also in intact cells, fused secretory vesicles do not form spherical structures during compound exocytosis (Douglas, 1974; Burwen & Satir, 1977).

Intervesicular fusion, as quantified by counting the percentage of twinned vesicles of the total number of vesicles, increases from 10^{-7} to 10^{-4} M Ca^{2+} and is half maximal around 10^{-6} M (Fig. 2). A similar dependence on Ca^{2+} concentration was reported by Milutinović *et al.* (1977) for the interactions of secretory vesicles with a cell membrane fraction isolated from the exocrine pancreas.

Other cations in concentrations up to 1 mM were found to be unable to replace calcium in inducing fusion. Mg^{2+} or Mn^{2+} even inhibited Ca-induced fusion (Gratzl & Dahl, 1978). Incubations of the vesicles with Ca^{2+} at various temperatures revealed a monotonous decrease of the extent of fusion with lowering the temperature. However, fusion can still be observed at 2 °C (Fig. 3*a*).

The first approach for identification of membrane components governing the fusion process was to study the effect of enzymatic modification of the membranes. It turned out that vesicles pretreated with neuraminidase, proteases or glutaraldehyde exhibited a reduced susceptibility to fusion depending on the concentration of enzymes

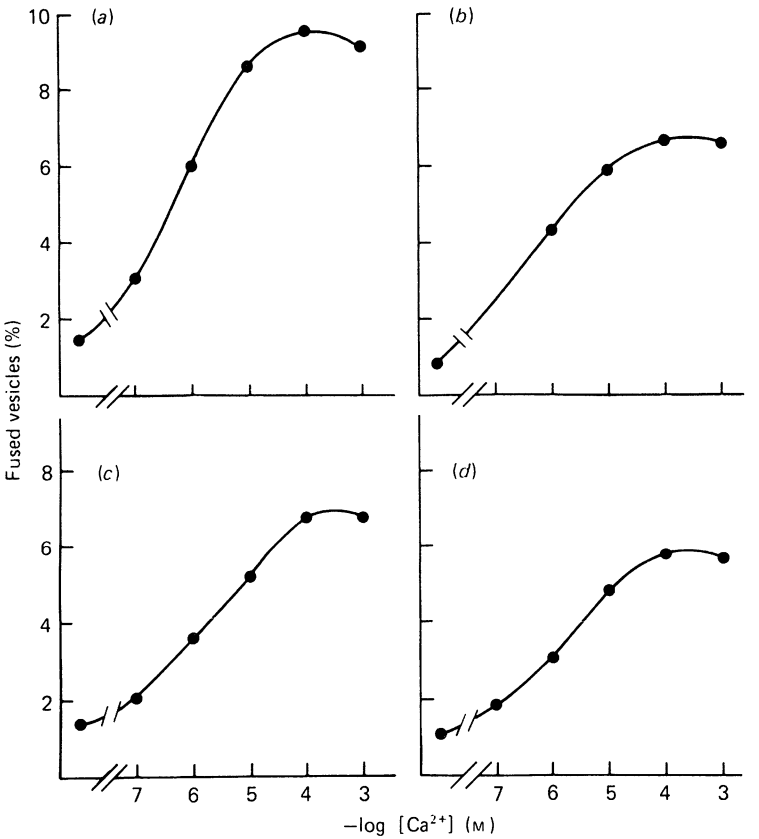


Fig. 2. Percentage of fusion of isolated secretory vesicles as a function of Ca^{2+} concentration. Fusion of vesicles isolated from liver (*a*), endocrine pancreas (*b*), adrenal medulla (*c*) and neurohypophysis (*d*) exhibit a similar dependence of Ca^{2+} concentration. Redrawn from Gratzl & Dahl (1978), Gratzl *et al.* (1977) and unpublished data.

used (Fig. 3*b*). An inhibitory effect of proteases on the interaction between secretory vesicles and a cell membrane fraction from exocrine pancreas has also been reported (Milutinović *et al.*, 1977).

