MEMBRANE FUSION OF SECRETORY VESICLES AND LIPOSOMES

TWO DIFFERENT TYPES OF FUSION

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Secretory vesicles isolated from adrenal medulla were found to fuse in vitro in response to incubation with Ca^{2+}. Intervesicular fusion was detected by electron microscopy and was indicated by the appearance of twinned vesicles in freeze-fractured suspensions of vesicles and in thin-sectioned pellet. Two types of fusion could be distinguished: Type I, occurring between 10^{-7} M and 10^{-4} M Ca^{2+}, was specific for Ca^{2+}, was inhibited by other divalent cations and was abolished by pretreatment of vesicles with glutaraldehyde, neuraminidase or trypsin. Fusion type I was linear with temperature. A second type of intervesicular fusion was elicited by Ca^{2+} in concentrations higher than 2.5 mM and was morphologically characterized by multiple fusions of secretory vesicles. This type of fusion was found to be similar to fusion of liposomes prepared from the membrane lipids of adrenal medullary secretory vesicles: Ca^{2+} could be replaced by other divalent cations, the effect of different divalent cations was additive and pretreatments attacking membrane proteins were ineffective. Fusion type II of intact secretory vesicles as well as liposome fusion was discontinuous with temperature. Liposome fusion could be detected within 35 ms and persisted for 180 min. Using liposomes containing defined Ca^{2+} concentrations we have not found a major influence of Ca^{2+} asymmetry on fusion. Incorporation of the ganglioside GM_3, which is present in the membranes of intact adrenal medullary secretory vesicles did not change the properties of liposomes fusion. Using a Ca^{2+}-selective electrode we have identified in secretory vesicle membranes both high affinity binding sites for Ca^{2+} ($K_d = 1.6 \cdot 10^{-6}$ M) and low affinity sites ($K_d = 1.2 \cdot 10^{-4}$ M).

Introduction

Data have accumulated over the past twenty years, corroborating independent evidence, that an exocytotic mechanism is involved in the release of hormones, neurotransmitters and enzymes by a great variety of secretory cells. The substances designed for export are stored in the cytoplasm within membrane-limited vesicles, which, upon stimulation during exocytosis, fuse with the cell membrane to release their (often macromolecular) content into the extracellular also fuse with each other resulting in 'compound exocytosis' [1]. In one of the first electron microscopical documents of the exocytotic process, Palade [2] has shown 'compound exocytosis' to occur in the exocrine pancreas. Subsequently, the observation of compound exocytosis has been extended to many other secretory cell types including adrenal medulla [3–11].

Based on these observations, we have used intervesicular fusion of isolated secretory vesicles as a model system for exocytosis, in order to obtain infor-

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mation about membrane fusion not readily available from studies on intact cells. Further justification of this approach is given elsewhere [12]. In previous studies we have shown that secretory vesicles isolated from endocrine pancreas, neurohypophysis and liver can be induced to fuse by Ca^{2+} [13–16]. We have concluded that a major role of Ca^{2+} is the initiation of exocytotic membrane fusion by direct interaction with the membranes. The goal of the present study was to extend this approach to secretory vesicles isolated form the adrenal medulla. The intention was to investigate whether Ca^{2+}-induced fusion of secretory vesicles is at root to various secretory cells and to continue studies on the contribution of the different membrane components to the fusion process. For this purpose we have extracted membrane lipids of the secretory vesicles and have reconstituted the lipids in small unilamellar vesicles (liposomes); the fusion characteristics of these liposomes are compared with those of intact secretory vesicles.

Preliminary reports of part of this work have appeared elsewhere [17,18].

Materials and Methods

Bovine adrenal glands, obtained from the local slaughterhouse, were kept in ice cold 0.9% NaCl solution containing 10 mM Mops (3-N-morpholino)-propanesulfonic acid) (pH 7.0) and 5 mM EDTA, and were used within 2 h of removal from the animals. The medullae were dissected out, minced with scissors and homogenized with the aid of a loose-fitting teflon-to-glass homogenizer. The homogenate, consisting of 20% of the wet tissue weight in 10 mM cacodylate (pH 7.0), 300 mM sucrose and 5 mM EDTA, was centrifuged at $1200 \times g_{max}$ for 10 min and a crude secretory vesicle fraction was recovered by centrifugation of the supernatant at $12\,000 \times g_{max}$ for 10 min. Purified secretory vesicles were obtained by sucrose density gradient centrifugation as described [19] with the following modifications: the sucrose step gradient consisted of 0.86 M; 1.6 M; 2.0 M; 2.2 M sucrose solutions, each buffered with 10 mM cacodylate (pH 7.0) and 5 mM EDTA. Aliquots of the resuspended crude secretory vesicle fraction were layered on top of this gradient and centrifuged at $190\,000 \times g_{max}$ for 180 min with an RPS 40 T rotor in a Hitachi ultracentrifuge. Secretory vesicles

were collected from the interfacial bands at 1.6/2 M sucrose and 2.0/2.2 M sucrose, dialyzed for 2 h against 10 mM cacodylate (pH 7.0), 300 mM sucrose, 1 mM EGTA and centrifuged at $12\,000 \times g_{max}$ for 10 min. The pellet was carefully resuspended in the dialysis buffer to obtain a protein concentration of approx. 10 mg/ml.

Purified secretory vesicles were also prepared in an iso-osmolal gradient using Percoll™ (unpublished). This preparation gave results similar to those reported in the present work.

