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Calcium-induced fusion of isolated secretory vesicles from the islet of Langerhans

Calcium-induzierte Fusion isolierter sekretorischer Vesikel der Langerhans'schen Insel

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Abstract

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(1) Isolated endocrine pancreatic secretory vesicles were exposed to series of cations. As revealed by freeze cleaving only Ca^{2+} was able to cause fusion of these vesicles forming a common lumen.

(2) Ca^{2+}-specific fusion was preceded by an aggregation of membrane associated particles. Particle aggregation also occured in vesicles incubated with the other cations (Ba^{2+}, Sr^{2+}, Mn^{2+}, Mg^{2+}, La^{3+}) tested in 10^{-4} M concentrations.

(3) Fusion occured in the assumed physiological range of free intracellular Ca^{2+} concentration. Fusion of secretory vesicles can already be observed at 10^{-6} M Ca^{2+} and is maximum at 10^{-5} M Ca^{2+}, indicating that small variations of intracellular free calcium concentration may trigger fusion of secretory vesicles.

(4) As the Ca^{2+}-specific fusion of isolated secretory vesicles takes place with the same morphological changes observed in intact cells during fusion of the vesicle membrane with the plasma membrane in the exocytotic process, it is concluded that Ca^{2+} may act as final intracellular trigger in stimulus-secretion coupling.

Introduction

Glucose is known to be the physiological stimulus to release insulin from the pancreatic B-cell. In the sequence of events leading to exocytosis calcium ions are of critical importance. This has been concluded from the requirement of calcium in the extracellular medium for the release of insulin from the islets of Langerhans [11, 19, 25]. Since glucose stimulates the net uptake of calcium by isolated pancreatic islets it has

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been suggested that an increase of intracellular calcium plays an important role in stimulus-secretion coupling [12, 22]. Recently calcium – introduced into the cells by means of an ionophore – has been shown to induce insulin release from pancreatic B-cells even in the absence of glucose [37]. Therefore, an increase of intracellular Ca^{2+} concentration seems to be not only a concomitant occurrence of stimulus induced secretion, but to be an essential link in the chain of events between the raise of extracellular glucose concentration and the release of insulin.

In a previous paper [3] fusion of secretory vesicles among themselves and with the plasma membrane has been shown to occur in glucose stimulated B-cells. Both events exhibit the same ultrastructural aspects. It therefore seemed useful to verify, whether by interaction of calcium with a suspension of isolated secretory vesicles fusions comparable to those observed in intact cells can be induced in the absence of other intracellular components.

Materials and methods

By microdissection under a binocular microscope about 100 islets were obtained from one pancreas of a mouse (NMRI/Han/20). We used about 400 islets for each experiment. To remove remnants of exocrine tissue the islets were incubated at 37° C with 2 mg/ml collagenase (type III, fraction A, obtained from Sigma Chemical Co., St. Louis, USA) in 10 mM cacodylate buffer (*p*H 7.0) containing 0.25 M sucrose. After about 10 min microscopic observations revealed that most of the exocrine tissue had been removed. The enzymatic treatment was then stopped by addition of an excess of icecold buffer.

Secretory granules were isolated by differential centrifugation as described by Howell *et al.* [14] with the following modifications: The islets were homogenized manually in 0.4 ml of 10 mM cacodylate buffer (*p*H 7.0) containing 0.25 M sucrose and 1 mM EGTA (CSE-solution) in a glass homogenizer with a loosely fitting teflon pistil. Remaining large fragments were rehomogenized in 0.2 ml CSE, and these homogenates were pooled before centrifugation. This and the subsequent procedure were performed at 4° C.

Nuclei, cell debris and the mitochondrial fraction were removed by centrifugation at 5500 g for 5 min. The supernatant yielded secretory granules after centrifugation at 24 000 g for 10 min as a yellowish fluffy pellet. The islets of Langerhans contain two major types of cells, about 80 % B-cells containing insulin and about 20 % A-cells containing glucagon [9, 27]. Therefore the isolated mixture of granules represented mainly B-granules and will be called secretory vesicles in this publication. The vesicle fraction was washed once in CSE, homogenized gently by hand in a small volume of CSE to get a protein concentration of about 0.05 mg/ml and used immediately for the experiments. Purity of secretory granule fraction was checked by thin section electron microscopy.