A further step was to extract the membrane lipids and to follow the fusion of liposomes prepared from the lipid extracts of adrenal chromaffin granule membranes. Small unilamellar liposomes (Plate 2*a*) were obtained by sonication of the lipids, which were extracted according to Folch, Lees & Sloane-Stanley (1957). They are rather homogeneous in size, with a mean diameter of 40 nm. After incubation of the liposomes with divalent cations, a dramatic increase of

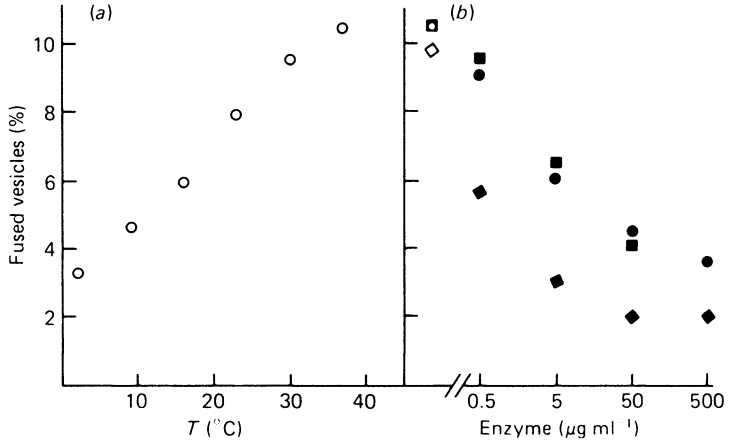


Fig. 3. Percentage of Ca^{2+}-induced fusion of rat liver secretory vesicles. (*a*) Temperature dependence of fusion induced by 10^{-4} M Ca^{2+}; (*b*) Before addition of Ca^{2+} (final concentration 10^{-4} M), vesicles have been pre-incubated in Ca^{2+}-free medium at 0 °C for 30 min with various concentrations of trypsin (■), pronase (●), neuraminidase (◆) or with the heat-inactivated enzymes (□, ○, ◇; 500 $\mu g\,ml^{-1}$). From Gratzl & Dahl (1978).

size of the liposomes with a concomitant reduction of their number are observed (Plate 2*b*).

The incubation time normally used was 5 min. Some preliminary experiments, however, have revealed a quantal increase of the liposome size after very short incubation times of some milliseconds (Plate 2*c*). This confirms the generation of large liposomes from fusion of the small vesicles and also shows that liposome fusion can be much more rapid than one might expect. For a quantitative evaluation of fusion, all liposome surfaces exposed in a defined area have been measured and their number counted (Fig. 4). Incubation with increasing concentrations of Ca^{2+} reveal that a threshold concentration of 2.5 mM is required to observe a minute increase in the mean of liposome surfaces, while this effect is more pronounced at 5 mM and higher concentrations. Mg^{2+} and other divalent cations can substitute for Ca^{2+}, Mg^{2+} being only slightly less effective.

One might argue that a specific function of membrane lipids has been lost by the procedure of liposome formation. Though the stoichiometry of lipids presumably is preserved, their orientation could be different from that in intact membranes with respect to sidedness on the lamellae or lateral distribution. This possibility seems unlikely, however, since a second type of fusion is exhibited