For Ca^{2+}-binding studies, membranes of secretory vesicles were isolated as follows: Secretory vesicles, stored at $-30°C$, were thawed by adding a 10-fold volume of an ice-cold medium containing 10 mM sodium citrate, 10 mM Mops (pH 7.0) and 10 g/l Chelex 100 (BioRad) and were centrifuged at $100\,000 \times g_{max}$ for 45 min. Membranes and Chelex 100 were resuspended with the aid of a syringe. Chelex 100 was removed by passing the suspension on through an nylon net. The washing procedure was repeated twice. The resulting membranes ('ghosts') were suspended twice in an excess of 190 mM KCl and 10 mM Mops (pH 7.0) (decalcified with Chelex 100) and centrifuged at $100\,000 \times g_{max}$ for 45 min. The Ca^{2+} concentration was measured with a Ca^{2+}-selective electrode, operating with a neutral carrier incorporated in a polyvinylchloride membrane [20]. Ca^{2+} binding was assayed in a total volume of 0.5 ml at 20°C under constant stirring.

In order to extract membrane lipids, the secretory vesicles were lysed with 10 mM cacodylate (pH 7.0), 5 mM EDTA. The membranes were pelleted at $100\,000 \times g_{max}$ (45 min), lyophylized and extracted according to Folch et al. [21] with the modification that the washing medium consisted of 0.74% KCl buffered with 10 mM cacodylate (pH 7.0) and 5 mM EDTA. The extracts (approx. 2 mg) were dried under a stream of nitrogen and under reduced pressure and the lipids were allowed to swell for 60 min in 200 ml buffer. Bath type sonication was carried out by placing the tip of a Branson Sonifier near the closed tube, which was immersed in a water bath thermostatically controlled at 37°C. After sonication at 150 W output for 30 min the almost-clear preparation was centrifuged at $100\,000 \times g_{max}$ for 60 min, and liposomes were collected from the middle of the tube. Incubation procedures, preparation for electron
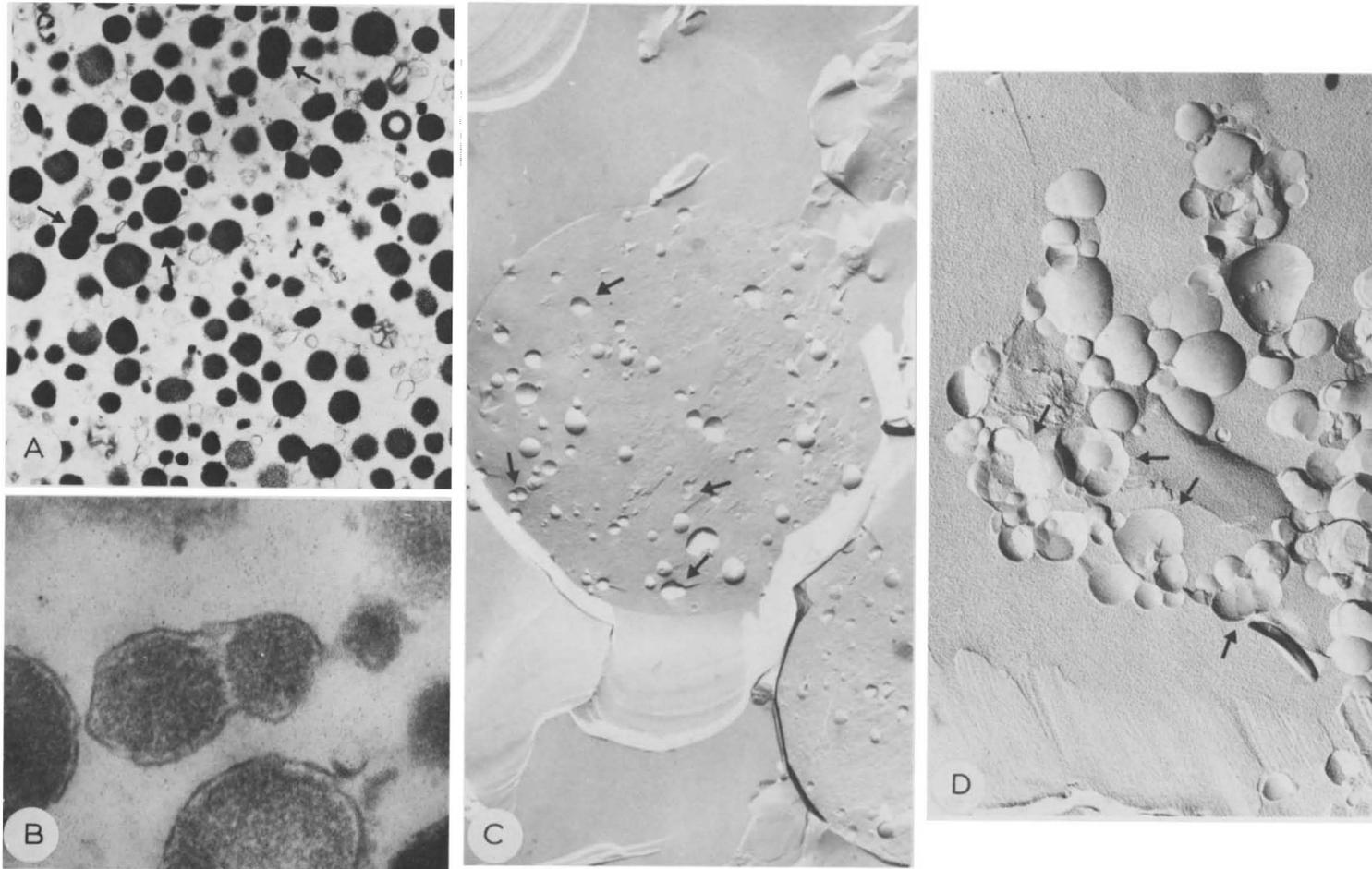
Fig. 1. Electron micrographs of isolated secretory vesicles. In thin-section electron micrographs twinned vesicles (arrows) are apparent after incubation with 10^{-4} M Ca^{2+} (A). At higher magnification, a continuous membrane is surrounding two electron-dense cores of a twinned vesicle (B). Twinned vesicles (arrows) are also apparent in spray droplets rapidly frozen in liquid propane after incubation of vesicles with 10^{-4} M Ca^{2+} (C). In a freeze-fracture electron micrograph, vesicles are heavily aggregated and multiple fusions of vesicles (arrow) occur, when incubated with 15 mM Ca^{2+} (5 min, 37°C) (D). Magnification: A, 14 000X; B, 80 000X; C, 7200X; D, 16 000X.

microscopy, and evaluation of fusion of secretory vesicles was done as described previously [16]. Quantitative analysis of liposome fusion was performed by randomly taking electron micrographs covering a total of 480 μm^2 of replica for each experimental condition. The cross-sectional area of each liposome was measured on prints with the aid of a curve digitizer MOP AM02 (Kontron Munich). Data are expressed as membrane area exposed in two dimensions.