Incubation procedure: In 1.5 ml polyethylene reaction vessels 0.3 ml of the vesicle suspension were mixed with 0.3 ml of CSE containing different concentrations of Ca^{2+} (the concentration of which was adjusted according to Portzehl *et al.* [28]), Mg^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+} or La^{3+} and incubated for 5 min at 37° C. Then 0.3 ml 2 % glutaraldehyde in CSE containing the corresponding concentration of ions used in the experiment were added for fixation. 5 min later 0.3 ml glycerol was added for cryoprotection and after 10 min at room temperature the vessels were centrifuged 4 min in a Model 3200 Eppendorff centrifuge at 12 000 rpm. The fixed vesicle fraction appeared as a small pellet which was resuspended in one drop of its supernatant.

Freeze-cleaving: Small droplets (less than 1 µl) of the suspension were frozen on golden specimens holders in Freon 22 cooled by liquid nitrogen, and afterwards stored in liquid nitrogen. The cleaving was performed according to the method of Moor and Mühlethaler [26] in a Balzers freeze-etch unit BAF 300. Fracturing and replication was performed at −100° C. Replicas were cleaned from organic material by sodium hypochloride. After washing in destilled water, replicas were picked up on formvar and carbon-coated single hole grids.

Replicas were examined in a Siemens-Elmiskop 101 at 80 or 100 kV. Photographs were taken as positives (platinum deposition: black). For identification of fracture faces the nomenclature of MCNUTT and WEINSTEIN [23] has been used.

Thin sections: Pellets were fixed with 2 % glutaraldehyde at room temperature and postfixed in a 1 % solution of osmium tetroxide. After dehydration in a graded series of alcohols and propylendioxide the pellet was embedded in Epon. Thin sections were cut on a Reichert-microtome OMU 3, and stained with lead citrate and uranylacetate. Protein concentration was determined according to LOWRY *et al.* [21]. Non specified chemicals were of the purest grade commercially available.

Results

An example of secretory vesicles prepared according to the method described is shown in Figure 1 a and b. In thin sections (Fig. 1 a) the electron-dense cores of the secretory vesicles can be observed which are believed to contain insulin. Some microsomal contamination is present. Only occasionally mitochondria can be observed (not shown in Fig. 1 a). The purity of the isolated vesicles appeared to be sufficient for the experiments and a density gradient step was omitted because of the reported low yield of vesicles [14]. Replicas of the freeze-cleaved preparations (Fig. 1 b) indicate that the fracture plane follows preferentially the membranes. The split membranes of isolated secretory vesicles exhibit the same features as secretory vesicles in intact B-cells [3]: Randomly distributed membrane associated particles (MAPs) stick more to the concave A-face than to the convex B-face. This may indicate preservation of intact membranes. For our investigations we have considered only vesicles with large diameter. The vesicles of smaller size may represent vesicles fractured in the apical part or microsomes. The presence of plasma membranes can be excluded, provided they appear as sheets of membranes or as rightside-out vesicles (convex A-face). If they form inside-out vesicles they may still be distinguishable from secretory vesicles by the smaller diameter of MAPs [3]. However, as indicated by the thin section (Fig. 1 a) a gross contamination with vesicles other than secretory vesicles is unlikely.

The distribution of MAPs in vesicle membranes as shown in Figure 1 b is typical for calcium concentrations below 10^{-8} M. Despite the close vicinity of the vesicles neither an aggregation of vesicles or of MAPs, nor fusion can be observed under these conditions. In contrast by raising the Ca^{2+} concentrations to 10^{-6} M or higher particle-rich B-faces become visible (Fig. 2). In this case MAPs are aggregated. They are observed in regions where neighbouring vesicles are attached to each other as well as in vesicles where a contact with a neighbouring one is not visible (Fig. 2 a, b). Besides the structural changes concerning the array of MAPs siamese twin vesicles are observed (Fig. 2 a, b). In the waist of these twin structures occasionally a ring of particles is visible in membrane B-faces (Fig. 3 a) and A-faces (Fig. 3 b). Both halves of the twin vesicles have the size of normal secretory vesicles. This formation was never found at low Ca^{2+} concentration. The cleveage plane from one to the other vesicle is continuous in the membrane B-face (Fig. 2 a) as well as in the A-face (Fig. 3 b). This indicates that these twin structures represent fused secretory vesicles with one common lumen.