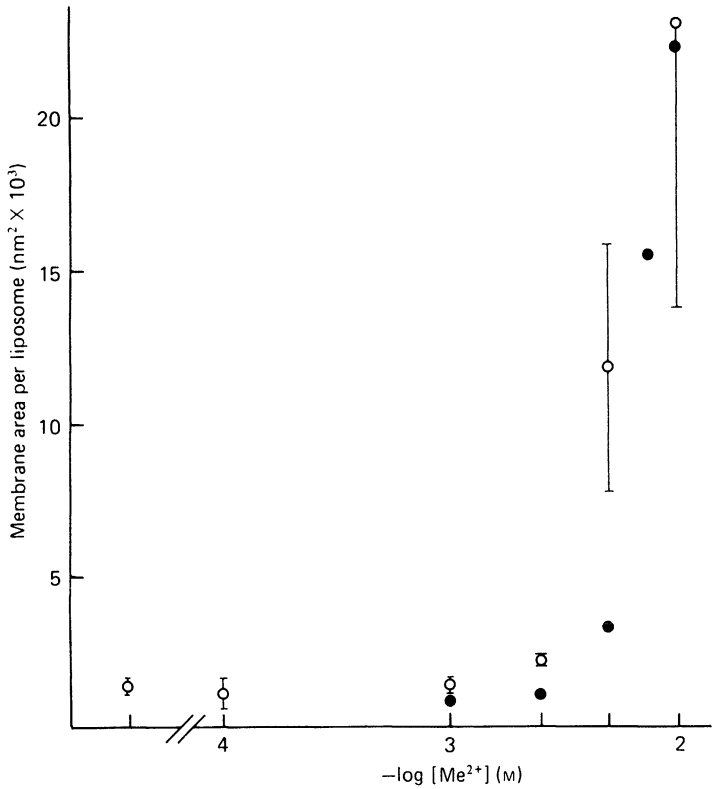


Fig. 4. Fusion of liposomes prepared from lipid extracts of adrenal chromaffin granule membranes as a function of divalent cation (Me^{2+}) concentration. Liposome fusion is quantified by measuring the areas of all membrane surfaces exposed in a defined area (480 μm^2) of a freeze-fracture replica and calculating the mean surface area per liposome. Liposomes have been incubated for 5 min at 37 °C with Ca^{2+} (○) or Mg^{2+} (●). The points for Ca^{2+} (○) are means ± SD of five different liposome preparations.

by intact secretory vesicles isolated from adrenal medulla in addition to that induced by Ca^{2+} in the micromolar range. While the fusion described before, and referred to as type 1 in the following, reaches a plateau level at 10^{-4} M Ca^{2+}, an excess of fusion can be observed starting from 2.5–5 mM Ca^{2+}. For the induction of this excess of fusion, which is called type 2, Mg^{2+} can replace Ca^{2+} and it is not inhibited by pretreatment of the vesicles with neuraminidase, proteases or glutaraldehyde (Fig. 5*a*, *b*). Morphologically, type 2 fusion is characterized by the occurrence of multiple fusions of the vesicles (Plate 1*d*), which contrasts sharply with fusion of type 1.

Fusion of chromaffin secretory vesicles induced by 5 mM Ca^{2+} or

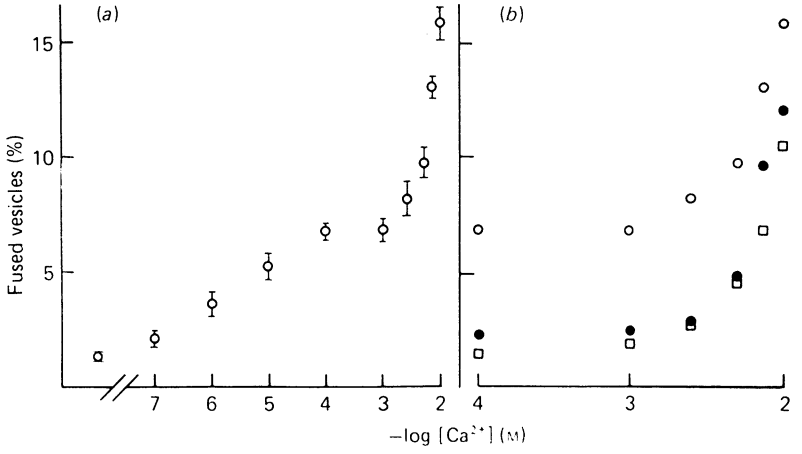


Fig. 5. Divalent cation concentration dependence of fusion of isolated adrenal secretory vesicles. (*a*) vesicles incubated with Ca^{2+} for 5 min at 37 °C; (*b*) vesicles incubated with Ca^{2+} (○), Mg^{2+} (□) or with Ca^{2+} (●) after pre-treatment of the vesicles with neuraminidase (500 $\mu g ml^{-1}$, 0 °C, 30 min).

Mg^{2+} was originally reported by Edwards, Phillips & Morris (1974). However, the same results were later interpreted as a reversible adhesion of vesicles by one of his co-authors (Schober, Nitsch, Rinne & Morris, 1977). We have observed that after addition of an excess of EGTA membrane contacts disappeared while the morphological changes indicating fusion types 1 and 2 persisted.

