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FUSION OF NEUROHYPOPHYSEAL MEMBRANES IN VITRO

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Summary

Freeze cleaving electron microscopy has shown that fusion of isolated secretory vesicles from bovine neurohypophyses was induced by Ca^{2+} in micromolar concentrations. Mg^{2+} and Sr^{2+} were ineffective. Mg^{2+} inhibited Ca^{2+}-induced fusion.

In suspensions containing secretory vesicles as well as sheets of cell membrane, release of vasopressin parallel to intervesicular fusion and fusion of secretory vesicles with sheets of cell membrane was observed after exposure to Ca^{2+}. Mg^{2+} and Sr^{2+} were ineffective in replacing Ca^{2+} as trigger for fusion or vasopressin release.

Intervesicular fusion and exocytotic profiles were observed when isolated neurohypophyses or neurosecretosomes were exposed to cold.

Introduction

It is well established that the passage of Ca^{2+} across the cell membrane is a critical step in stimulus-secretion coupling in the neurohypophysis [1,2,3]. Although Ca^{2+} influx is apparently too small to be reliably quantitated [4], experiments with Ca^{2+} transport inhibitors [2] bear out this contention. Also, treatment of isolated neurohypophyses with ionophores, which increase intracellular, free Ca^{2+} concentration by increasing transmembrane transport, can induce secretion [5,6]. Both ultrastructural [7] and biochemical [8,9] studies suggest that secretion in the neurohypophysis, as in various other endocrine systems [10], occurs by exocytosis. However, the events that are triggered by

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Ca^{2+} leading to exocytosis are not resolved.

Fusion of neurosecretory vesicles with the cell membrane in stimulated cells is well documented in thin section [7] and freeze cleaving electron microscopy [11,12,13]. Using the latter technique, intervesicular fusion of isolated secretory vesicles from the islets of Langerhans [14], liver [15] and adrenal medulla [16] was demonstrated when Ca^{2+} in micromolar concentrations was added. Intervesicular fusion was also observed intracellularly in stimulated pancreatic B-cells [17], and other secretory cells [18—24].

In this study we report the fusion of isolated neurosecretory vesicles with each other after exposure to low concentrations of Ca^{2+}. In addition we investigated the interaction of neurosecreotry vesicles with sheets of cell membrane as well as the release of vasopressin.

Materials and Methods

Preparation and cold stimulation of rat neurohypophyses

Rats were decapitated with scissors. The neural lobe was dissected out under a binocular microscope and incubated for 15 min in ice-cold Krebs-Henseleit solution.

Preparation and cold stimulation of bovine neurosecretosomes

Neurohypophyses were obtained within 20 min of the death of the animals from the Copenhagen Public Slaughterhouse and were transported within 20 min at 23° C to the laboratory. A fraction enriched in neurosecretosomes was isolated according to the method described by Bindler et al. [25] with a few modifications: the neural lobes were freed of connective tissue as well as adherent intermediate lobe tissue and minced in a medium containing sucrose (250 mM), and *N*-Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (20 mM), pH 7.3. The mince was homogenized in a Potter Elvehjem type homogenizer using a teflon pestle (clearance 0.279 mm) which was modified as described previously [25]. This homogenization involved 6 up and down strokes at 1000 rev./min using a Technidrive English Electric DC motor. The homogenate was centrifuged at $700 \times g$ for 10 min in an MSE high speed centrifuge. The supernatant was recovered using a Pasteur pipette and stored. The pellet was suspended in 2 ml homogenization medium, rehomogenized using a pestle with a clearance of 0.102 mm (4 strokes, 1000 rev./min) and centrifuged again at $700 \times g$ for 10 min. The supernatant was collected, pooled with the initial supernatant and centrifuged at $1700 \times g$ for 15 min. The pellet contained the neurosecretosome enriched fraction. The supernatant was discarded. During the homogenization steps the tube was surrounded by ice. All the centrifugation procedures were carried out at room temperature to reduce cold-induced release of vasopressin [26—28].

The neurosecretosome pellet was suspended in a small volume (0.5 ml/2 neurohypophysis, 15—20 mg protein/ml) of incubation medium (NaCl 126 mM/KCl 4.8 mM/$CaCl_2$ 2.8 mM/$MgSO_4$ 1.3 mM/*N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulphonic acid (HEPES) 26 mM (pH 7.0)/dextrose 10 mM). 10 μl aliquots of this suspension were transferred to an Eppendorff Microfuge tube (1.5 ml capacity) and cooled in crushed ice for 15 min.

Preparation of isolated bovine neurosecretory vesicles

The procedure for the isolation of a crude secretory vesicle fraction (fraction 3) and purified vesicle fraction (fraction D) has been described previously [29]. In the present experiments, however, 10 mM sodium cacodylate was used instead of 20 mM TES to buffer all solutions during the isolation of neurohypophysial fractions. To maintain low Ca^{2+} concentrations, 5 mM ethyleneglycol-2-(2-aminoethyl)-tetraacetic acid (EGTA) was added to all solutions. The fractions obtained were dialyzed against 10 mM cacodylate buffer (pH 7.0) containing 250 mM sucrose and 1 mM EGTA (CSE medium). The dialyzed fraction was centrifuged at $2600 \times g$ for 15 min in a Beckman L2-65K ultracentrifuge using a 65 rotor. The pellet was suspended in CSE medium, to give a protein concentration of approximately 20 mg/ml.