Spray-freezing was performed with a Balzers spray-freeze apparatus using the spray device of this apparatus or by directing a jet of suspension into liquid propane as described [22]. Short incubation times were achieved with a stopped flow apparatus either by sequential rapid mixing of the vesicle suspension with Ca^{2+} solution followed by EGTA or by jet-freezing of the incubation mixture in liquid propane.

Protein was determined according to Lowry et al. [23]. Prior to the determination the vesicles were precipitated with 10% trichloroacetic acid and the pellets redissolved in NaOH/desoxycholate (2%/3%) (w/w).

Results and Discussion

Fusion of isolated secretory vesicles

Secretory vesicles isolated from the adrenal medulla, freeze-fractured in suspension after exposure to different Ca^{2+} concentrations, have morphological characteristics similar to those found for various other types of secretory vesicles [13–16]: The vesicles are dispersed in the medium when no Ca^{2+} has been added, while vesicle-vesicle contacts and twinned vesicles (and a few triplets) are apparent in the presence of 10^{-4} M Ca^{2+}. The continuous fracture plane in E and P-faces of twinned vesicles indicates that they are products of intervesicular fusion (for interpretation see also Ref. 16). Addition of an excess of EGTA reversed the adhesion of vesicles to each other but there was no effect on the frequency of twinned vesicles, indicating that in contrast to adhesion, vesicle fusion is not reversible by EGTA. Vesicles have usually been incubated for 5 min at 37°C. The frequency of twinned vesicles remained unchanged when shorter (1 min) or longer (30 min) incubation times have been applied. This suggests that the fusion process is fast and completed in less than 1 min. Thin-sections of secretory vesicles incubated

with Ca^{2+} also reveal the presence of twinned vesicles, where two electron-dense cores are surrounded by a continous membrane (Fig. 1).

Fig. 1 shows also as a control a spray-frozen droplet containing the vesicle suspension after incubation with 10^{-4} M Ca^{2+}. Several twin vesicles can be observed in the vesicle suspension, which has undergone no other treatment than cryofixation before fracturing and replication.

A quantitative evaluation of the frequency of intervesicular fusion shows a sigmoidal relation with the Ca^{2+} concentration over the range $10^{-7}-10^4$ M reaching a plateau (Fig. 2). Half maximal effect is observed at about $5 \cdot 10^{-6}$ M. Thus, fusion of chromaffin vesicles exhibit an almost identical dependence on Ca^{2+} concentration as found for various other secretory vesicles [13–16]. The highest percentage of fusion observed with 10^{-4} M Ca^{2+} is about 8%, which is a 5-fold increase over the control. It may be argued that this is not a dramatic effect. However, besides inherent in the morphological assay used [16], most secretory cells including adrenal medulla [24] release only a very small fraction of their secretory product stored in secretory vesicles even when maximally stimulated for a long period of time. It is not conceivable therefore to assume that all secretory vesicles are in the same state of maturation.

Additional intravesicular fusion can be observed exceeding the plateau level just described, when the Ca^{2+} concentration is increased from 2.5 mM to 10 mM (Figs. 1 and 2). At these divalent cation concentrations vesicles are heavily aggregated. For ease of interpretation therefore, the quantitative analysis was done on samples which, after incubation with divalent cations, are mixed with EDTA-containing buffer.

In contrast to Ca^{2+}, other divalent cations (Mg^{2+}, Mn^{2+} or Sr^{2+} were able to induce fusion of secretory vesicles only in concentrations higher than 2.5 mM (Fig. 2). Pretreatment of secretory vesicles with neuraminidase (Clostridium perfringens) abolished the Ca^{2+}-induced fusion at low concentration (Fig. 2) and the concentration dependence for Ca^{2+} is now indistinguishable from the effects of Mg^{2+}, Mn^{2+} or Sr^{2+}. Thus two types of fusion with characteristic ionic requirements can be distinguished: Type I is specific for Ca^{2+} at concentrations lower than 10^{-4} M; type II is induced by several divalent cations at concentrations above 10^{-3} M.
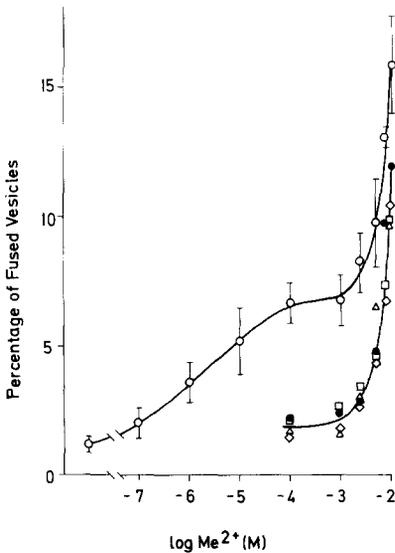
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Fig. 2. Percentage of fused secretory vesicles as a function of divalent cation concentrations: Ca^{2+} (○), Mg^{2+} (◇) and Sr^{2+} (△). Ca^{2+}-induced fusion is also given after pretreatment with neuraminidase (500 µg/ml) for 30 min at 0°C (●). Incubation: 5 min at 37°C. The experiments were evaluated by counting 500 vesicles for each cation concentrations. The values represent the mean of two experiments.

Ionic conditions

The types of in vitro fusion described here occurred in highly simplified media as compared to the complex composition of cytoplasm. In the following we compare the characteristics of the in vitro fusion system with the events known occurring in secretory cells.