In some twin vesicles the waists do not show the aggregation of MAPs described above (Fig. 4). These structures may represent the corresponding membrane faces to the fused vesicles shown in Figure 3 or a later stage of fusion development were particles are randomly redistributed. The different features of fusion described above have been also observed in concentrations as low as 10^{-6} M Ca^{2+} but the frequency of these events

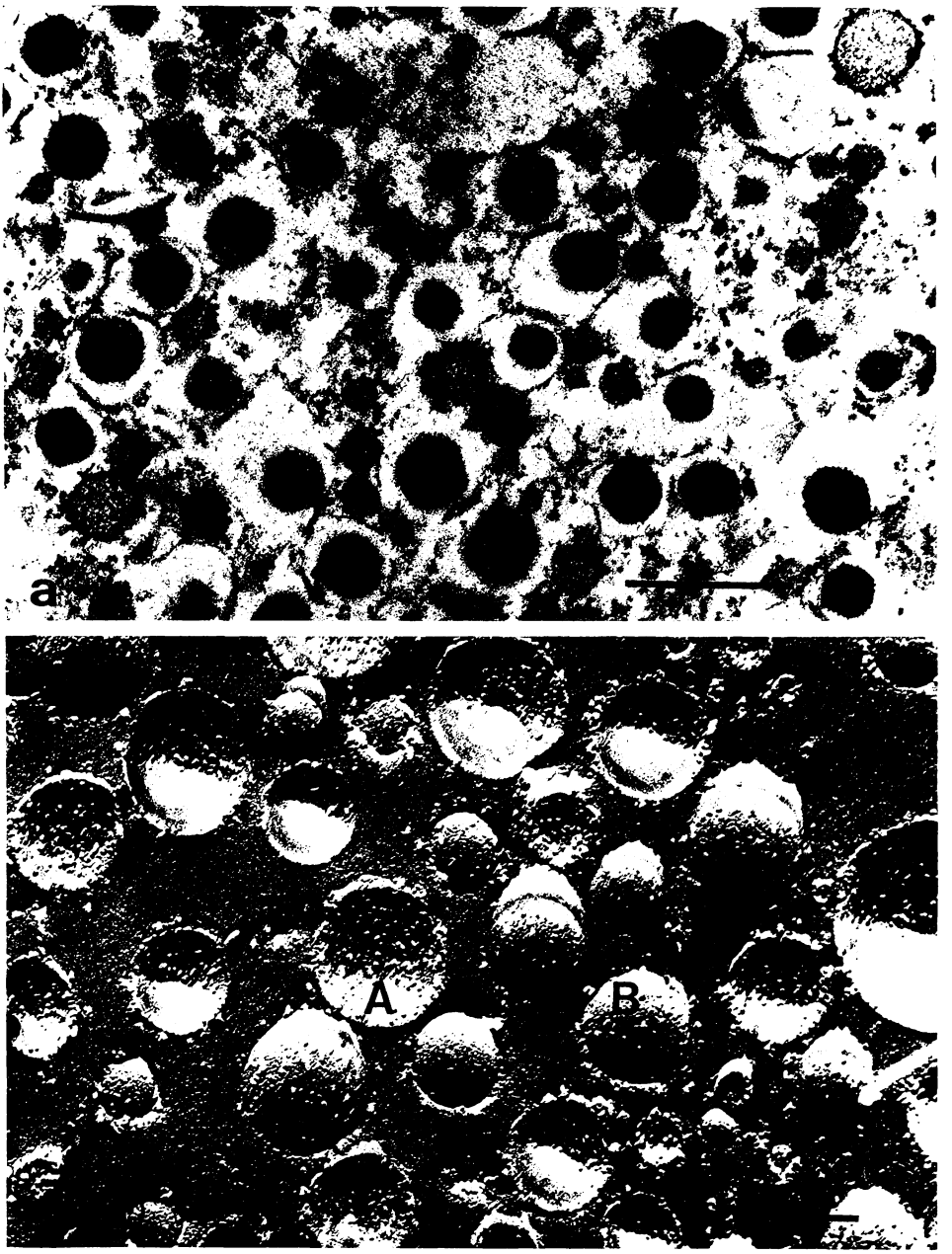

Fig. 1 a. Electron micrograph of a thin sectioned pellet from the vesicle preparation. It consists mostly of B-granules characterized by the dense core surrounded by a halo and the limiting membrane. Some microsomal contamination is present. – 45 000 ×. – Scale 0.5 μm. – **b.** Electron micrograph of a freeze-fractured vesicle preparation incubated in low Ca^{2+} concentration ($< 10^{-8}$ M). Randomly distributed membrane associated particles (MAPs) stick more to the concave A-faces (A) than to the convex B-faces (B) of the limiting membranes. Encircled arrowhead indicates direction of shadowing. – 80 000 ×. – Scale 0.2 μm.