Incubation of isolated neurosecretory vesicles with media of different cation concentrations

CSE-medium containing various concentrations of Ca^{2+} ranging from 10^{-8} to 10^{-3} M was prepared as described by Portzehl et al. [30]. Mg^{2+}- and Sr^{2+}-containing solutions were prepared from stock solutions made in CSE medium. To start the reaction 10 µl aliquots of fraction 3 or fraction D was added to 10 µl CSE-medium containing the cations under study. Incubation (at 37°C) was carried out for 5 min and then stopped by the addition of 1.5 ml ice-cold medium of the same cation concentration used during incubation. The total content of the incubation tube was filtered through a millipore filter (pore size 0.22 µm). The filtrate was assayed for vasopressin released. The amount so determined was compared with the total amount of vasopressin, in the original fraction after extraction with 0.25% acetic acid in 0.9% NaCl [31].

When incubating vesicle fractions for ultrastructural studies the incubation procedure was identical. The reaction was stopped by the addition of the same solution used in the experiment containing, as a fixative, 2% glutaraldehyde in place of an equal amount of sucrose. The resulting mixture was incubated for 5 min at 37°C. Then for cryoprotection 10 µl glycerol was added. After 10 min at room temperature small droplets (≈ 0.5 µl) of the suspension were frozen on golden specimen holders in Freon 22 cooled by liquid nitrogen.

Radioimmunoassay of vasopressin and neurophysin

Antisera raised against synthetic arginine vasopressin (Sandoz, Basel, Switzerland) was kindly donated by Mr. J. Dencker (Royal Pharmaceutical High School, Copenhagen). Arginine vasopressin was iodinated with ^{125}I by the chloramine T technique [32] and the labelled hormone was purified on DEAE Sephadex-A 25. The assay mixture contained 100 µl ^{125}I-labelled arginine vasopressin (2000 cpm), 200 µl sample or standard and 300 µl antiserum in assay buffer (final dilution in assay tube 1 : 100000). The assay buffer consisted of 0.1 M sodium phosphate (pH 7.4) containing bovine serum albumin 1.8 mg/ml. The usual incubation time was 24 h. The antibody-bound arginine vasopressin was separated by ethanol precipitation (67% v/v final) and centrifugation. Precipitates were counted in a Selectronic (Copenhagen) γ-spectrometer. All procedures were carried out at room temperature. The standard curves, appropriate blanks and samples were assayed in duplicate. The synthetic arginine vaso-

pressin used for iodination and as standards was a generous gift by Ferring AB (Malmö, Sweden), (antidiuretic activity 345 I.U./mg). The routine sensitivity of the assay was between 25 and 50 μU/ml (72—144 pg/ml). The cross reactivity of the antiserum with oxytocin was 1/300 of that with arginine vasopressin. Radioimmunoassay of neurophysin was performed as described previously [33]. Protein was assayed by the method of Lowry et al. [34] with crystalline bovine serum albumin as a standard.

Freeze fracturing

Freeze fracturing and replication were performed in a Balzers BAF 300 device at −100°C. Replicas were cleaned in sodium hypochlorite solution and washed in distilled H_2O. They were picked up by Formvar- and carbon-coated 1 hole grids and examined in an electron microscope (Siemens Elmiskop 101) at 100 kV. All photographs were printed as positives (platinum depositions: black). The direction of shadowing is indicated by an encircled arrowhead. Fracture faces are denoted according to the nomenclature introduced recently [35].

Results

Exocytosis in cold-stimulated isolated neurohypophyses and neurosecretosomes

Neurhypophyses are known to release hormones via exocytosis. This process is initiated physiologically by depolarization of the cell membrane. Intense