The difference in ionic strength between model system and intact cells seems to be negligable, since replacing sucrose by potassium-chloride (100 mM) has no influence on either types of fusion (data not shown.

It is conceivable that in the adrenal medulla the intracellular Ca^{2+} concentration rises in a manner similar to that demonstrated in other secretory cells, where free Ca^{2+} concentrations have been measured to rise from the resting conditions of less than 10^{-7} M [25,26] to about 10^{-5} M during secretion [27,28]. Mg^{2+} has been shown to have an inhibitory effect on Ca^{2+} induced release, when Ca^{2+} and Mg^{2+} together are injected in giant synapses [29], which indicates that Mg^{2+} has an intracellular target.

From the different fusion types observed in this study, therefore, only fusion type I matches the ionic requirements of intact cells. Fusion of secretory vesicles occurs in the µM range of Ca^{2+} concentrations and is inhibited by Mg^{2+}: If Mg^{2+} (10^{-3} M) is added simultaneously with Ca^{2+} (10^{-4} M) the percentage of fused vesicles is decreased (Table I). This inhibition is balanced in the presence of Mg^{2+}, at concentration ($5 \cdot 10^{-3}$ M) which can elicit fusion by itself (compare Fig. 2, Table I). The specificity of Ca^{2+} in inducing fusion of secretory vesicles in vitro is even greater than in intact cells, where Sr^{2+} and Ba^{2+} can partially replace Ca^{2+} during secretion. However, as discussed recently, injection of Sr^{2+} could very well result in a redistribution and enhancement of free intracellular Ca^{2+}-concentration [30].

Adrenal medullary cells rendered leaky by high-voltage discharge have been shown to release adrenalin, with the same Ca^{2+}-concentration dependence and with the same antagonistic effect of Mg^{2+} [31] as shown for the intervesicular fusion of secretory vesicles of adrenal medulla and of other secretory tissues [13–16].

Fusion of liposomes

In order to determine the contribution of the various membrane components to the fusion process we studied the fusion of phospholipid vesicles (liposomes). prepared from hole lipid extracts of membranes of chromaffin secretory vesicles. The lipid composition of those membranes and consequently of the liposomes derived from them is complex [32, 33].

TABLE I

EFFECT OF Mg^{2+} ON Ca^{2+}-INDUCED FUSION OF SECRETORY VESICLES FROM ADRENAL MEDULLA

The experiments were evaluated by counting 500 vesicles for each incubation. Each value gives the mean of two experiments.

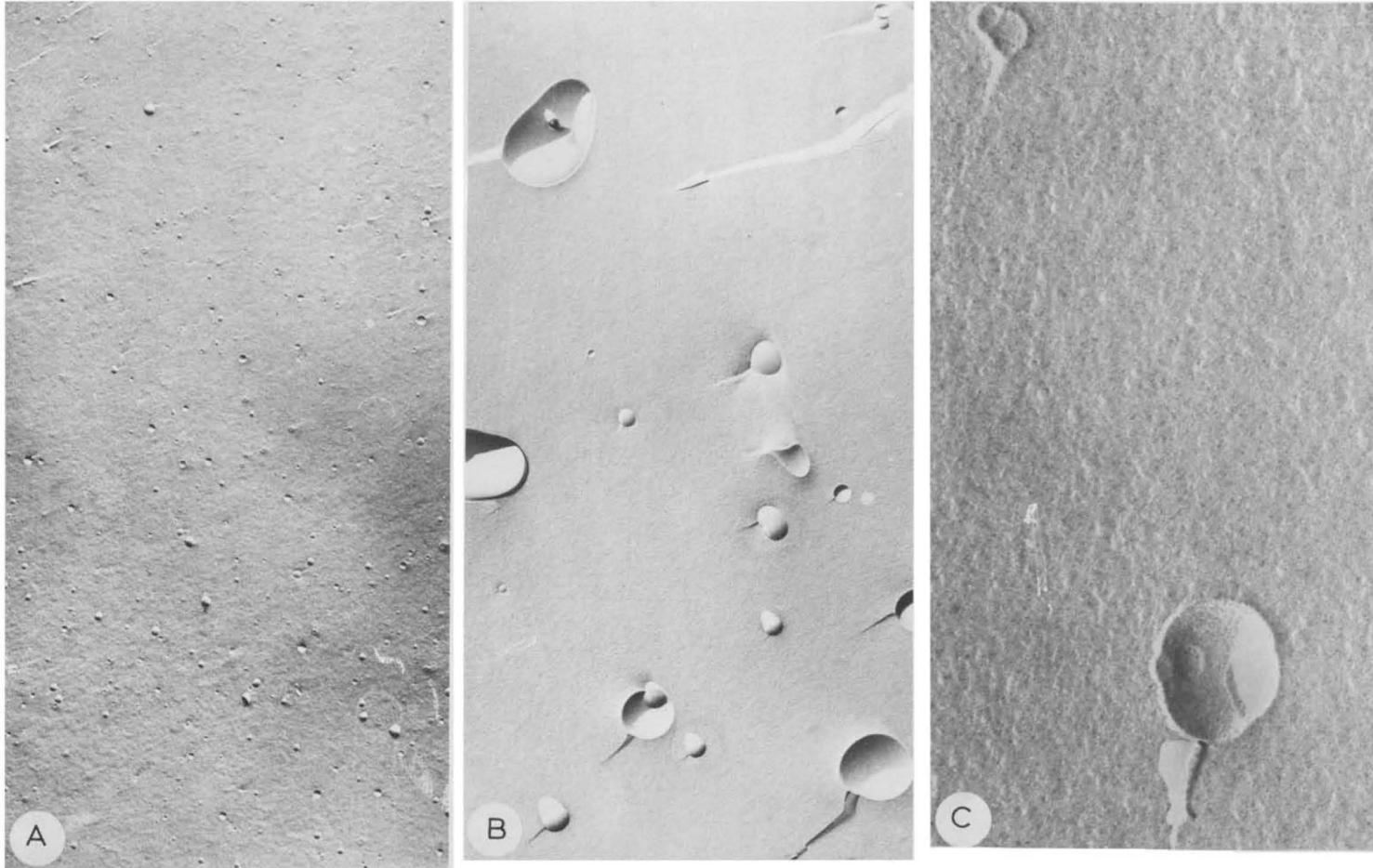| Ca^{2+} (M)	Mg^{2+} (M)	Percentages of fused vesicles
–	–	1.0
10^{-4}	–	6.5
10^{-4}	10^{-3}	4.3
$5 \cdot 10^{-3}$	–	10.5
$5 \cdot 10^{-3}$	5×10^{-3}	11.9