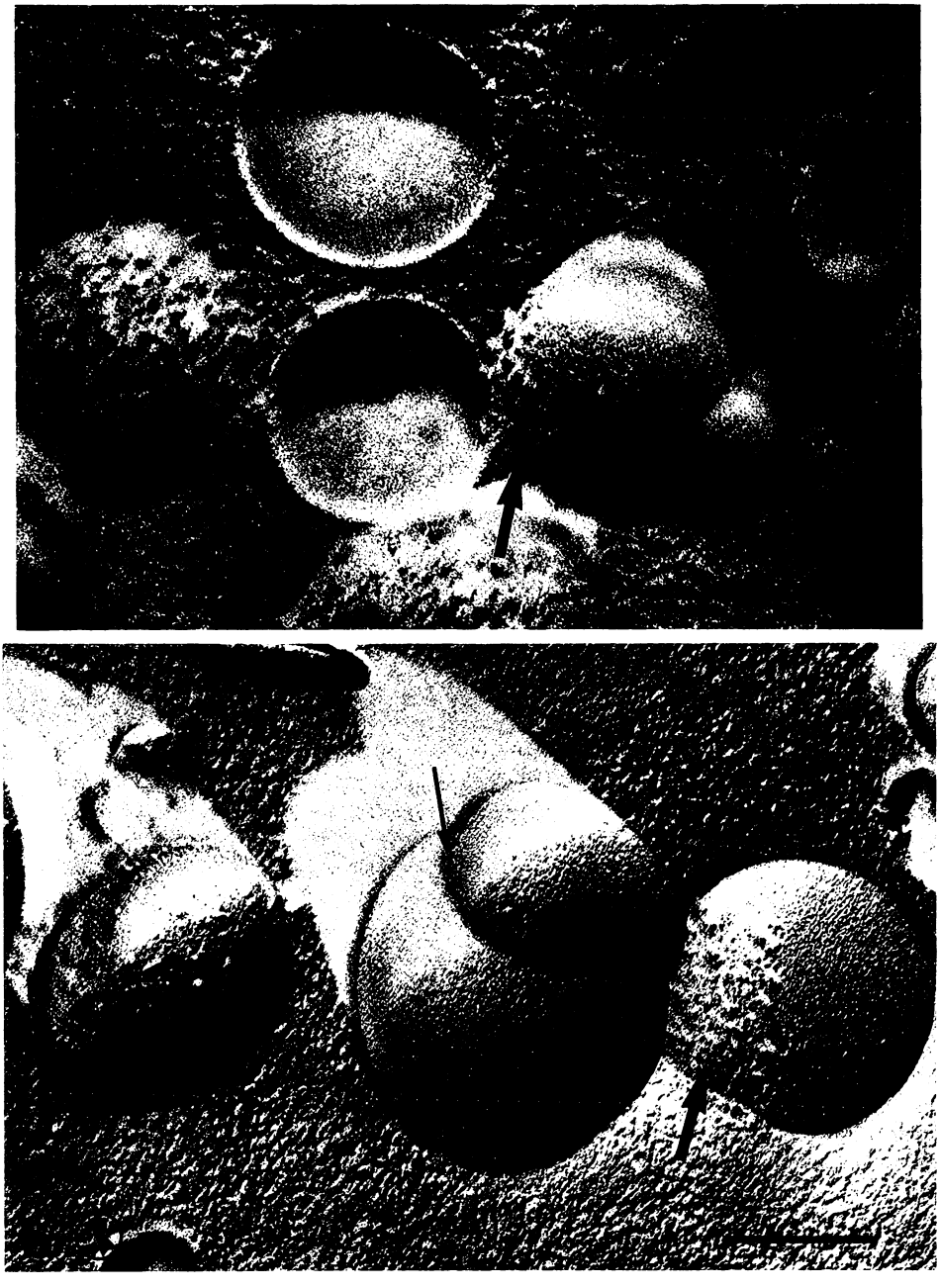

Fig. 2. Freeze-fractured vesicles incubated in high Ca^{2+} concentration. MAPs are frequent in membrane B-faces and are accumulated. This accumulation is present in zones of contact between neighbouring vesicles *(large arrow)* but is also visible on top of convex vesicles. Twin vesicles with a waist *(small arrow)* are visible and the continuous cleavage plane of the mem-

was highest at Ca^{2+} concentrations of 10^{-5} M and did not change up to 10^{-3} M Ca^{2+} (Tab. 1).

To determine whether the processes observed are specific for Ca^{2+} we tested various other cations. As shown in Table 1 no fusions of vesicles could be induced by other cations, although aggregation of MAPs – less pronounced – occured in membrane B-faces. Attachments of distinct vesicles were also present under these conditions.

Discussion

The isolation of secretory vesicles from endocrine pancreatic tissue is rather laborious and the difficulties arising during their isolation have been reported in various publications [5, 14, 15, 17]. As described above, the population of secretory vesicles obtained are derived mainly from B-cells and the effects observed should be attributed to B-granules rather than to A-granules. The presence of microsomes does not affect the experiments described, since microsomes were shown to be unable to fuse under the conditions described in this investigation (GRATZL and DAHL, 1975 – manuscript in preparation).

Aggregation of MAPs is a prominent feature of secretory vesicle membranes, exposed to increasing Ca^{2+} concentrations. This change in the array of intramembranous particles was found to be induced also by other cations, though to a lower extent. The observed aggregation of MAPs appears on the B-face of the membrane, which is practically devoid of MAPs at low Ca^{2+} concentrations. Aggregation of MAPs may arise from a lateral