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## FUSION OF ISOLATED MYOBLAST PLASMA MEMBRANES AN APPROACH TO THE MECHANISM

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### Summary

Fusion of plasma membranes isolated from myoblasts grown in culture has been investigated.

1. Membrane fusion was specifically dependent on  $\text{Ca}^{2+}$  at physiological concentrations. However, at higher concentrations of cations, fusion could be triggered not only by  $\text{Ca}^{2+}$ , but by  $\text{Mg}^{2+}$  and  $\text{Sr}^{2+}$  as well.

2. The amount of fusion was directly proportional to temperature.

3. Fusion was found to depend on the state of maturation of the myoblast membranes.

4. Experiments with chemically and enzymatically modified membranes and with membranes derived from myoblasts grown in the presence of inhibitors of protein biosynthesis suggest the participation of proteinaceous membrane components in the fusion mechanism.

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### Introduction

During maturation of skeletal muscle fibers, myotubes are formed by fusion of mononucleated myoblasts. This process has been studied in myoblast cultures which have been grown in media containing artificially low  $\text{Ca}^{2+}$  concentration. Under this condition fusion competence was acquired, but fusion did not occur [1,2]. Cell fusion can be triggered by the addition of physiological amounts of  $\text{Ca}^{2+}$  to the medium [1,5]. The influence of several parameters such as pH [3], cations [5] and lipid composition [3,6] on myoblast fusion has been studied in detail. However, the interpretation of these studies has been ham-

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pered by interfering processes such as cell motility, cell aggregation and cell metabolism.

This difficulty can be avoided by using a simplified system. Several model systems for membrane fusion have been developed recently, such as chemically-induced fusion of membranes which do not fuse physiologically [7,8], virus-induced cell fusion [9,10], liposome-cell fusion [11–14] and liposome-liposome fusion [15–18]. As opposed to artificial model systems, tissue fractionation techniques to isolate membranes which have the physiological capacity for fusion have been utilized in the present study. It has been shown that isolated secretory vesicles [19–23] and isolated plasma membranes of fusion competent myoblasts grown in culture can be induced to fuse [24]. This provides the possibility of following membrane fusion in the absence of other cellular components.

The aim of the present study is to obtain further information about fusion of plasma membranes isolated from myoblasts. As an approach to the understanding of underlying mechanisms, the fusion of myoblast membranes with respect to states of maturation and the influence of various enzymes, chemicals and inhibitors of protein biosynthesis has been studied.

## Materials and Methods

Preparation of muscle cell cultures and the isolation of myoblast membranes was performed as described previously [2,24,25]. With the exception of one series of experiments myoblasts were grown for 50 h in cultures before harvesting and fractionation. Fibroblasts were derived from 6-day-old muscle cell cultures after one transfer and plasma membranes were isolated by the same fractionation method used for the isolation of myoblast plasma membranes.

The methods for incubation and freeze-fracturing of the plasma membrane vesicle suspensions have been described previously [24]. Freeze-fracturing of intact myoblasts in culture has been performed according to the methods described by Pauli et al. [26].

Vesicle fusion was quantified by counting 500 vesicles from one incubation in a randomly selected scanned replica and expressed as the percentage of twinned vesicles as compared to the total number of vesicles. Twinned vesicles have been shown earlier to result from fusion of two vesicles [24]. All membrane preparations used were assayed for their percentage of fusion with no  $\text{Ca}^{2+}$  and with 1.4 mM  $\text{Ca}^{2+}$ . To compare different membrane preparations with varying fusion percentages, the fusion with 1.4 mM  $\text{Ca}^{2+}$  after 5 min at 37°C was set to 100%, and the data were related to this value as the “relative percent of fusion”.

Preincubation of myoblasts with cycloheximide (10  $\mu\text{g/ml}$ ) was performed by addition of this drug to the culture medium 3, 4 or 5 h prior to cell harvest. For cholesterol preincubation, half of the medium was collected, 2 mg/ml of cholesterol were added and the suspension was sonicated [3]. This suspension was added to the cultures for additional 5 h of incubation.