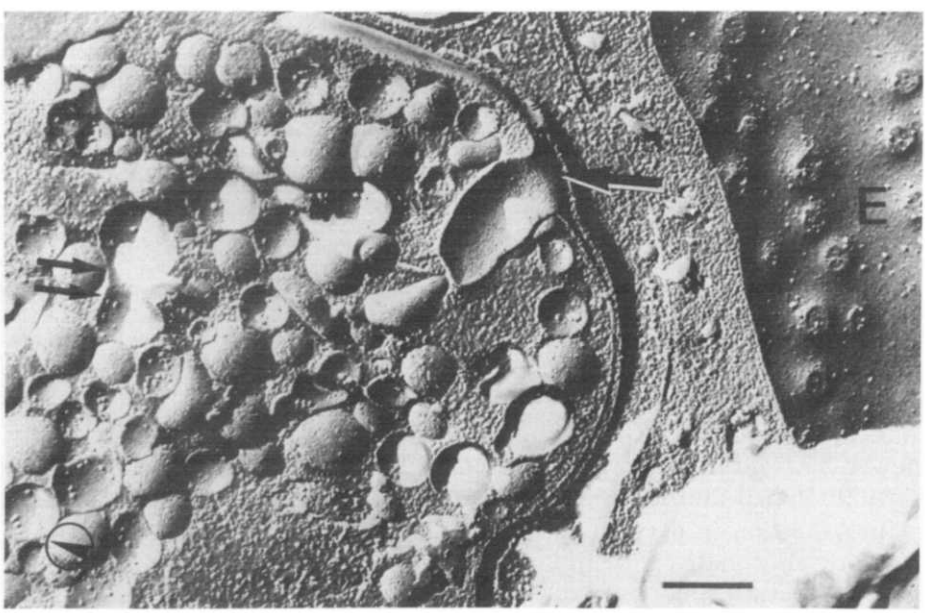

Fig. 1. Freeze-fracture replica showing a nerve ending in the neurohypophysis of a rat. The isolated neurohypophysis was exposed to cold (2°C) for 15 min. The arrow points to an exocytotic orifice. The large area of the exposed vesicle membrane indicates compound exocytosis. Fused vesicles not in obvious contact with the plasma membrane can also be observed (double arrows). E, Endothelial cell. Magnification × 60000, scale 0.2 μm.

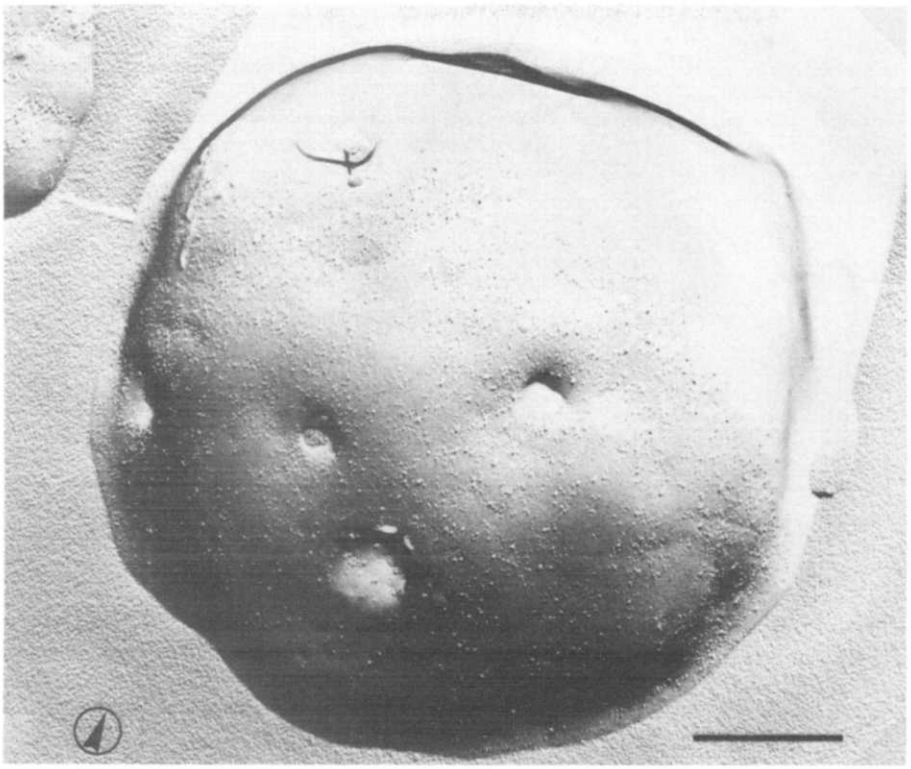

Fig. 2. Electron micrograph of a freeze-fractured preparation of nerve endings from bovine neurohypophyses (neurosecretosomes) exposed to cold (2°C) for 15 min. The fracture has exposed a large area of the plasma membrane P-face. The presence of several holes indicates exocytotic activity. Magnification × 40000, scale 0.5 μm.

release of neurohypophyseal hormones also occurs on exposure of the neural lobe or isolated neurosecretosomes to cold [26—28]. In the present experiments depolarization of isolated rat neurohypophyses by K^+ (56 mM) or cooling to 0°C resulted in the appearance of exocytotic profiles in freeze-fractured neurohypophyses. Exocytosis is characterized by fusion of secretory vesicles with the cell membrane. In addition, fusion of secretory vesicles with other vesicles undergoing exocytosis was observed, as well as fusion of secretory vesicles with each other, where no connection between vesicles and cell membrane was evident. The coincidence of vesicle-cell membrane fusion and intervesicular fusion is called "compound exocytosis". This phenomenon is shown for a cold-stimulated rat neurohypophysis in Fig. 1.

In the neurohypophyses hormones are released from nerve endings of hypothalamic cells. These terminals of the neurosecretory cells can be isolated and are called "neurosecretosomes" [25]. Like the intact cells, neurosecretosomes respond to cold with release of hormones [28]. We have found membrane alterations typical for exocytosis ("necks" and "holes") in the cell membrane of neurosecretosomes after exposure to high K^+ (56 mM) or cold (Fig. 2).

Stimulation of neurosecretosomes also results in intervesicular fusion of secretory vesicles as shown in a crossfractured neurosecretosome (Fig. 3).