movement and intramembranous particles in this case may adhere more to the B-face than to the A-face of the membrane upon exposure to Ca^{2+}. However, the high particle density on the B-face could also be due to an integration of peripheral particles in the membrane which are usually not visible in freeze-cleaving.

Particle aggregation was observed in zones of contact between neighbouring vesicles as well as in other membrane areas. In the latter case the material above the exposed membrane faces had been removed by the cleavage process. Therefore, it cannot be decided whether the aggregation of MAPs occurs only in contact regions or also independently of membrane contact. This implicates the inability to conclude whether an aggregation of MAPs favours a membrane contact or whether a contact induces an aggregation of MAPs.

In fused vesicles a band of MAPs was found in the region of the remaining waist. This may be a relict of the aggregation of MAPs in contacting vesicles and their existence in B- and A-faces may be an indication for a different distribution of MAPs at various stages of fusion development.

There is accumulative evidence for the key role of MAPs in the fusion process of biological membranes (cf. ref. [29]): The aggregation of MAPs in the B-face of the membrane of vesicles contacting each other as well as neighbouring the plasma membrane was already described in glucose stimulated B-cells by BERGER, DAHL and MEISSNER [3] as an event preceding membrane fusion. The results of these authors also

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brane. Encircled arrowhead indicates direction of shadowing. – **a.** Ca^{2+}-concentration: 2×10^{-3} M. – 150 000 ×. – Scale 0.2 μm. – **b.** Ca^{2+}-concentration: 2×10^{-5} M. An aggregation of MAPs *(large arrow)* is visible on the B-face of a vesicle attaching two fused vesicles *(small arrow)*. – 100 000 ×. – Scale 0.2 μm.