Enzymatic modification of the membranes was performed prior to the addition of  $\text{Ca}^{2+}$ . Vesicles (2 mg protein/ml) were preincubated in 0.25 M sucrose and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES),

pH 7.0, at 0°C for 30 min either with bovine trypsin (Boehringer Mannheim, G.R.F.), with pronase (*Streptomyces griseus*) (Serva Heidelberg, G.F.R.), with dispase (*Bacillus polymyxa*) (Boehringer) or with neuraminidase (*Clostridium perfringens*) (Boehringer) in concentrations specified under Results. Protein concentration was determined fluorometrically [60].

Cytochalasin B and cycloheximide were obtained from Serva. Purified cholesterol was a gift of Mr. Janko, Konstanz, G.F.R., Department of Biology. The ionophore A23187 was a gift of Eli Lilly GmbH, Bad Homburg, G.F.R. All other chemicals were of the purest grade commercially available.

## Results

### *Fusion of intact myoblasts and of isolated myoblast cell membranes*

Myotube formation from myoblasts which does not occur in cultures with  $\text{Ca}^{2+}$  concentrations below  $2 \cdot 10^{-5}$  M, can be triggered by rising the  $\text{Ca}^{2+}$  concentration to 1.4 mM [1,2]. Micrographs of freeze-fractured myoblast monolayers show that after addition of  $\text{Ca}^{2+}$ , individual cells contact each other (Fig. 1a) and multinucleated myotubes are formed. In the contact areas (Fig. 1b) gap junctions are frequently found. Aggregations of intramembranous particles have been observed also in preparations of membrane vesicles after the addition of  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  to the incubation medium [24].

Fusion of isolated membrane vesicles is characterized by the appearance of twinned vesicles (Fig. 2) [24]. The capacity of myoblasts to form myotubes with respect to time in culture was quantified and found to increase between 30 and 60 h [2]. Fusion competence of the corresponding myoblast membranes at these different states of maturation was examined by investigating the fusion of plasma membrane vesicles. Table I demonstrates that the percentage of fusion in 24 h myoblast membranes after the addition of 1.4 mM  $\text{Ca}^{2+}$  was no different from that without  $\text{Ca}^{2+}$ . However, in membrane preparations derived from myoblasts after 30, 50 or 60 h in culture,  $\text{Ca}^{2+}$ -specific fusion increases progressively. In contrast, no fusion was observed in membranes isolated from fibroblasts derived from the same primary cultures after the addition of  $\text{Ca}^{2+}$ .

TABLE I

FUSION OF PLASMA MEMBRANE VESICLES ISOLATED FROM MYOBLASTS OF DIFFERENT STATE OF MATURATION AND FROM FIBROBLASTS

Values are mean ( $\pm$ S.D. where  $n$  equals 3 or more). The number of membrane preparations studied is given in parentheses.

Cells (time in culture)	Percentage of fused vesicles	
	No $\text{Ca}^{2+}$ added	1.4 mM $\text{Ca}^{2+}$
Myoblasts (24 h)	1.3 (2)	2.4 (2)
Myoblasts (30 h)	$1.6 \pm 0.5$ (3)	$4.8 \pm 2.3$ (3)
Myoblasts (50 h)	$1.0 \pm 0.3$ (10)	$7.0 \pm 2.0$ (10)
Myoblasts (60 h)	0.6	8.8
Fibroblasts	2.2	2.6