Fig. 3. Freeze-fracture electron micrograph of liposomes prepared from lipid extracts of membranes of secretory vesicles isolated from adrenal medulla. Small unilamellar vesicles obtained by sonication are homogeneous in size (A). After incubation with 10^{-2} Ca^{2+} (5 min, 37°C) the number of liposomes is reduced, while their size is increased considerably (B). Liposomes are enlarged and have a bumpy appearance, where the bumps are of the original size, after incubation of liposomes incubated with Ca^{2+} (10 mM) for 35 ms (C). Magnification: A and B, 14 000X; C, 80 000X.

Unilamellar liposomes prepared by sonication of an aqueous dispersion of this lipid extract are rather homogeneous in size (Fig. 3). The measured membrane area per liposome was 1510 ± 17.6 nm^2 ($n = 7334$) from which one can calculate mean diameter of 438 Å (43.8 nm). No change in size or number of liposomes was observed when incubated at 37°C for several minutes or even when stored over night at about 4°C. This is despite of the high content of lysolecithin, which has been reported to induce fusion of erythrocytes [34] and liposomes [35] or alternatively to induce exchange diffusion [36].

With 2.5 mM Ca^{2+} a moderate increase in size of the individual liposomes was observed as measured by the exposed membrane area per liposome. Concomitantly the number of liposomes within a defined area of replica was reduced. Those effects progressively increased with 5, 7.5 and 10 mM Ca^{2+} (Figs. 3 and 4). The constancy of the total membrane area exposed in the defined, scanned field of replica (Fig. 4) indicates that the large liposomes are derived from the original population of small vesicles. Although molecular diffusion cannot be excluded completely, the most likely interpretation for generation of the larger liposomes is a fusion process. In contrast to fusion of intact secretory vesicles, the fusion of liposomes does not result in twin vesicles; the fusion products are much larger structures which can be
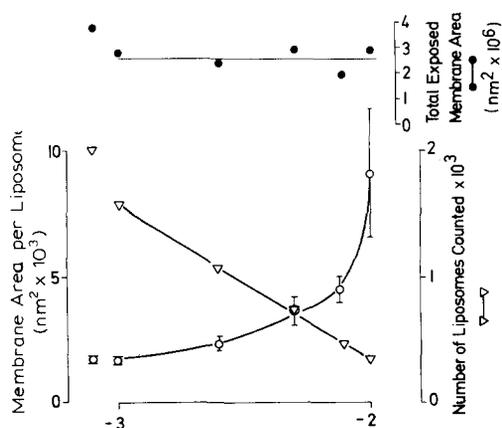
spheres or irregular in shape (Fig. 3). While in the majority of experiments the liposomes suspensions were cryoprotected with glycerol for freeze-fracturing, control experiments with the spray-freezing method have shown identical results suggesting that the observed results are not artifactual. Withdrawal of Ca^{2+} by adding an excess of EGTA does not further affect the liposomal structure.

After incubation with 10 mM Ca^{2+} (37°C for 5 min) the membrane area per liposome was increased to 23050 ± 1652 nm^2 ($n = 838$) equivalent to a mean diameter of 1713 Å (171.3 nm). This implies that 10–25 vesicles have fused to form larger spheres. Since a larger sphere comprises a larger volume than the sum of small spheres with the same surface area it has to be assumed that volume changes take place. This assumption is suggested by the constancy of the total membrane area. The coexistence of spheres and irregular structures however indicates, that such volume changes only partially compensate the disproportion between surface area and volume of vesicles following fusion.

If the membrane area per liposome is plotted as a function of different cation-concentrations, a threshold requirement of more than 2.5 mM of these ions to induce fusion is obvious (Fig. 5). Mg^{2+} and Mn^{2+}
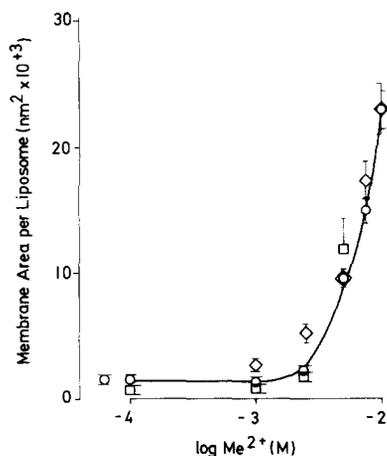

Fig. 4. Membrane area per liposome (mean ± S.E.) (o), total exposed membrane area (●) and number of liposomes (∇) as a function of the Ca^{2+} concentration. Incubation: 5 min at 37°C. The data represent the evaluation of one characteristic experiment with one liposome preparation.

Fig. 5. Liposome fusion as a function of divalent cation concentration: Ca^{2+} (o), Mg^{2+} (◊) or Mn^{2+} (□). Incubation: 5 min at 37°C. On the ordinate the mean membrane area per liposome (S.E.) is given. The Ca^{2+}, Mg^{2+} and Mn^{2+} data represent the evaluation of three, two, and one characteristic experiment, respectively.

are almost as effective as Ca^{2+} (Fig. 5). If Ca^{2+} and Mg^{2+} are added to the liposomes simultaneously, the effect of both ions is additive (Table II). These characteristics are very similar to properties found with divalent cation-induced liposome fusion in other model systems [37–44].