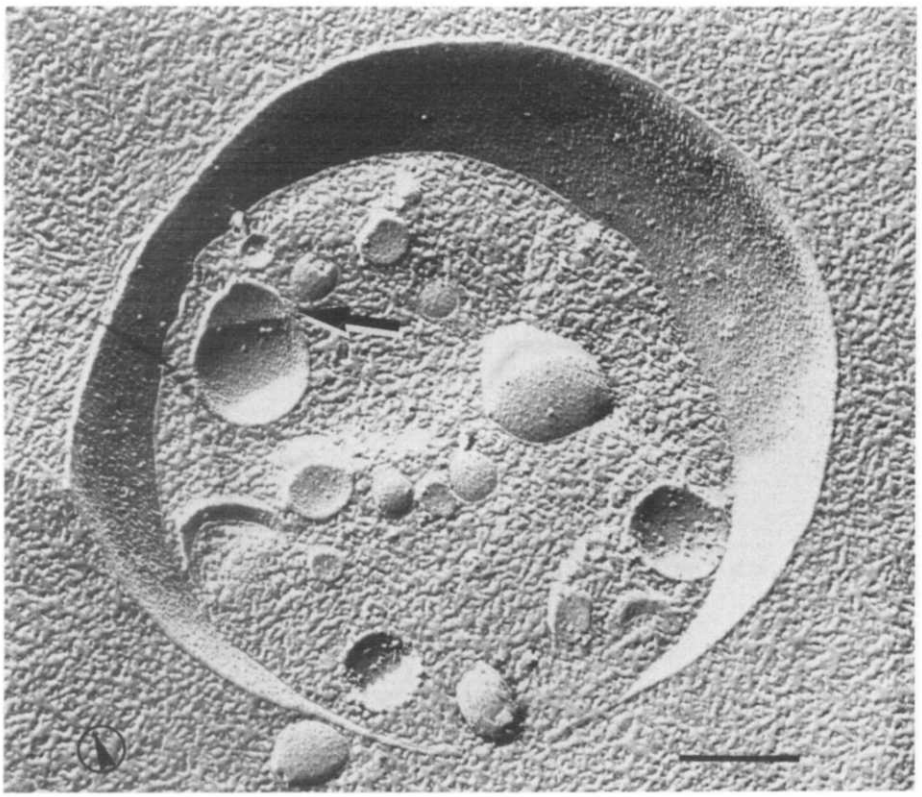

Fig. 3. Cross fractured neurosecretosome after exposure to cold (2°C) for 15 min. Fused secretory vesicles (arrow) can be observed. Magnification × 80000, scale 0.2 μm.

Membrane fusion of neurosecretory vesicles

Secretory vesicles, isolated as described in Methods by differential and sucrose density centrifugation were found to be dispersed in buffered solutions containing EGTA. This is shown in a freeze fractured vesicle suspension in Fig. 4. The presence of divalent cations (Ca^{2+}, Mg^{2+}, Sr^{2+}) in the incubation medium resulted in the formation of vesicle clusters. Aggregation of membrane associated particles was observed in the areas of membrane contact (Fig. 5). Furthermore, Ca^{2+}, but not Mg^{2+} or Sr^{2+}, induced the formation of twinned vesicles. These structures represent fused vesicles since the cleavage plane was continuous from one to the other vesicle both in membrane P- and E-faces. The transition area from one to the other vesicle may or may not exhibit aggregates of membrane associated particles (Fig. 6 a—d).

The percentage of fused vesicles as a function of the free Ca^{2+}-concentration is shown in Fig. 7. Vesicle fusion increases between 10^{-7} M Ca^{2+} and 10^{-4} M Ca^{2+} and is half maximal around 10^{-6} M Ca^{2+}.

Mg^{2+} and Sr^{2+} were not effective in inducing fusion of isolated secretory vesicles. Mg^{2+} even inhibited the Ca^{2+}-induced fusion apparently in a concentration dependent manner (Table I).

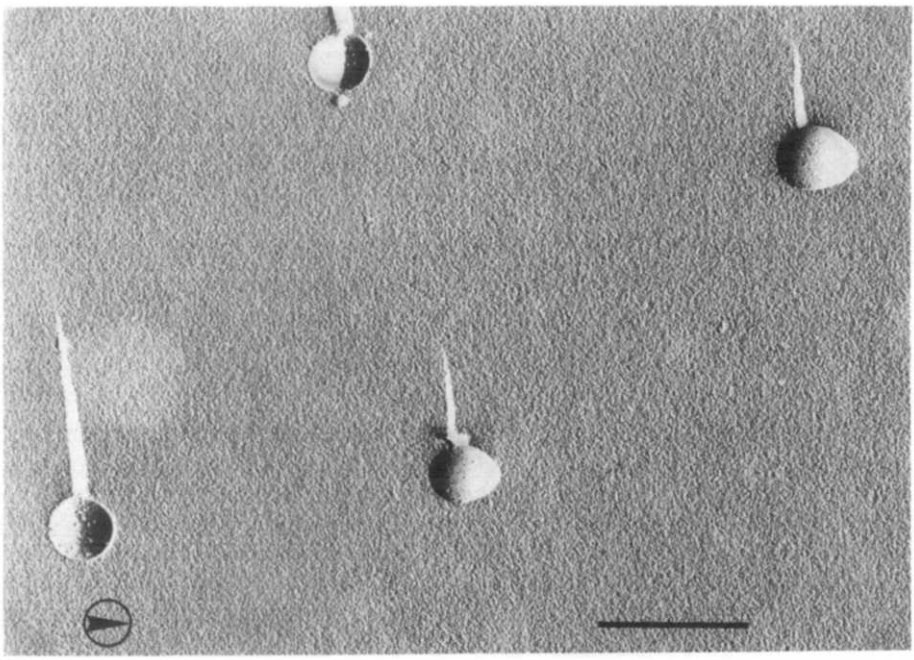

Fig. 4. Electron micrograph of a freeze-fractured suspension of isolated secretory vesicles of the neurohypophysis (fraction D) incubated in low Ca^{2+} concentration ($<10^{-8}$ M). The vesicles are dispersed in the medium. The membrane-associated particles are randomly distributed and adhere more to the concave P-faces than to the convex E-faces. Magnification X 40000, scale 0.5 μm.

