CYTOBIOLOGIE

European Journal of Cell Biology

Volume 13 · Number 1 · June 1976

WISSENSCHAFTLICHE VERLAGSGESELLSCHAFT MBH

BOOK REVIEWS

CYTOBIOLOGIE
13, 211–223 (1976)

Calcium-induced fusion of plasma membranes isolated from myoblasts grown in culture

Calcium-induzierte Fusion von Plasmamembranen, isoliert aus Myoblasten in Kultur

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Received September 20, 1975
in revised form February 26, 1976
and April 28, 1976

Abstract

Membrane fusion – calcium – isolated plasma membranes – myoblasts

1. Plasma membrane vesicles isolated from myoblasts grown in vitro were found to fuse in the presence of calcium ions.
2. As revealed by freeze cleaving techniques, addition of 1.4 mM $CaCl_2$ or 1.4 mM $MgCl_2$ resulted in attachment of vesicles and aggregation of intramembranous particles.
3. Plasma membrane vesicles fuse solely in the presence of calcium, and fusion is identified by the appearance of twinned vesicles.
4. Pore formation was preceded by an aggregation of intramembranous particles in the area of membrane contact.
5. 20 mM $MgCl_2$ oder 10^{-6} M lysolecithin both decreased the amount of vesicle fusion in a similar manner to the inhibition of the fusion of myoblasts.

Introduction

Membrane fusion appears to be essential in several biological processes taking place at the cellular and subcellular level [28, 31]. One of these is the spontaneous cell fusion which occurs during the development of striated skeletal muscle [24]. Muscle cell fusion can be inhibited in vitro if the cells are grown in media containing low calcium concentrations [37]. After 50 hours growth and proliferation, fusion can be triggered by raising the calcium concentration in the medium to about 2 mM. Under these conditions myoblasts form polynuclear myotubes in a monolayer within 6 to 8 hours [7, 37].

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Although it has been suggested that calcium ions act on sites of plasma membranes accessible to ions of the extracellular fluid [36], no information about the site of specific calcium binding is available. Furthermore, the question of the involvement of certain proteins, phospholipids or intracellular messengers in the mechanism of cell fusion remains to be resolved.

Morphological observations and studies by RASH et al. [29, 30] concerning the electrical coupling of fusing myogenic cells in vitro as well as in vivo indicate that intramembranous particles (IMPs) play an important role in myoblast fusion. The particles aggregate in the area of contact, form gap junctions and thus may mediate the initial events of fusion.

Since methods for the isolation of plasma membranes from muscle cells are now available [34], the interaction of these membranes can be studied in the absence of cytoplasmic components. It is of particular interest to show whether plasma membranes isolated from myoblasts are still able to fuse in a cell-free system. Moreover, it is important to establish whether a fusion of isolated plasma membrane vesicles depends on parameters similar to those affecting myoblast fusion [7, 32, 37] and whether a rearrangement of IMPs is related to the fusion process.

Materials and methods

Cell cultures of chick embryo breast muscle were prepared as described previously [6]. Cells were grown in a medium containing low calcium concentrations (10^{-5} M $CaCl_2$) to keep fusion below 5 % [6, 37]. After 52 hours myoblasts are competent for fusion, and on increasing the calcium concentration to 1.4 mM the cells form myotubes within 6 to 8 hours [7, 37]. Plasma membranes were prepared from myoblasts competent for fusion according to the method described by KENT et al. [19]. The membrane fraction floating to the interphase between 13 % and 27 % sucrose was collected by centrifugation at 100 000 g for 30 minutes and resuspended in 200 µl of a 0.25 M sucrose medium buffered by 20 mM HEPES at *p*H 7.3. Phase contrast microscopy showed that the membrane fraction consisted of vesicles as well as membrane sheets. To transform sheets into vesicles, the preparation was passed 5 times through a syringe needle, inner diameter 0.2 mm.