From the results discussed so far it is obvious (compare Figs. 2 and 5; Tables I and II) that in respect to cation concentration and specificity the requirements for liposome fusion and fusion type II of intact secretory vesicles are very similar, although there are also some noteworthy differences. While heavy aggregation of secretory vesicles at mM Ca^{2+} concentration was a typical feature of fusion type II, this was not found for liposomes. Also liposome fusion was much more extensive, the great majority of liposome having performed at least one round of fusion (Figs. 3 and 5). Thus, upon collision of liposomes, fusion seems to be favored over development of stable membrane adhesion. That this does not apply for fusion type II of secretory vesicles could be due to several factors like size and curvature of vesicles, different vesicle contents, or protein components on the vesicle surface inhibitory to fusion by enabling stable membrane contacts.

Fusion of secretory vesicles isolated from the adrenal medulla induced by 2.5 mM Ca^{2+} has already been reported earlier [45]. However, the membranes of vesicles on the published micrographs are fragmented so that it remains unclear to what extent intervesicular fusion has taken place. In subsequent publications the same results have been interpreted as fusion, reversible fusion and adhesion [46–48].

Ca^{2+} binding

A conceivable first step in Ca^{2+}-induced membrane fusion is the binding of this cation to structures on the membrane surface. Due to the high content of Ca^{2+} in intact adrenal medullary secretory vesicles (cf. Ref. 24) and due to the sensitivity of these vesicles to osmotic changes the determination of Ca^{2+}-binding to intact vesicles bears several pitfalls. These we avoided in the present work by a membrane preparation which was thoroughly depleted of Ca^{2+} (see Materials and Methods). As shown in Fig. 6 membranes of secretory vesicles exhibit two different types of binding sites, one characterized by high affinity for Ca^{2+} ($K_d = 1.6 \cdot 10^{-6}$ M), the other by an affinity for Ca^{2+} which is two orders of magnitude lower ($K_d = 1.2 \cdot 10^{-4}$ M).

Binding studies on chromaffin granule preparations have been reported earlier. From data on the electrophoretic mobility of intact secretory vesicles one can calculate a binding constant for Ca^{2+} in the mM range [49]. Investigating the Tb^{3+} binding to membranes of secretory vesicles, a binding constant in the mM range for Ca^{2+} was reported [50]. A recent
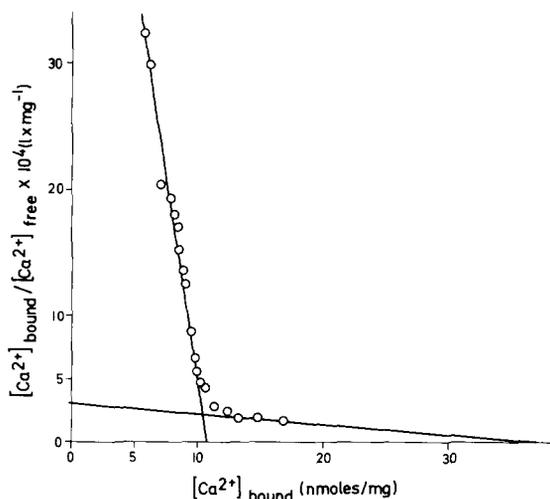
TABLE II

EFFECT OF Mg^{2+} ON Ca^{2+}-INDUCED FUSION OF LIPOSOMES

The data represent the evaluation of one characteristic experiment. All experiments were done with the same liposome preparation.

Cations	Membrane area per liposome, mean ± S.E. (nm²) (×10³)
–	1.38 ± 0.03
2.5 mM Ca^{2+}	1.93 ± 0.12
2.5 mM Ca^{2+} + 2.5 mM Mg^{2+}	3.55 ± 0.30
5 mM Ca^{2+}	15.39 ± 0.10
5 mM Ca^{2+} + 5 mM Mg^{2+}	39.60 ± 2.88
10 mM Ca^{2+}	35.87 ± 2.79
10 mM Mg^{2+}	22.27 ± 1.60

Fig. 6. Scatchard plot of Ca^{2+}-binding of secretory vesicle membranes. Binding was measured with a Ca^{2+}-selective electrode. The data represent the evaluation of one characteristic experiment.

reinvestigation [51] reported a temperature sensitive Ca^{2+} binding on membranes of secretory vesicles with a binding constant of $9.1 \cdot 10^{-6}$ M. Except for the last data cited, the reported binding constants show close similarities with the low affinity binding site reported in the present investigation that affinity being comparable to values reported from binding data of Ca^{2+} to negatively charged phospholipid membranes [52–54].

We conclude therefore, that the low affinity binding site for Ca^{2+} reported here represents the binding ability of phospholipids; and we interpret the additional found high affinity binding site as a characteristic property of a proteinaceous component of the membrane.

It is noteworthy that fusion of secretory vesicles (type I) parallels the Ca^{2+} binding to the high affinity sites. By contrast, all low affinity sites for Ca^{2+} must be occupied in order for fusion type II or fusion of liposomes to proceed.

Time course and Ca^{2+} gradient

The secretory event in intact cells is known to be a fast process. Neurotransmitter release can be complete in milliseconds and a similar fast discharge of vesicle contents was shown for mast cells [55].

As mentioned, fusion of intact secretory vesicles is complete after 1 min. For technical reasons we have applied a method for short incubation times to study liposome fusion only. Extensive fusion was already observed after 35 ms (Figs. 2 and 7). This is faster than the time course for the fusion of other types of liposomes reported so far [37,39,43,44]. Therefore the fast time course of secretion can be very well compatible with a membrane fusion event. Fusion of liposomes was observed to be fast initially and declining progressively with longer incubation, but continued for hours (Fig. 7). The later observation is in contrast to some other investigations, where complete cessation of liposome fusion was observed [41,44]. An explanation for the time couse observed in this study would be that fusion capacity is inversely related to the diameter of liposomes.