The binding of Ca^{2+} to secretory vesicle membranes

Secretory vesicles have been shown to exhibit a net negative surface charge which is influenced by divalent metal ions (Banks, 1966; Matthews, Evans & Dean, 1972). The concentration of the divalent cations required to change the electrophoretic mobility of the vesicles is in the millimolar range. Since with this method only the overall charge can be estimated, minor components which bind ions with different affinity might be easily overlooked. Recently, the Ca-sensitive dye, Arsenazo III, has been used to follow minute variations of the free Ca^{2+} concentrations even in EGTA buffers (Di Polo *et al.*, 1976). In preliminary experiments with this method, a high-affinity binding site with a dissociation constant of 5×10^{-6} M could be identified on chromaffin granule membranes (R. Ekerdt, M. Gratzl & G. Dahl, unpublished). The density of this binding site was found to be 3 nmol Ca^{2+} per milligram of protein. With

murexide, an additional binding site with much lower affinity (K_{diss} 2×10^{-4} M) could be found. The density of these sites is 100 times higher. Liposomes, prepared from the lipid extracts of these membranes, only exhibited the low-affinity binding site (K_{diss} 2×10^{-4} M).

The relation of the model systems to exocytosis

The model systems used in both the inductive and the deductive approaches seem to exhibit a congruence. The type 2 of vesicle fusion, which is also found for liposomes derived from these membranes, exhibits characteristics similar to those found for fusion of liposomes composed of single or mixed phospholipids. If the results obtained from the inductive and deductive approaches are compared with the cellular processes during secretion, however, it can be concluded that this convergence is achieved at a stage where some of the specific functions are lost.

Ionic requirement

In resting cells, the cytoplasmic free Ca^{2+} concentration has been estimated to be $\leqslant 10^{-7}$ M (Baker, 1976). It rises to about 10^{-5} M in response to stimulation (Llinás & Nicholson, 1975; Gilkey, Jaffe, Ridgway & Reynolds, 1978). As shown by studies where Ca^{2+} or Mg^{2+} has been applied intracellularly by microelectrodes (Kanno, Cochrane & Douglas, 1973; Miledi, 1973), the action of Ca^{2+} to elicit secretion is specific for this ion. Mg^{2+} or Mn^{2+} even inhibited the action of Ca^{2+} (Miledi, 1973). Only fusion type 1 of secretory vesicles exhibits such properties, whereas fusion type 2 and fusion of liposomes require much higher concentrations of Ca^{2+}, which in most cases can at least partially be replaced by Mg^{2+}. For this type even an additive action of Ca^{2+} and Mg^{2+} was observed.

Temperature

Liposome fusion has been shown to depend on the physical state of the membranes, with fusion occurring only at temperatures above the thermal phase transition of the phospholipids. Recent reports (Lagunoff & Wan, 1974; Marsh, Radda & Ritchie, 1976) of phase transitions occurring in membranes involved in exocytosis at ambient temperatures (18 °C, 33 °C) should, therefore, exclude significant fusion of secretory vesicles at low temperatures. However, vesicle fusion, although reduced, was found to be present at low temperatures. A calorimetric study of the membranes of chromaffin granules

and of their lipid extract did exclude a phase transition of the bulk of the lipids (Blume & Ekerdt, unpublished). This is not surprising if the high content of cholesterol and lysolecithin is considered. Furthermore, exocytotic release, even induced by cold, was shown to occur in intact cells of the neurohypophysis and endocrine pancreas with biochemical (Hong & Poisner, 1974) and morphological evidences (Dahl & Henquin, 1978).

Time course

Neuronal transmission can be complete within one millisecond. From Dr Douglas's kinematographic study of mast cell secretion, a similar time course of the exocytotic event can be estimated (Douglas, 1974). The fusion type 1 of secretory vesicles is complete in something less than one minute. A more rapid technique for detection of fusion has as yet only been applied to liposome fusion, and preliminary results indicate that incubation times as short as some milliseconds are sufficient to detect fusion. This is one of the few cases where marked differences can be found between liposomes prepared from lipid extracts of secretory vesicle membranes and liposomes composed of pure phospholipids. The latter require several minutes for fusion (Papahadjopoulos *et al.*, 1976). However, an intermediate time course has also been reported (Miller & Racker, 1976).

Specificity of membranes for fusion

The fact that secretory vesicles fuse with the cell membrane or with each other during compound exocytosis, but not with other membranes, implies the existence of factors which govern the specificity of interaction. The various acidic phospholipids under study for liposome fusion are components of all cellular and subcellular membranes (Hörtnagl, 1976). Specificity of membrane interactions therefore might depend on the content of these components, as indicated by the high threshold concentration required for the induction of liposome fusion (40–60%).