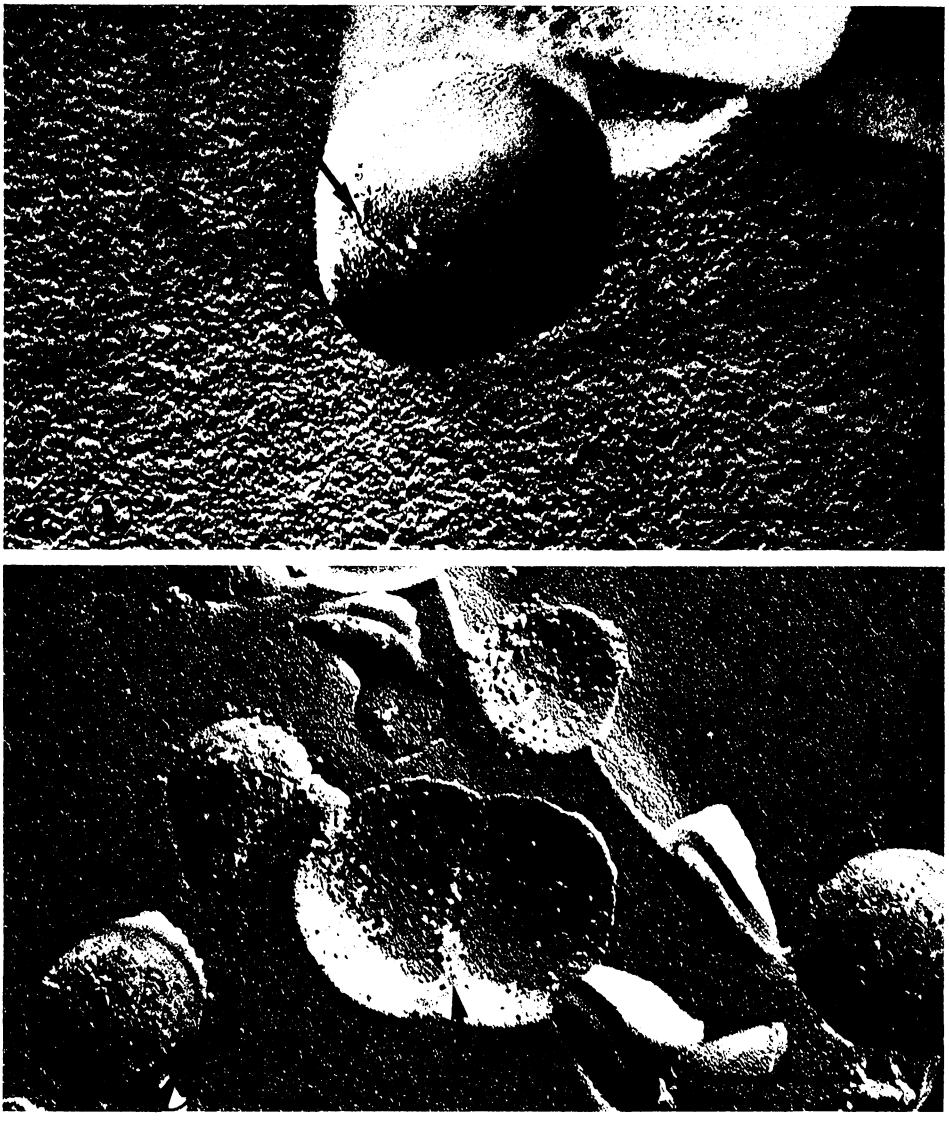

Fig. 3. Freeze-fractured vesicles incubated in a solution containing 2×10^{-5} M Ca^{2+}. Encircled arrowheads indicate direction of shadowing. – **a.** B-face of fused vesicles with a ring-like aggregation of MAPs *(arrow)*. – **b.** Aggregation of MAPs on the A-face of fused vesicles *(arrow)*. – 100 000 ×. – Scale 0.2 μm.

indicated that the aggregation of MAPs may represent a zone of increased permeability. A similar behaviour was shown for the fusion of myoblast plasma membranes, where fusion is preceded by the development of gap junctions coupling myoblasts electrically [31, 32]. Vesicles of plasma membranes isolated from myoblasts also fused in the presence of Ca^{2+} with transitory formation of an aggregation of MAPs (SCHUDT, DAHL