Plasma membrane suspension (0.01 ml, protein concentration 2.0 mg/ml) was incubated at 37° C with 0.01 ml HEPES-buffered sucrose solution (0.25 M) containing different concentrations of $CaCl_2$, $MgCl_2$ or synthetic lysolecithin. Incubations were carried out in 0.5 ml polyethylene reaction vessels. After 30 minutes, 0.01 ml 2 % glutaraldehyde containing the components used in the experiment was added. After 5 minutes of fixation at 37° C, 0.01 ml glycerol was added for cryoprotection.

Freeze cleaving: Small droplets (less than 1 µl) of the suspension were frozen on gold specimen holders in freon 22 which was cooled in liquid nitrogen and were then stored in liquid nitrogen. The cleaving process was performed according to the method of MOOR and MÜHLETHALER [22] in a Balzers freeze-etch unit BAF 300. Fracturing and replication were performed at −100° C. Organic material was removed from the replicas using sodium hypochlorite. After washing in distilled water, replicas were picked up on formvar- and carbon-coated single hole grids and examined in a Siemens Elmiskop 101 at 80 or 100 KV.

Photographs were taken as positives (platinum deposition: black). Direction of shadowing is indicated by an encircled arrowhead.

For identification of fracture faces we have used the nomenclature introduced recently by BRANTON et al. [8]: The membrane faces are denoted as P and E, where P corresponds to the earlier A-face and E to the earlier B-face.

Aggregation of intramembranous particles was quantitatively evaluated as described by DUPPEL and DAHL [11]: Vesicles containing particle-free membrane areas of 0.01 µm² (cor-

responding to 12 × 12 mm in micrographs with total magnification of 120 000 ×), with an unchanged total number of particles, were designated as vesicles exhibiting particle aggregation. An aggregation index was obtained from the ratio of vesicles with and without particle aggregation. As a control the maximum number of particles within 0.01 μm^2 was determined. In addition, the distribution of intramembranous particles was checked using standard error of mean (SEM) of the mean density of particles (Tab. II).

Protein concentration was determined fluorometrically according to FAIRBANKS [13].

Glycerol-3-phosphate dehydrogenase (mitochondrial) activity was determined according to GARDENER [16].

α-neurotoxin isolated from the venom of *Naja naja siamensis* was a kind gift from DR. F. HUCHO, Fachbereich Biologie der Universität Konstanz, and was labelled with iodine-125 according to MARCHALONIS [20]. Its binding properties and competition with decamethonium was determined according to PATERSON [27]. Synthetic lysolecithin (ET_{16}-H) was a generous gift from DR. U. WELTZIEN, Max-Planck-Institut für Immunbiologie, Freiburg. Unspecified chemicals were of analytical grade.

Results

Plasma membranes of cultured chick embryo muscle cells were purified according to the method described by SCHIMMEL et al. [34] and slightly modified by KENT et al. [19]. After 52 hours growth in a medium containing low calcium concentration, cells were removed from the culture dishes by scraping with a rubber policeman. Enzymatic