The content of phospholipids bearing a net negative charge in secretory vesicle membranes, however, is much lower. Their percentage of the total phospholipids is about 10% (Winkler, 1976; Dreyfus, Aunis, Harth & Mandel, 1977). Surprisingly, liposomes prepared from lipid extracts of these membranes fuse despite their low content of acidic phospholipids, as did liposomes prepared from lipid extracts of a mitochondrial and, to a lower extent, of a microsomal fraction. Also, liposomes derived from soybean lipids

can be induced to fuse by 5 mM Ca^{2+} (Ingolia & Koshland, 1978). Thus, it is very unlikely that the specificity of membranes for physiological fusion processes is determined by membrane lipids. Fusion type 1 of secretory vesicles, on the other hand, seems to account for membrane specificity, since neither a microsomal fraction nor mitochondria exhibit this type of fusion.

Hypothetical fusion sites and the nature of these sites

As outlined before, fusion type 1 of secretory vesicles is compatible with the exocytotic event. This type of fusion is affected by pre-treatment of the membranes with neuraminidase, proteases and glutaraldehyde and is not mimicked by liposomes derived from these membranes. Interestingly, transmitter release can also be inhibited after presynaptic injection of neuraminidase into the giant synapse (Tauc & Hinzen, 1974). These data suggest that specific membrane components, presumably glycoproteins, are of major importance for the fusion process.

Recently, however, the ganglioside GM3 has been shown to be present in secretory vesicle membranes of adrenal medulla (Dreyfus *et al.*, 1977; Geissler *et al.*, 1977). This component, however, applying the Folch extraction procedure, is absent in the lipid extract we have used. Since the action of neuraminidase also could be attributed to this component, it cannot be ruled out at present that the ganglioside might also be a candidate for governing fusion type 1.

The data on Ca^{2+} binding to secretory vesicles suggest that only a limited portion of the membrane surface is capable of interacting with these ions. The surface occupied by the low-affinity binding sites can be roughly estimated to be about 6% of the total vesicle surface if phospholipids with a cross-sectional area of 0.5 nm^2 are considered to represent these sites. This is plausible, since the low-affinity binding site is present both in intact vesicles and in liposomes prepared from their membrane lipids. Furthermore, charged phospholipids represent nearly 10% of the phospholipids (Winkler, 1976; Dreyfus *et al.*, 1977) in the adrenal medulla secretory vesicle membranes. The data are in agreement with those obtained from the electrophoretic mobility studies reported by Matthews *et al.* (1972).

The high-affinity binding sites, on the other hand, which are found in intact vesicles would occupy only about 1% of the vesicle surface if they were built up of proteins of about 4 nm in diameter. While