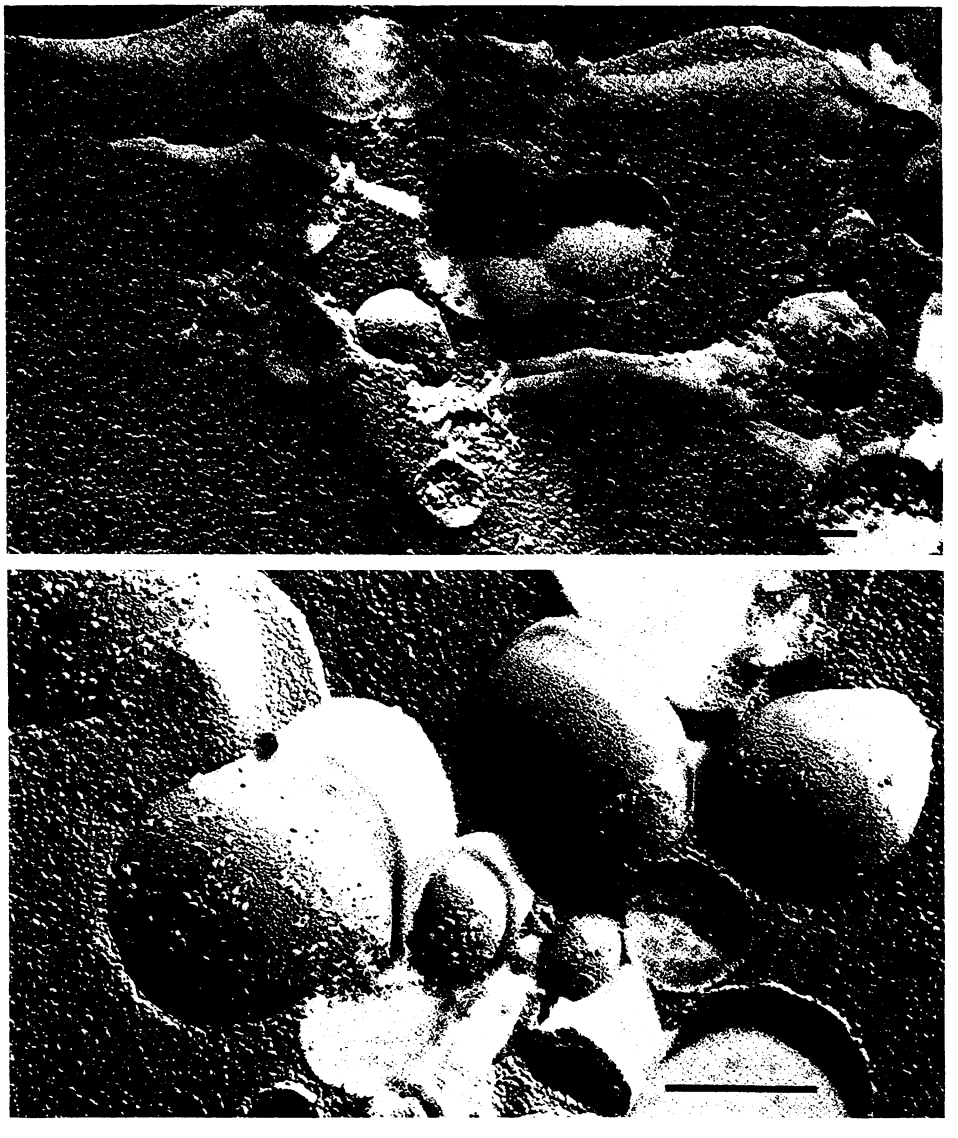

Fig. 4. Fused vesicles without aggregation of MAPs neither on A-face (**a**) nor on B-face (**b**). Encircled arrowheads indicate direction of shadowing. – 100 000 ×. – Scale 0.2 μm.

and GRATZL, 1975 – manuscript in preparation). A particular formation of MAPs is also described by SATIR *et al.* [34] for membrane fusion during mucocyst secretion in tetrahymena. Finally, aggregations of MAPs were found to accompany the virus induced fusion of hen erythrocytes [2].

Glycerol was suggested by SCHWAB-STEY *et al.* [36] to cause fusion of mitochondria from tetrahymena pyriformis. This was also reported by AHKONG *et al.* [1] for the

fusion of hen erythrocytes. We used in our experiments glycerol for cryoprotection during the freeze-fracture process. However, glycerol was added after prefixation with glutaraldehyde. Therefore, in our experiments glycerol should not have induced fusion. Furthermore Ca^{2+}-specific fusion of Golgi vesicles was observed even in the absence of glycerol (GRATZL and DAHL, 1975 – manuscript in preparation).