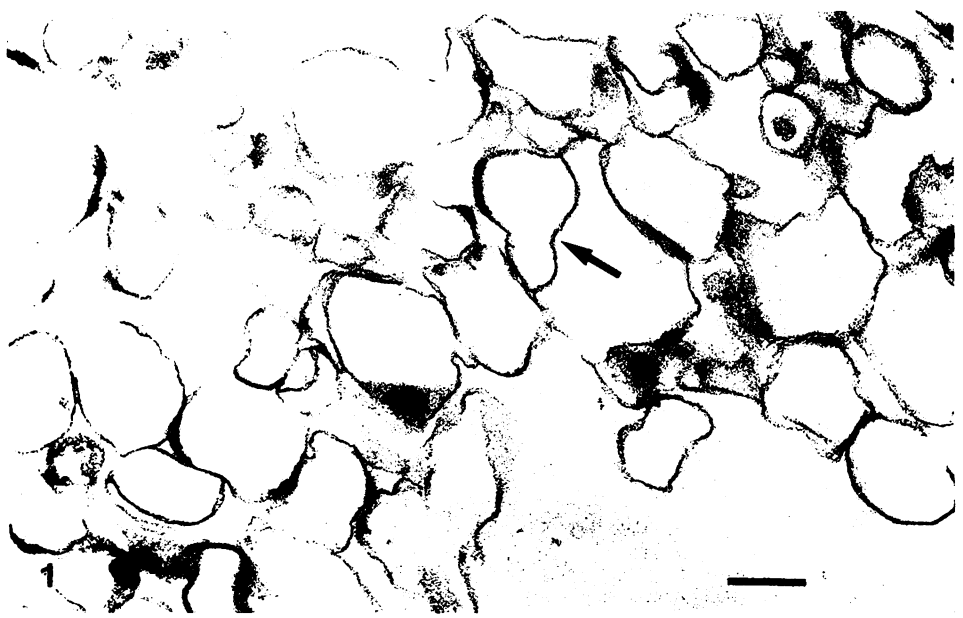

Fig. 1. Electron micrograph of a thin sectioned pellet from a preparation of plasma membranes. Membranes have been incubated in a solution with 1.4 mM $CaCl_2$. The shape of the membrane profiles varies and fused vesicles may be observed *(arrow)*. – 50 000 ×. – Scale 0.2 μm.