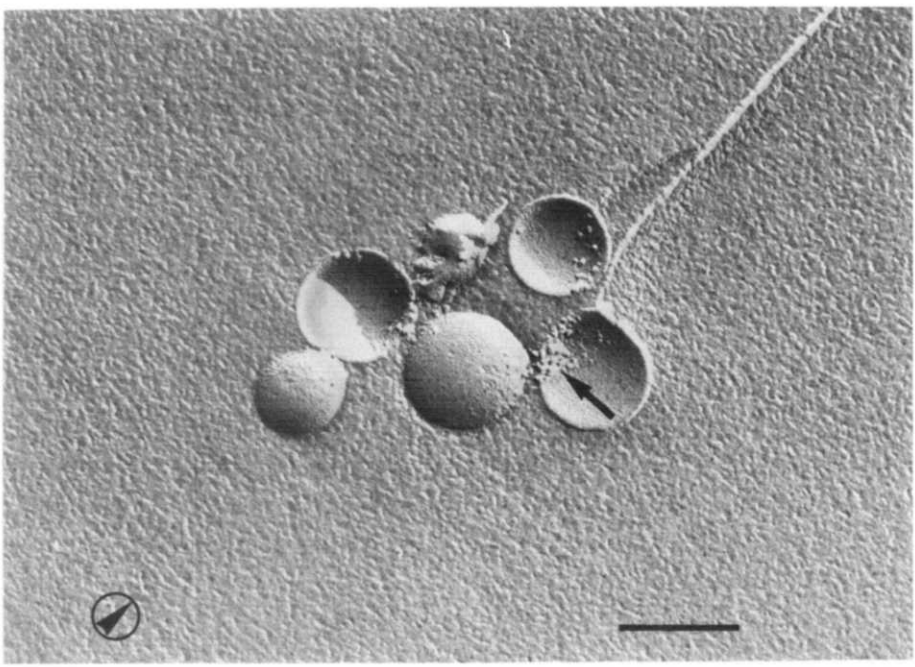

Fig. 5. Freeze-fractured isolated secretory vesicles (fraction D) incubated with $2 \cdot 10^{-5}$ M Ca^{2+}. The vesicles contact each other forming clusters. In areas of contact, aggregations of membrane associated particles (arrow) can be observed. Magnification X 80000, scale 0.2 μm.