Tab. 1. Ca^{2+}-dependence and specificity of the fusion of pancreatic endocrine secretory granules.

Cation	Concentration (M)	Particle Aggregation	Fusion
Ca^{2+}	$< 10^{-8}$	-	-
	2×10^{-6}	+	+
	2×10^{-5}	++	++
	2×10^{-4}	+++	++
	2×10^{-3}	+++	++
Mg^{2+}	1×10^{-4}	+	-
Sr^{2+}	1×10^{-4}	+	-
Ba^{2+}	1×10^{-4}	+	-
Mn^{2+}	1×10^{-4}	+	-
La^{3+}	1×10^{-4}	+	-

\- < 1 %
\+ 1 - 5 %
++ 5 - 10 %
+++ > 10 %

These figures were evaluated by counting 200 vesicles for each cation concentration in a scanned replica.

During the stimulation of pancreatic B-cells with glucose two processes are known to take place: Fusion of secretory vesicles among each other and fusion of secretory vesicles with the plasma membrane. Both membrane fusions exhibit the same morphology [3]. The coincidence of both events is described for different cells and called "compound exocytosis" [7] or "tandem exocytosis" [18]. The similarity of intervesicular fusion and the fusion of secretory vesicles with the plasma membrane in intact B-cells [3] led us to the conclusion that the study of intervesicular fusion may give indications about the mechanism of membrane fusion during the exocytotic process.

The results presented show, that Ca^{2+} is able to induce fusion of isolated secretory vesicles with the same morphological changes observed during membrane fusion in intact cells [3]. Exposure of vesicles to other cations led also to a certain extent to aggregation of MAPs and attachment of vesicles. However, other cations were inefficient to trigger the fusion of these vesicles.

Specificity of Ca^{2+} to induce exocytosis was observed by WOLLHEIM *et al.* [37]. In these studies externally added Ca^{2+} with the aid of an ionophore was able to cause insulin release from monolayer cultures of endocrine pancreas. Mg^{2+} and Sr^{2+} could not replace Ca^{2+}. Though Ba^{2+} alone had a stimulatory effect on insulin release, in the authors' conditions Ba^{2+} was not shown to be effective in the presence of the ionophore.

Specificity for Ca^{2+} and lack of response to other cations applied intracellularly were reported also upon other systems including mast cells [16] and from the giant synapse of the squid [24]. Similarily the use of ionophores in different systems suggest that Ca^{2+} is a link between stimulus and secretion [4, 10, 30, 38].

In our studies fusion of secretory vesicles can be observed at very low concentrations of Ca^{2+} (10^{-6} M). The concentration of free Ca^{2+} in different cells was reported to be in the same range (cf. ref. [33]). Therefore, small variations in the concentration of free intracellular Ca^{2+} may trigger exocytosis in pancreatic B-cells. Structural changes of isolated chromaffin granules associated with a reversible aggregation of vesicles have been described [8] using very high concentration of Ca^{2+} (4 mM). This concentration is far from physiological conditions. These effects have been obtained also with Mg^{2+}. We therefore hesitate to compare these results with the Ca^{2+}-specific fusion of secretory vesicles reported in this paper.

From our experiments it becomes evident that fusion of the pancreatic endocrine secretory vesicles requires the interaction of Ca^{2+} with the vesicular membrane. Binding of Ca^{2+} to pancreatic secretory vesicles has been shown by DEAN [6] using microelectrophoresis. The binding of calcium to secretory vesicles in stimulated B-cells was also found in histochemical studies and elemental X-ray analysis [13, 35]. Preliminary results recently described by LAZARUS and DAVIES [20] suggest a Ca^{2+}-induced insulin release occuring during incubation of isolated B-granules with purified plasma membranes in the presence of Ca^{2+} and ATP.

In conclusion the experiments described in this publication indicate that Ca^{2+} in the pancreatic B-cell is able to act as final intracellular trigger in stimulus-secretion coupling causing the fusion of membranes. It will be a tempting task for the future to elucidate the nature and interaction of the missing links between glucose stimulus and Ca^{2+}-induced exocytosis in the pancreatic islet.

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