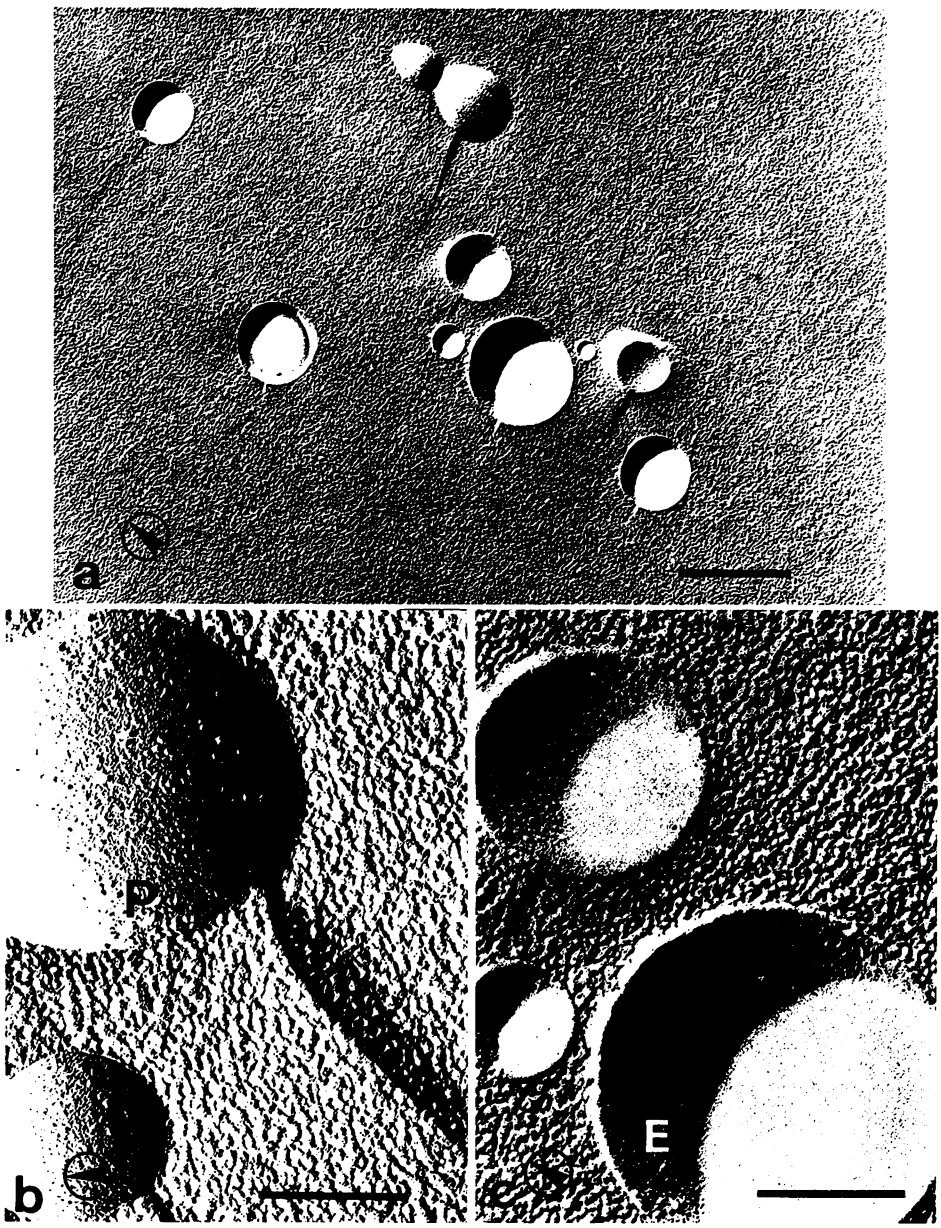

Fig. 2. Electron micrograph of a freeze fractured suspension of plasma membranes in low calcium concentration. Spherical vesicles are dispersed in the medium (**a**). The intramembranous particles are distributed randomly and adhere more to the convex P-faces (**b**) than to the concave E-faces (**c**). – **a.** 35 000 ×. – Scale 0.5 μm. – **b and c.** 100 000 ×. – Scale 0.2 μm.

detachment was avoided in order to prevent any modification of the cell surface. Cells were homogenized and plasma membranes isolated according to [19]. The purity of plasma membranes was determined by the distribution of acetylcholine receptor-bound α-neurotoxin in the various membrane fractions. This has been reported to be a reliable marker of myoblast plasma membrane [21, 28]. Table I shows the protein content and distribution of two markers in several fractions taken from different stages of the separation procedure. Thin sections of the isolated membrane fraction collected from the interphase between 13 % and 27 % sucrose show smooth-surfaced membranes containing no electron dense material. Nuclei, mitochondria or membranes originating from rough endoplasmic reticulum were rarely observed (Fig. 1).

Tab. I. Distribution and relative specific activities of subcellular markers in the fractionation of fusion-competent myoblasts.

Fraction	Protein	Glycerolphosphate dehydrogenase		α-neurotoxin binding	
	(%)	(%)	relative specific activity	(%)	relative specific activity
Homogenate, particulate	100	100	1	100	1
1700 g pellet	86	80	0.93	36	0.42
27 % gradient fraction	1.3	0.2	0.15	8	6.2
32 % gradient fraction	2.3	1.7	0.74	5	2.2

Freeze cleaving of the isolated vesicular preparation (Fig. 2) showed that the intramembranous particles (IMPs) adhere more to the convex P-faces (1000/μm²) than to the concave E-faces (200/μm²) (Tab. II). A few concave vesicles with high particle density may represent inside-out plasma membranes or vesicles derived from endoplasmic reticulum. In agreement with the low glycerol-3-phosphate dehydrogenase activity, mitochondria were rarely observed. Contamination by nuclear membranes, which can easily be detected by their pores, was negligible. From the biochemical as well as the morphological data it can be concluded that most of the isolated vesicles represented plasma membranes orientated right-side-out.

Tab. II. Aggregation of intramembranous particles and total number of intramembranous particles of isolated myoblast plasma membranes under different conditions.

Additives in the incubation medium	Aggregation index *) (P-face) a	b	c	Maximum number of particles within a defined area of P-face (0.01 μm²)	Mean density per 0.01 μm²	Number of particles per μm² in P-face	E-face
none	7	32	0.22	12.6 ± 5.2 (n = 28)	10.3 ± 3.6 (n = 39)	1031	235
1.4 M $CaCl_2$	78	35	2.03	18.5 ± 5.9 (n = 58)	10.5 ± 9.3 (n = 55)	1050	275
1.4 M $MgCl_2$	37	20	1.85	17.3 ± 4.9 (n = 24)	9.9 ± 8.2 (n = 47)	996	293

*) Number of vesicles with (a) and without (b) particle-free areas of 0.01 μm². Ratio a : b = c. A total area of 0.4 to 0.6 μm² was counted of each preparation. For details see Methods.

When plasma membranes were incubated in a medium to which no calcium was added, vesicles were dispersed (Fig. 2 a) and IMPs were distributed randomly in the convex P-face (Fig. 2 b). Increasing the concentration of calcium in the incubation medium to 1.4 mM resulted in a clustering of vesicles (Fig. 3). Concomitantly, IMPs were found to be aggregated in a large proportion of the vesicle preparation (Tab. II).