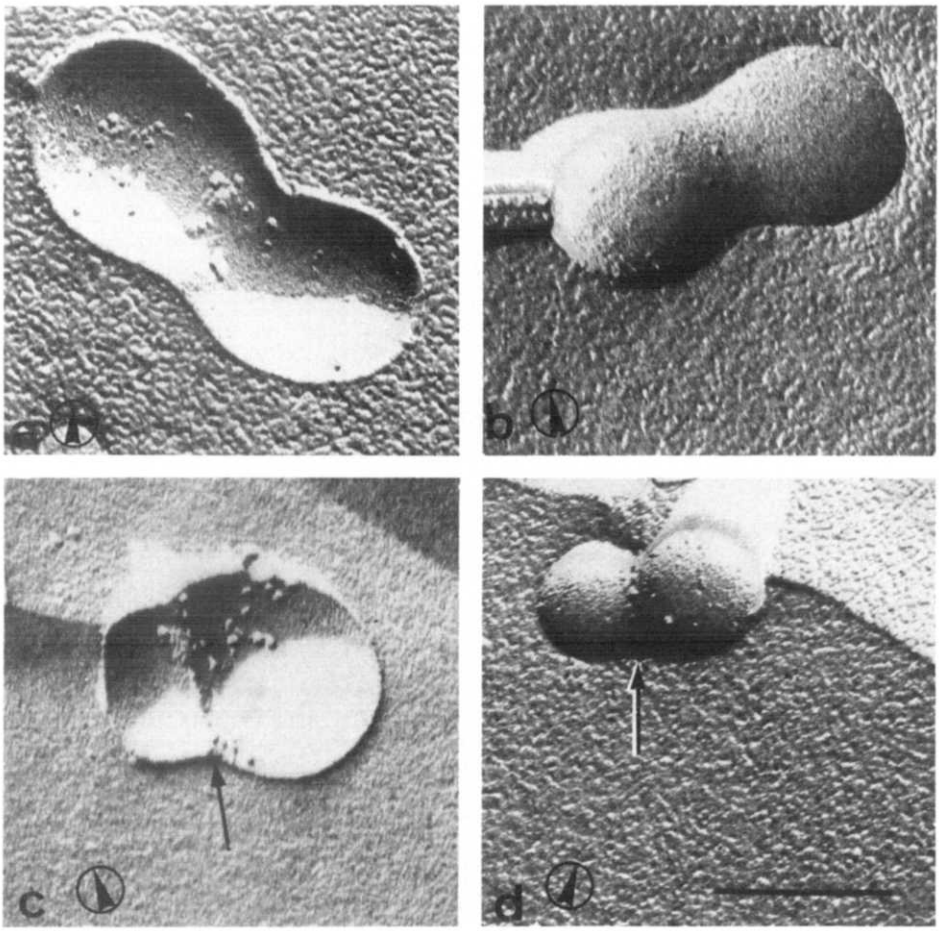

Fig. 6, a—d. Freeze-fractured isolated secretory vesicles (fraction D) incubated in a solution containing Ca^{2+} ($2 \cdot 10^{-5}$ M). Twinned vesicles with a continuous cleavage plane from one to the other vesicle in membrane P- (a and c) and E-face (b and d) indicate vesicles fusion. Aggregates of membrane associated particles can be observed (arrows). Magnification × 120000, scale 0.2 μm.

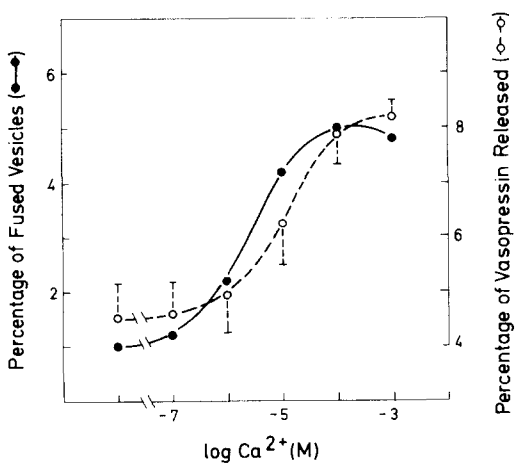

Fig. 7. Fusion of secretory vesicles (fraction 3) and release of vasopressin induced by increasing concentrations of Ca^{2+}. Fraction 3 was incubated in a medium containing 0.25 M sucrose, 10 mM sodium cacodylate (pH 7.0) and 1 mM EGTA (CSE medium). Ca^{2+} concentrations were adjusted as described [30]. Vesicle fusion was determined by counting 500 vesicles for each incubation. Vasopressin release was measured by radioimmunoassay after millipore filtration of the incubate as described under Methods. The data for vasopressin release are identical to the ones in Table II.

TABLE I

CATION DEPENDENCE OF THE FUSION OF VESICLES FROM FRACTION D

The values represent two typical sets of experiments. The experiments were evaluated by counting 500 vesicles for each incubation.

Cations	Percentage of fused vesicles	
Control ($<10^{-8}$ M Ca^{2+})	1.2	0.8
10^{-4} M Ca^{2+}	5.4	6.0
10^{-4} M Mg^{2+}	1.6	1.0
10^{-4} M Sr^{2+}	1.4	1.4
10^{-4} M Ca^{2+} + 10^{-4} M Mg^{2+}	3.8	3.6
10^{-4} M Ca^{2+} + 10^{-3} M Mg^{2+}	2.6	2.8

TABLE II

VASOPRESSIN RELEASED (% OF TOTAL CONTENT) FROM FRACTION 3 EXPOSED TO DIFFERENT CONCENTRATIONS OF Ca^{2+}, Sr^{2+} AND Mg^{2+}

For procedures see legend under Fig. 7. Values are mean (± S.E.M. where n equals 3 or more). The number of experiments (n) is given in parentheses.

	Percentage of vasopressin released		
Cations in the incubation medium (M)	Ca^{2+}	Sr^{2+}	Mg^{2+}
Control ($<10^{-8}$ M Ca^{2+})		4.25 ± 0.6 (5)	
10^{-7}	4.60 ± 0.6 (3)	—	—
10^{-6}	4.99 ± 0.7 (5)	3.26 (2)	3.82 (2)
10^{-5}	6.24 ± 0.9 (3)	3.35 (2)	3.24 (2)
10^{-4}	7.89 ± 0.8 (5)	4.20 ± 0.8 (3)	4.21 ± 0.7 (3)
10^{-3}	8.20 ± 0.3 (5)	3.26 (2)	4.39 (2)

Membrane fusion and release of secretory product in fraction 3

In conjunction with the electronmicroscopic evaluation we also followed the amount of hormone in the vesicles as well as in the incubation medium. Fraction D, used mainly for the electron microscope studies described above, was a preparation purified from fraction 3 by a density-gradient step [29]. This procedure rendered secretory vesicles fragile as indicated by loss of hormone. Fraction 3 was found to be more stable. As described later this secretory vesicle fraction released vasopressin upon addition of Ca^{2+} but not of Mg^{2+} or Sr^{2+}. Electron microscopic inspection of this fraction revealed the presence of sheets of cell membranes among other contaminants of this vesicle preparation. After addition of Ca^{2+} to fraction 3, vesicles were found in close contact with membrane sheets and in the area of membrane attachment aggregations of membrane associated particles were observed (Fig. 8a). In addition, vesicles were found to have fused with membrane sheets connecting the vesicle lumen with the surrounding medium (Fig. 8b, c).