From experiments with phospholipid vesicles it has been concluded that Ca^{2+} asymmetry across the liposomal membrane is necessary for fusion [38,56]. Liposomes, derived from chromaffine granule membranes, preloaded with defined Ca^{2+} concentrations,

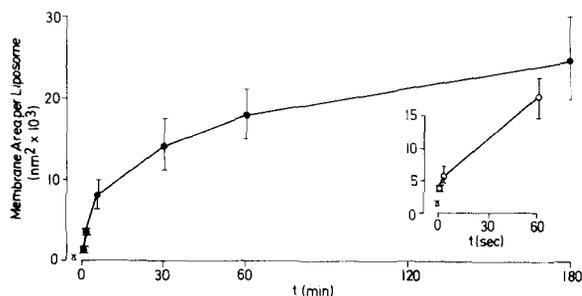Fig. 7. Time course of the Ca^{2+}-induced fusion of liposomes. Incubation: 37°C with $5 \cdot 10^{-3}$ M (●); 20°C with 10^{-2} M Ca^{2+} (○); and control values without Ca^{2+} (△) added. The two shortest incubation periods (10^{-2} M Ca^{2+}) (○) were carried out with a rapid mixing device. The data represent the mean membrane area per liposome ± S.E.) of one characteristic experiment.

did not exhibit differences in fusion capacity relative to unloaded liposomes (Table III). These results therefore do not support the postulated asymmetric cation requirement for fusion.

Temperature

Secretagogue induced secretion ceases with the lowering of temperature. From the temperature dependence of secretion studied on intact mast cells [57], it was concluded that the fusion event during exocytosis depends strongly on the physical state of membrane lipids. However the temperature dependence of secretion need not reflect necessarily the temperature dependence of fusion since other pro-

TABLE III

FUSION OF LIPOSOMES, CONTAINING VARIOUS CONCENTRATIONS OF Ca^{2+}

The data represent the evaluation of one characteristic experiment. All experiments were done with the same liposome preparation.

Ca^{2+} in liposomes	Ca^{2+} added	Membrane area per liposome mean ± S.E. (nm^2) ($\times 10^3$)
10^{-4} M	–	1.89 ± 0.15
10^{-3} M	–	2.14 ± 0.17
10^{-4} M	$5 \cdot 10^{-3}$ M	6.34 ± 1.07
10^{-3} M	$5 \cdot 10^{-3}$ M	6.42 ± 0.88
10^{-4} M	10^{-2} M	16.60 ± 3.32
10^{-3} M	10^{-2} M	26.82 ± 4.43

cesses involved in exocytosis may exhibit temperature sensitivity.

Phase transitions and phase separations have been postulated to be necessary prerequisits for the fusion of pure phospholipid membranes induced by divalent cations [37]. While such studies have been performed on idealized membrane systems where interpretation is readily available, the complex composition of biological membranes prevents an easy interpretation of experimental results.

Fig. 8 shows a linear increase with temperature of chromaffin granule fusion induced by 10^{-4} M Ca^{2+}. This linear dependency of fusion type I argues against a temperature sensitive change of the physical state of the membrane lipids induced by Ca^{2+}-concentrations sufficient to induce fusion. However, when incubated with 10^{-2} M Ca^{2+} or Mg^{2+}, secretory vesicles exhibit a discontinuous temperature dependence (Fig. 8) which has close similarities to the temperature dependence of liposomes (Fig. 9). Thus, fusion type II and liposome fusion have a very similar temperature dependence, in addition to the requirements already discussed. At first glance, such an observation could be interpreted as revealing the involvement of phase-changes of the membrane lipids. Indeed, from investigations of both the properties of fluorescent probes
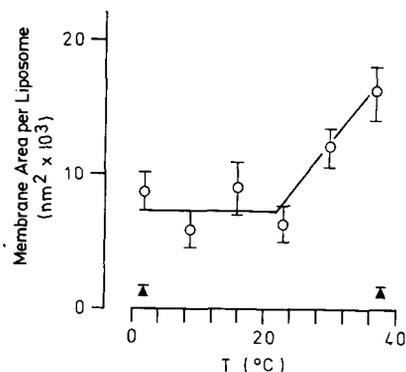
Fig. 9. Ca^{2+}-induced fusion of liposomes as a function of temperature. Incubation: 5 min with 10^{-2} M Ca^{2+} (o) or control values without Ca^{2+} (▲) added. On the ordinate the mean membrane area per liposome ± S.E.) of one characteristic experiment.

or spin labels bound to adrenal medullary secretory vesicles and the activities of membrane bound enzymes as a function of temperature, it has been concluded that structural transitions occur in membrane lipids at 33°C [58–60]. Furthermore minute deviations in the baseline of differential scanning calorimetry thermographs have been interpreted as reflecting melting peaks [61].

In contrast, there are also experimental data which exclude a phase transition in the temperature range 5°–40°C, at least for the bulk of the phospholipids. These were obtained from measurement of fluorescence polarization on intact secretory vesicles [62]. These results were confirmed by applying the same method to liposomes composed of the total lipid extract of the same membranes [63]. Furthermore, differential scanning calorimetry of both intact secretory vesicles and of liposomes exhibited straight baselines, thus giving no hint for a phase transition for the bulk of membrane lipids [63]. Such an observation may be expected considering the complex mixture of lipids in secretory vesicle membranes and the high content of cholesterol [32,33]. Finally, the discontinuity in the temperature dependence of fusion type II and fusion of liposomes differs from that described in other systems, where a relation between phase transition and fusion was proposed. Fusion was reported to cease below transition temperature [64], but was also reported to peak at the transition temperature [65,66]. In contrast, fusion type II and
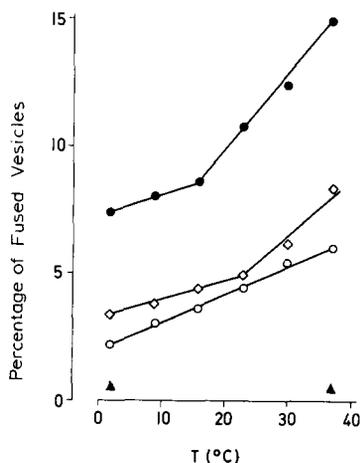
Fig. 8. Effect of temperature on the fusion of secretory vesicles induced by different divalent cation concentrations. Incubation for 5 min was carried out with 10^{-4} M Ca^{2+} (o), 10^{-2} M Mg^{2+} (◇) or 10^{-2} M Ca^{2+} (●) or without divalent cations added (▲). The experiments were evaluated by counting 500 vesicles for each experimental condition.

fusion of liposomes show also reduced but still excessive fusion at low temperature. The observed nonlinear temperature-dependence deserves further investigation.