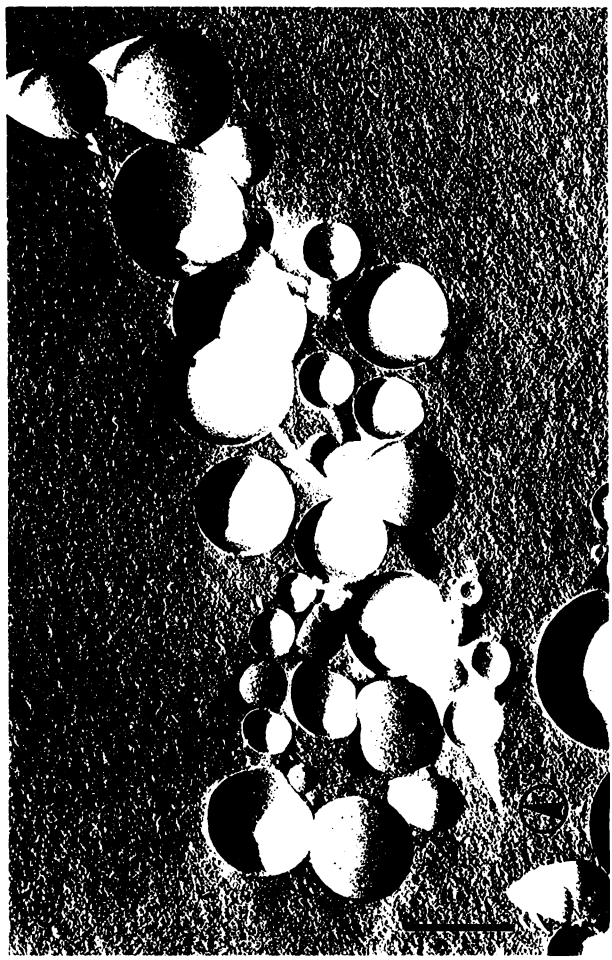

Fig. 3. Freeze-fractured vesicles of myoblast plasma membranes incubated in 1.4 mM $CaCl_2$. Vesicles are clustered and some are fused. – 30 000 ×. – Scale 0.5 μm.

Fig. 4. Higher magnification of plasma membrane vesicles incubated in 1.4 mM $CaCl_2$. Intramembranous particles are aggregated in zones of contact between vesicles *(small arrows)* and in zones where the fracturing procedure has removed the overlying material. Twinned vesicles and tripled vesicles indicate vesicle fusion *(large arrows)*. – 100 000 ×. – Scale 0.2 μm.


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Vesicles in tight apposition were observed frequently and in the areas of contact, aggregations of IMPs appeared in membrane P-faces (Fig. 4). In addition to an attachment of vesicles and the aggregation of IMPs, twinned vesicles were observed in the presence of 1.4 mM calcium (Fig. 4). Within the resolution of freeze fracture electron