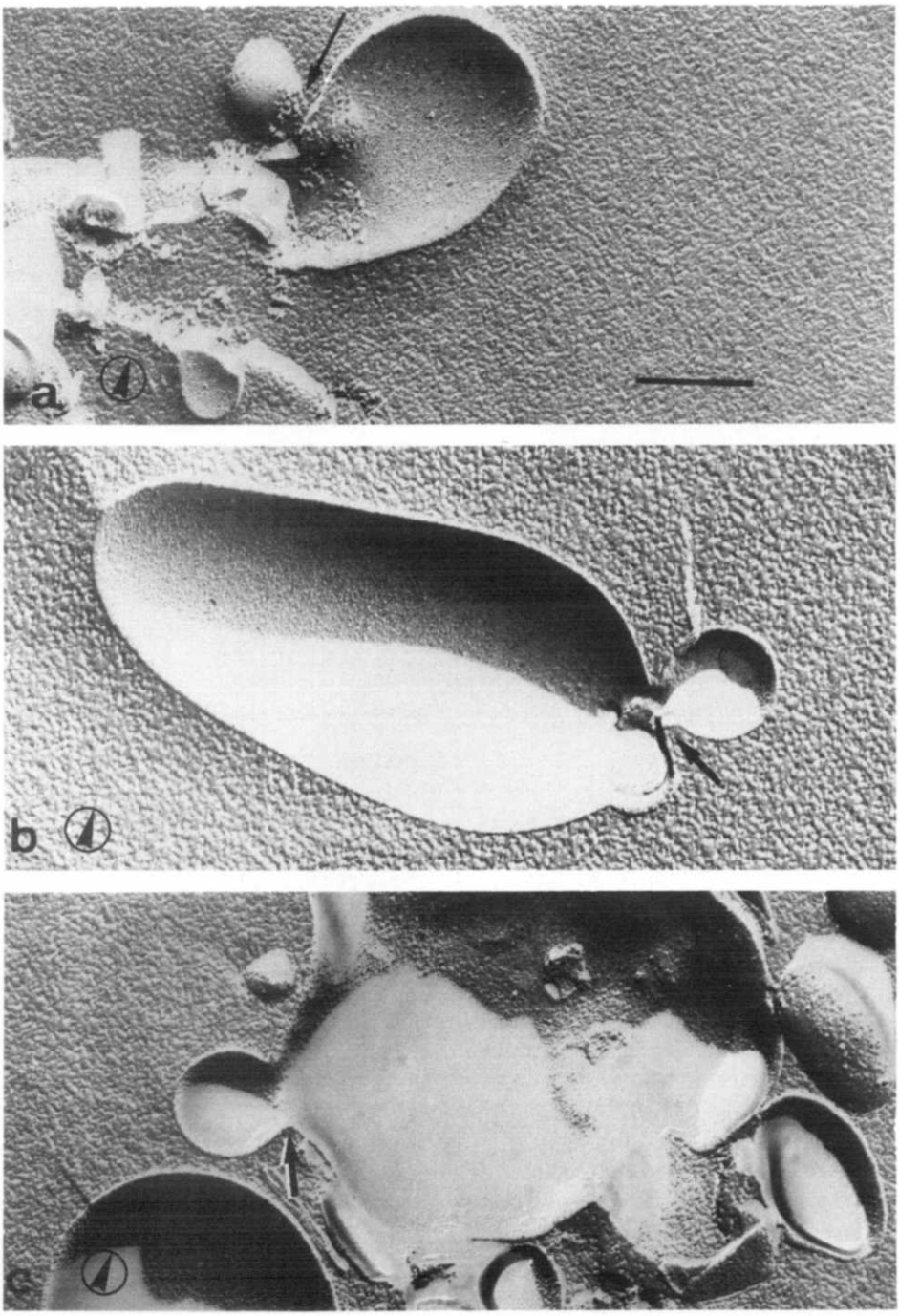

Fig. 8, a—c. Electron micrograph of a freeze-fractured preparation of the neurohypophysis (fraction 3) incubated in Ca^{2+} (10^{-4} M). Secretory vesicles and some sheets of plasma membrane are present in this fraction. An aggregation of membrane associated particles can be observed on a vesicle contacting a membrane sheet (a). Fusions of vesicles with membrane sheets are shown in (b) and (c) (arrows). Magnification × 80000, scale 0.2 μm.

The amount of vasopressin released into the medium increased as a function of the Ca^{2+} concentration (Fig. 7). Half maximal effect was obtained between 10^{-6} and 10^{-5} M Ca^{2+}. Fig. 7 shows that fusion of secretory vesicles parallels the observed hormone release when fusion and vasopressin were determined using identical incubation procedures.

In two experiments a similar increase in the release of neurophysin (the intravesicular hormone-binding protein) into the medium was observed. However, the percentage neurophysin released into the medium was significantly lower than that of vasopressin released.

In another set of experiments Sr^{2+} and Mg^{2+} instead of Ca^{2+} was included in the CSE medium. These cations, in the concentrations tested, did not induce vasopressin release from the vesicles (Table II). When Mg^{2+} (10^{-4} M or 10^{-3} M) was added to an incubation medium containing 10^{-4} M Ca^{2+} the release caused by Ca^{2+} was slightly inhibited.