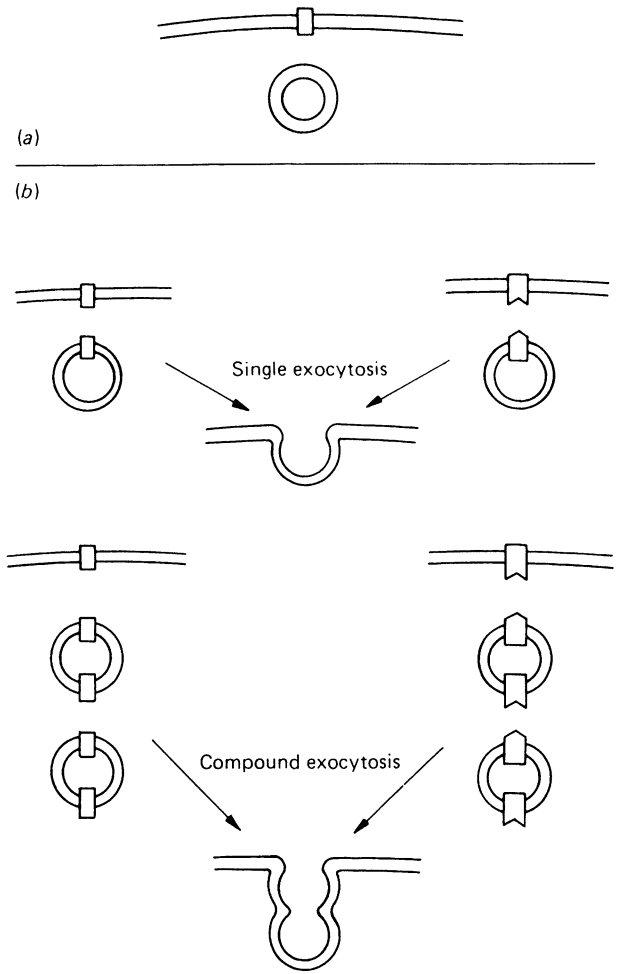


Fig. 6. Hypothetical fusion sites. Instead of sites being exclusively present on the cell membrane (*a*), it is more likely for there to be partner sites in both membranes for the process of recognition (*b*). These sites could be symmetric (identical) or asymmetric (complementary) in both membranes. The number of such sites on secretory vesicles could determine whether a cell will secrete by single or by compound exocytosis.

the major part of anionic sites seem to be randomly scattered over the vesicle membrane (Eagles, Johnson & Van Horn, 1976; Howell & Tyhurst, 1977), it is tempting to assume that the high-affinity binding sites are concentrated at one or two poles of the vesicle. This might be supported by the absence of multiple fusions for fusion type 1 in contrast to type 2. Interestingly, the occurrence of fusion sites

was postulated by Del Castillo & Katz in 1957 from theoretical considerations on the transmitter release by the motor end-plate. In this case, collision and contact of vesicles could be possible despite the repulsive forces of an overall net negative charge at low cation concentrations.

For recognition and interaction, the occurrence of partner sites in both membranes is more likely than a site in one membrane only (Fig. 6), for, otherwise, intracellular membranes different from secretory vesicles would also be accepted by these sites.

Whether the sites are symmetric (identical) in both membranes or whether they are asymmetric (complementary) cannot be distinguished at present. In the case of asymmetric sites, we have to expect the presence of both types on a single vesicle for compound exocytosis to take place. If only the complement to the site of the cell membrane existed on the vesicles, intervesicular fusion would be impossible. Thus the coexistence or non-coexistence of both types of sites might determine whether a cell will secrete by compound or by simple exocytosis. However, the number of cells recognized to use compound exocytosis is still increasing. Thus, very recently, compound exocytosis has been demonstrated in the adrenal medulla (Fenwick *et al.*, 1978) which for a long period of time has been considered to be a representative for simple exocytosis (Douglas, 1974). Compound exocytosis was first observed in cells containing secretory vesicles of large diameter, such as salivary gland, exocrine pancreas and mast cell. Since the chance of passing through the series of fused vesicles with thin sections is greater in large vesicles, a technical reason for the non-observation in cells with small vesicles has also to be taken into account.

Another possibility for a prevalence of one of the two forms of exocytosis could be the location of the increase of the Ca^{2+} concentration. For example, in the pancreatic β-cells, where glucose-stimulated secretion is dependent on extracellular Ca^{2+} and exocytosis is triggered by the transmembrane flux of Ca^{2+} which creates a gradient of the intracellular Ca^{2+} that declines very rapidly towards the cell centre (see Matthews, this symposium), intervesicular fusion is restricted to a small layer just beneath the cell membrane. By contrast, intervesicular fusion can be observed throughout these cells when they are incubated at low temperatures of 2 °C, a procedure which seems to result in a generalized rise of cytoplasmic free Ca^{2+} concentration (Dahl & Henquin, 1978). Likewise, compound exocytosis is most prominent in the mast cell

stimulated by polyamine 48–80, which is considered to make use of intracellular Ca^{2+} sources for exocytotic secretion (Douglas, 1974).

Conclusion

The purpose of this report has been to summarize what we have learned about the mechanism of exocytotic membrane fusion by means of model systems. While some details seem to have been clarified, a major part – especially that concerning the molecular mechanism – remains obscure. With respect to the two complementary model systems, we can notice that the coexistence of both approaches was, and presumably will continue to be, very fruitful.

At present, there is still a gap in the information provided by the model systems. Liposomal fusion seems not to account fully for events taking place during exocytotic secretion. On the other hand, it is not clear in what way membrane proteins are involved in the fusion process. Whereas a function as recognition sites is rather suggestive, conformational changes of membrane proteins might also directly interfere with the melting process of the lipid bilayers. However, proteins might also create the microenvironment for events to take place, comparable to those considered to be responsible for liposome fusion. The role for membrane proteins is still a matter of speculation and thus, at this stage of knowledge, a detailed description of the molecular events of exocytotic membrane fusion must await additional information from experiments.

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