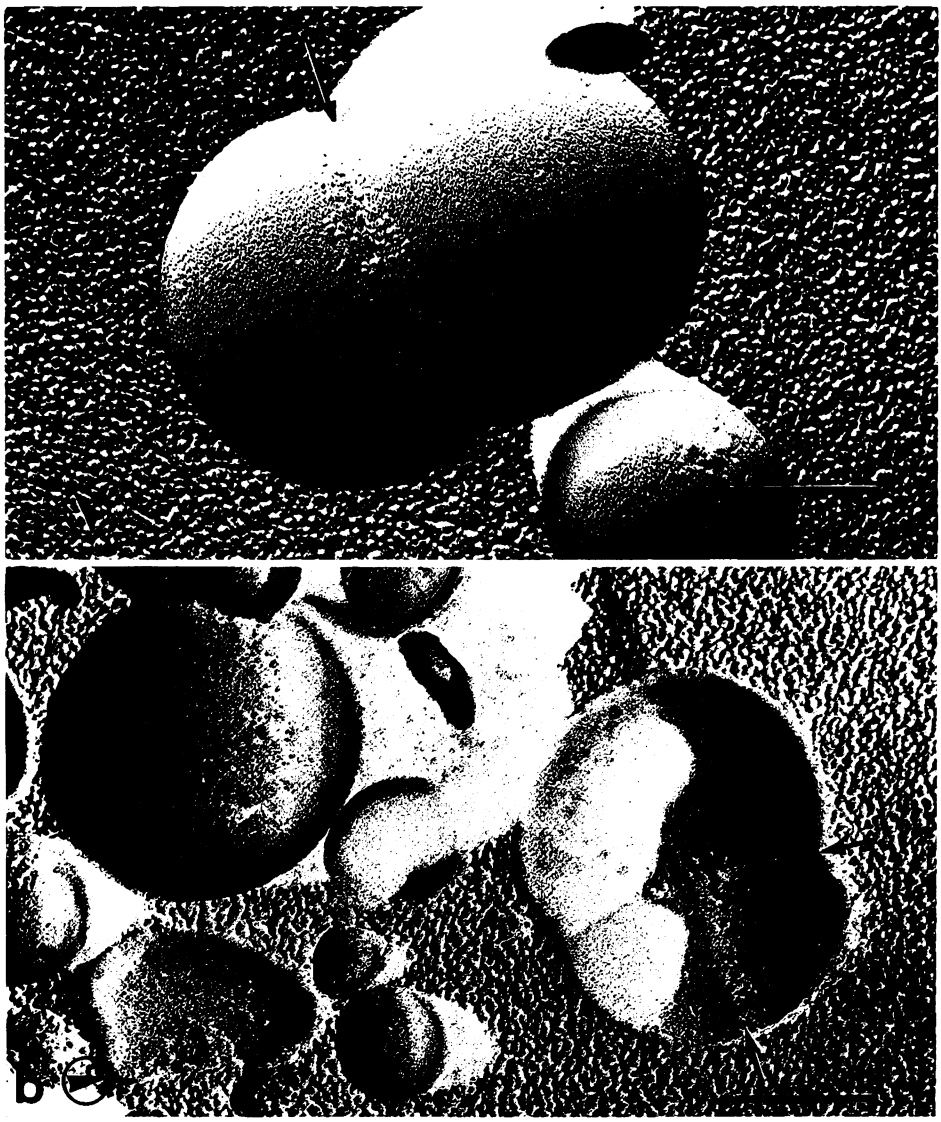

Fig. 5. Plasma membrane vesicles incubated in a solution containing 1.4 mM $CaCl_2$. – **a.** Convex P-face of fused vesicles with a seamline occupied by an aggregation of IMPs *(arrow)*. – **b.** Concave E-face of fused vesicles with an aggregation of IMPs along the waists *(arrows)*. – 100 000 ×. – Scale 0.2 μm.

microscopy (30 Å) no ridge in the membrane which might eventually separate the two vesicles could be detected. The cleavage plane in these structures is continuous in the P-face as well as in the E-face (Fig. 4 and 5), and in addition, thin sections (Fig. 1) reveal that twinned vesicles were not separated by a septum. This indicates that two vesicles have formed a common lumen by fusion. Only a very low percentage of fused vesicles were observed after incubation in the absence of calcium (Tab. III).