Discussion

The physiological stimulus for the release of hormones from the neurohypophyses is depolarization of the cell membrane. Isolated neural lobes and the isolated nerve endings (neurosecretosomes) release large amounts of vasopressin on exposure to K^+ and cold [26—28]. Biochemical data indicate that this release is an exocytotic event [27,28]. This was confirmed in this study by the appearence of exocytotic profiles in isolated neural lobes and neurosecretosomes when exposed to K^+ or cold. Furthermore, secretory vesicles fuse with each other during this treatment. This may suggest an accumulation of an intracellular effector leading to membrane fusion.

The requirement of Ca^{2+} in stimulus-secretion coupling in the neurohypophysis is well documented [1—6]. Experiments with isolated secretory vesicles from endocrine pancreatic tissue [14] liver [15] and adrenal medulla [16] have indicated that Ca^{2+} is able to act as a final trigger of exocytosis initiating membrane fusion. The present study with secretory vesicles from the neural lobe of the hypophysis makes it seem probable that in this system also Ca^{2+} controls membrane fusion leading to release of hormone.

The experiments indicate that Ca^{2+} in micromolar concentrations causes membrane fusion in a "minimal" medium. To compare this in vitro situation with the intracellular environment the influence of Mg^{2+} and K^+ on Ca^{2+}-induced fusion needs to be considered.

Despite the fact that monovalent cations such as K^+ inhibit Ca^{2+} binding to phospholipid membranes [36] the presence of 60 mM KCl did not change Ca^{2+}-induced fusion of secretory vesicles isolated from rat liver (Gratzl, M. and Dahl, G., unpublished). The Mg^{2+} concentration of intact cells is assumed to be high [37]. Transmitter release induced by injection of Ca^{2+} into the giant synapse was found to be inhibited by simultaneous intracellular application of Mg^{2+} [38]. As shown in the present study Mg^{2+} is able to inhibit Ca^{2+}-induced membrane fusion in 10^{-4} M or higher concentrations. As a consequence in intact cells, a shift of the sigmoidal curve, which describes the Ca^{2+} dependence of membrane fusion, to higher concentrations of Ca^{2+} can be expected. However, an effect of Mg^{2+} in the intact cells is difficult to evaluate since the local

free concentration of Mg^{2+} is unknown. The free intracellular concentration of Ca^{2+} is also difficult to determine. Recent experiments using aequorin as intracellular indicator of Ca^{2+} indicate that the free concentration is 10^{-7} M or less [39—41] but increases during stimulation of the cell [39]. Thus, the Ca^{2+} concentration dependence of intervesicular fusion is compatible with secretion by intact cells.

In parallel to the fusion of secretory vesicles we have observed the release of vasopressin. There are a number of studies (cited in ref. 42) in which attempts were made to release vasopressin from isolated neurosecretory vesicles by adding Ca^{2+} to the suspension medium. In all these studies the Ca^{2+} concentrations employed were high and the reproducibility of the biological assays was problematic.

It would appear unlikely that the increase in hormone release observed in this study could have originated from neurosecretosomes present in the crude granule fraction for the following reasons: the half maximal concentration of Ca^{2+} in the extracellular medium required for K^+ stimulated release from neurohypophysis is $5 \cdot 10^{-4}$ M [1] which is 10—100 times higher than the concentrations used here. Furthermore, under the incubation conditions employed no depolarization occurs. In fact, increasing Ca^{2+} concentrations should hyperpolarize neurosecretosomes [43]. Under the centrifugation conditions used, neurosecretosomes would have been spun down at lower *g*-values than secretory vesicles, and indeed neurosecretosomes could not be detected in the fractions examined by electron microscopy. Lastly, the purified vesicle fractions were devoid of lactate dehydrogenase, a marker enzyme for such elements [29,44].

The almost identical Ca^{2+} concentrations' dependence for both fusion and release suggests a cause and effect phenomenon. The specificity of the Ca^{2+} effect and the antagonism by Mg^{2+} argues against the release being a reflection of a general leakiness.

Fusion of two vesicles should lead to formation of a common lumen and not to any loss of material into the surrounding medium. Sheets of cell membrane have been found in fraction 3. Interactions of secretory vesicles with such sheets have been shown to occur (Fig. 8). Allthough the infrequency of this observation prevented a quantitative evaluation, the fusion of secretory vesicles with sheets of cell membrane at least would contribute to the observed release of hormone.

The fact that Sr^{2+} cannot induce membrane fusion is interesting since injection of Sr^{2+} into giant synapses did cause transmitter release [38]. Also, in studies of the release of oxytocin from rat neurohypophyses Sr^{2+} could replace Ca^{2+}. when applied extracellularly [45]. However, in both these investigations cell systems were used so that an intracellular redistribution of Ca^{2+} by Sr^{2+} cannot be ruled out.

In conclusion, both the release of hormone from neurosecretory vesicles in the presence of sheets of cell membrane and membrane fusion are characterized by the requirement of low concentration of Ca^{2+}, Ca^{2+}-specificity, and inhibition by Mg^{2+}. These results suggest an in vitro exocytosis type reaction. The molecular mechanisms involved in this Ca^{2+}-induced release are currently under investigation in our laboratories.

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