Involvement of different membrane components

The only data about involvement of different membrane components in the exocytotic process of intact cells stems from morphological studies. In earlier studies it was reported that the exocytotic fusion process is characterized by a clearing of membrane particles from the fusion site [67–69]. This was interpreted as evidence against protein involvement in exocytotic membrane fusion. Recent studies indicate that the particle clearing has originated artifactually during processing for electron microscopy [70,71].

Membrane fusion type I is characterized by its sensitivity to neuraminidase and glutaraldehyde (Fig. 10) which implies a leading role of a protein in membrane fusion. Similar properties have been described for fusion of secretory vesicles isolated from rat liver [16]. Also the loss of characteristic features of fusion type I in liposomes from extracted membrane lipids stresses the involvement of proteinaceous membrane components in fusion. However it has to be assumed,
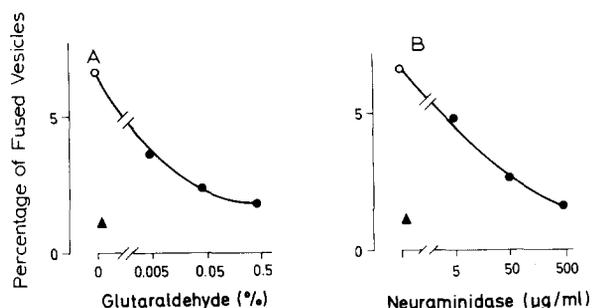
that in liposomes the orientation of lipids with respect to sidedness on the two membrane lamellae and to lateral distribution within the plate of the membrane is different from that in intact membranes. Sidedness of phospholipids could be of functional importance and an asymmetric distribution of phospholipids has in fact been shown for several biological membranes [72–75]. These arguments however, may not be of relevance for the fusion process since intact secretory vesicles isolated from the adrenal medulla exhibit a second type of fusion which shows several characteristics also found for liposome fusion. The above-mentioned sensitivity of fusion type I could indicate the involvement of gangliosides in this type of fusion. However, incorporation of up to 10% of the ganglioside in this type of fusion. However, incorporation of up to 10% of the ganglioside GM_3 into liposomes originally present in intact membranes [33], did not change the Ca^{2+} concentration dependence of liposome fusion (not shown).

Involvement of proteins in fusion processes also become known for virus-cell membrane fusion, where it even was possible to isolate the fusion promoting protein [76]. Acquisition of fusion competence has been demonstrated in cell membranes of maturing myoblasts, which fuse during muscle development [77].

Binding of isolated secretory vesicles from exocrine pancreas tissue to cell membranes has been observed with a specificity for μM Ca^{2+}-concentrations [78]. The described cation dependence and the sensitivity to proteolytic enzymes is in agreement with the results presented here.

Fig. 10. (A) Effect of glutaraldehyde on Ca^{2+}-induced fusion of secretory vesicles. Vesicles were preincubated (10 min) in the absence of Ca^{2+} at 0°C with various glutaraldehyde concentrations. Incubation: 5 min at 37°C with 10^{-4} M Ca^{2+} (●). Controls were carried out without pretreatment with glutaraldehyde (10^{-4} M Ca^{2+} (○) or no Ca^{2+} (▲)). (B) Effect of neuraminidase on Ca^{2+}-induced fusion of secretory vesicles. Vesicles were preincubated in the absence of Ca^{2+} at 0°C for 30 min with various neuraminidase concentrations. Incubation: 5 min at 37°C with 10^{-4} M Ca^{2+}. Controls were carried out with no Ca^{2+} (▲) added, or with 10^{-4} M Ca^{2+} (○). The experiments were evaluated by counting 500 vesicles for each experimental condition.

Conclusion

Of the different types of fusion described here only fusion type I of intact secretory vesicles is compatible with the cellular properties. It is specifically induced by Ca^{2+} in the μM concentration range, is inhibited by Mg^{2+}, shows a linear temperature dependency and is sensitive to various modifying treatments. Fusion type II and fusion of liposomes composed of lipid extracts of these membranes have various common characteristics: both require Ca^{2+} in the mM concentration range; Ca^{2+} can be replaced by other divalent cations; Ca^{2+} and Mg^{2+} added simultaneously have an additive effect; they exhibit the

same discontinuous temperature dependencies; neither are affected by neuraminidase, proteases and glutaraldehyde. This indicates that the function of proteins in fusion type I is to guarantee the specificity of membranes to interact and to provide sensitivity for low Ca^{2+} concentrations and specificity for this ion.

Various contributions of proteins in the mechanism of fusion are conceivable. Proteins very likely could serve as recognition sites or they could form and maintain domains of phospholipids being susceptible to fusion. However, none of the phospholipid species present within the membrane of adrenal medullary secretory vesicles is known to exhibit fusion characteristics similar to fusion type I, if studied in liposomes composed of single phospholipid or simple mixtures. However proteins, binding Ca^{2+} with high affinity could create a Ca^{2+} environment for such domains different from the bulk concentration. Finally, proteins may be involved actively in the 'melting' of the two bilayer of complex interaction and conformation changes. It is tempting to attribute the reported high affinity of secretory vesicle membrane for Ca^{2+} to such membrane constituents.

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