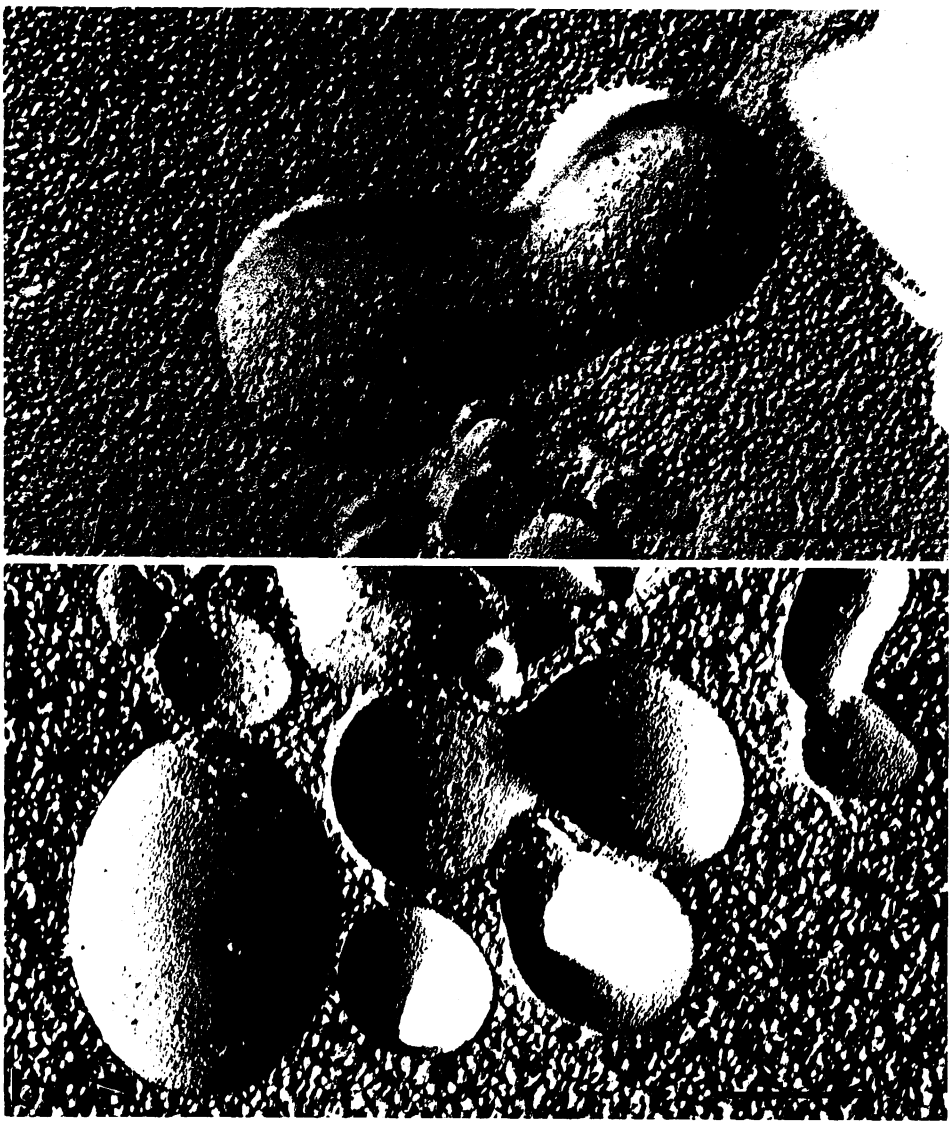

Fig. 6. Fused plasma membrane vesicles exhibiting a softened waist. IMPs are randomly distributed in membrane P-faces (**a**) and E-faces (**b**). – 100 000 ×. – Scale 0.2 μm.

Tab. III. Percentage of vesicle fusion under different incubation conditions.

Additives in the incubation medium	Percentage of vesicle fusion (%)	Number of counted vesicles
none	1.1	1000
1.4×10^{-3} M $CaCl_2$	15.3	1200
1.4×10^{-3} M $MgCl_2$	1.8	1000
1.4×10^{-3} M $CaCl_2$ 2×10^{-2} M $MgCl_2$	6.2	1000
1.4×10^{-3} M $CaCl_2$ 10^{-6} M lysolecithin	7.4	400

Among the fused vesicles two populations could be distinguished. One of them was characterized by an accumulation of IMPs which followed a seamline and were found in both P- and E-faces of the membrane (Fig. 5 a and 5 b). In the second population this seamline was not detectable and fused vesicles exhibited soft waists (Fig. 6 a and 6 b). From these figures it can be seen that IMPs were distributed in a manner similar to their array before fusion took place. Incubation of vesicles in the presence of 1.4 mM $MgCl_2$ instead of $CaCl_2$ resulted in a clustering of vesicles and an aggregation of IMPs. However, in contrast to calcium, magnesium did not induce fusion (Tab. III).

Comparable to monolayer myoblast fusion [26, 35], isolated membrane vesicles fused at 1.4 mM $CaCl_2$ and were effectively inhibited from fusing by 20 mM $MgCl_2$ and 10^{-6} M lysolecithin (Tab. III).

Discussion

The membrane fraction used in this study consisted mainly of plasma membranes as shown by biochemical analysis, thin section and freeze fracture electron microscopy. The presence of membranes originating from sarcoplasmic reticulum (which might be also present in this preparation) cannot be ruled out in view of the methods used. However, BASKIN [4] found intramembranous particles at a density of 86/μm^2 in sarcoplasmic membranes prepared from 14-day old chick embryos. The high average particle density of 1000/μm^2 found in the present investigation makes it unlikely that sarcoplasmic membranes are present to a significant extent.

Furthermore, ultrastructural studies of FISCHMAN [14] showed that sarcoplasmic reticulum was not observed in unfused myoblasts.

Addition of divalent cations such as magnesium or calcium to the isolated plasma membranes resulted in an attachment of vesicles to form clusters. A concomitant aggregation of IMPs was also often observed in the zone of contact between adjacent vesicles. In contrast to vesicle attachment and particle aggregation – both of which can be evoked by either calcium or magnesium – the fusion of vesicles observed as twin structures can only be demonstrated after incubation with calcium. Therefore the pore formation resulting in a common lumen between two vesicles seems to be calcium-specific, as is the fusion of isolated secretory vesicles [10, 17], mast cell secretion [18], and transmitter release [21].

The calcium-induced fusion of vesicles was inhibited by 20 mM $MgCl_2$. This calcium-magnesium antagonism has already been shown in myoblast fusion [35] and secretion [9, 15, 21].

Calcium-magnesium antagonism and lysolecithin inhibition suggest that the mechanism of myoblast fusion differs from that of artificially induced fusion processes. Virally induced erythrocyte fusion [38], fusion of nascent membranes of *Echinosphaerium nucleofilum* [39] and fusion induced by fusogenic lipids [2] do not show these ion selectivities.

Aggregation of IMPs has been shown in many systems and has been correlated with cell to cell contact [12], agglutination of erythrocytes [23], fusion processes in mucocyst secretion [33], and to myoblast fusion [30]. Furthermore, aggregation of IMPs preceeds the fusion of secretory vesicles with plasma membrane in intact pancreatic islet cells [5] as well as calcium-specific fusion of isolated secretory vesicles [10, 17]. However, from experiments described in this paper, it cannot be determined whether the aggregation of IMPs is due to intramembranous bridge formation (induced by divalent cations) between components in one vesicular membrane, or whether it is a consequence of intermembranous bridge formation between neighbouring vesicles. It can however be concluded that aggregation of IMPs is related to membrane attachment and may be a necessary prerequisite for fusion. This is indicated by the appearance of IMPs in the waists of fused vesicles. These IMPs may represent remnants of aggregates observed in the zones of vesicular contact.

Low molecular weight compounds such as cyclic nucleotides thought to participate in the mechanism of fusion [31], are not present in our system. In addition, the amount of cytoplasmic enzymes should also have been greatly reduced by the membrane isolation procedure. Therefore it seems likely that components other than those fixed on the membrane do not contribute to the basic process of fusion.

AHKONG et al. [1] suggested (without morphological evidence) that aggregation of membrane proteins and fusion may be induced by a micellising agent such as lysolecithin. In our experiments lysolecithin produced just the opposite effect; a decrease in the amount of fused vesicles was observed.

In conclusion, we suggest that the following steps occur in the course of fusion:

1. Dependent on divalent cations, vesicles contact each other and IMPs aggregate.
2. Under the specific influence of calcium ions, vesicles fuse at the zones of particle aggregation forming twin structures.
3. This early stage of fusion is characterized by a seamline which may be stabilized by the presence of IMPs.
4. Subsequently, IMPs redistribute randomly and the waist softens.

Acknowledgements. We thank Mrs. I. KÜMMEL, Mrs. E. JARDEL, and Mr. R. WEISS for excellent technical assistance. We are grateful to DR. M. A. CAHILL and DR. C. R. WULF for reading the manuscript. – This work was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 138 "Biologische Grenzflächen und Spezifität" and Sonderforschungsbereich 38 "Membranforschung".

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