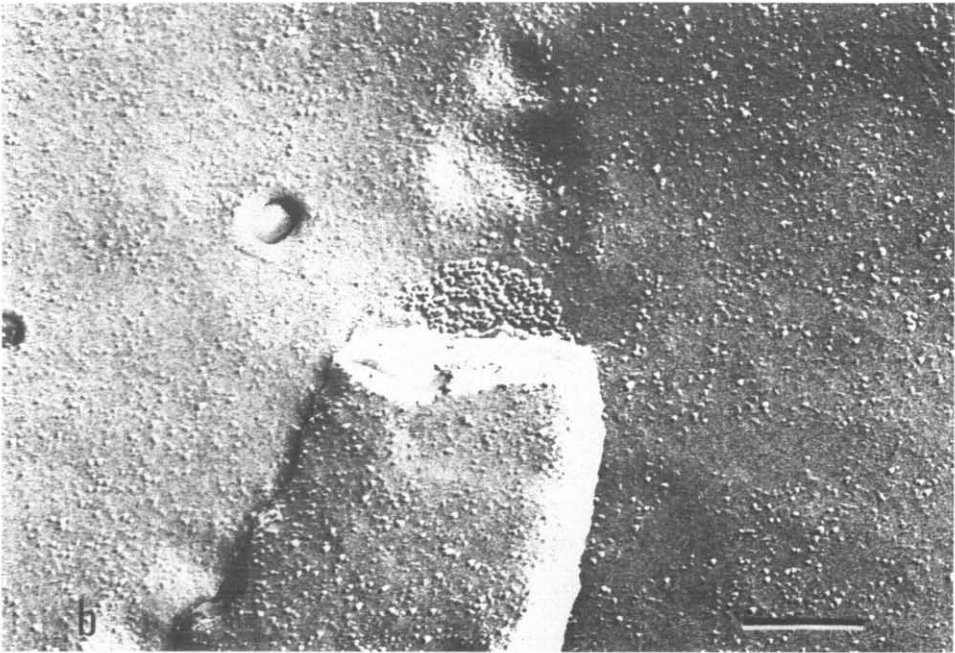
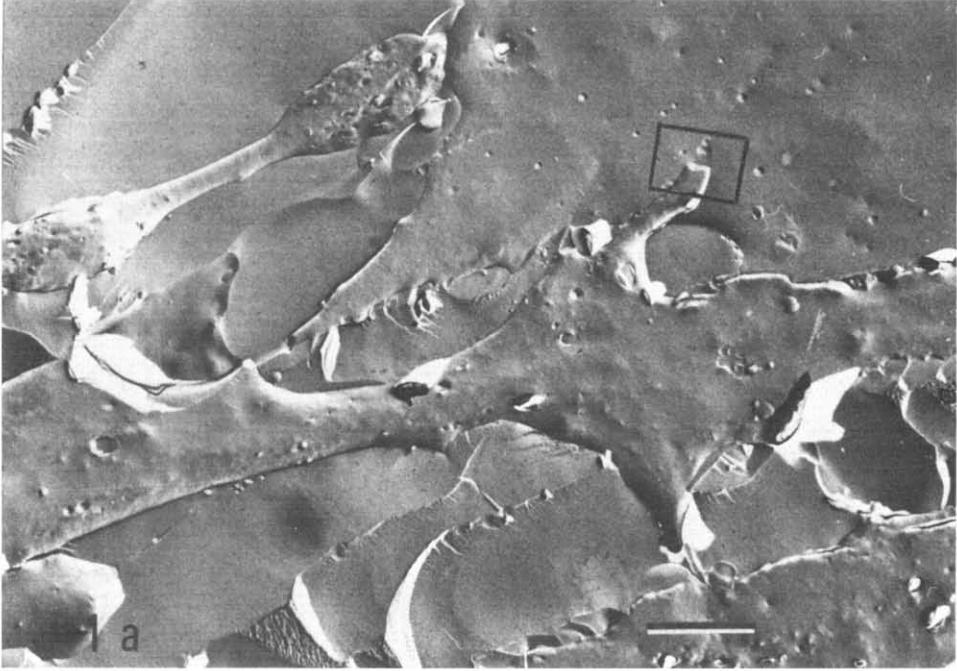


Fig. 1. Freeze-fracture electron micrograph of myoblasts in culture (50 h). 15 min after addition of 1.4 mM  $\text{Ca}^{2+}$ . Individual myoblasts contact each other (a). Higher magnification of the contact area reveals the presence of a gap junction between a finger-like process of one cell and the neighboring cell (b). Magnification: (a), 7200X, bar = 2  $\mu\text{m}$ ; (b), 80 000X, bar = 0.2  $\mu\text{m}$ .

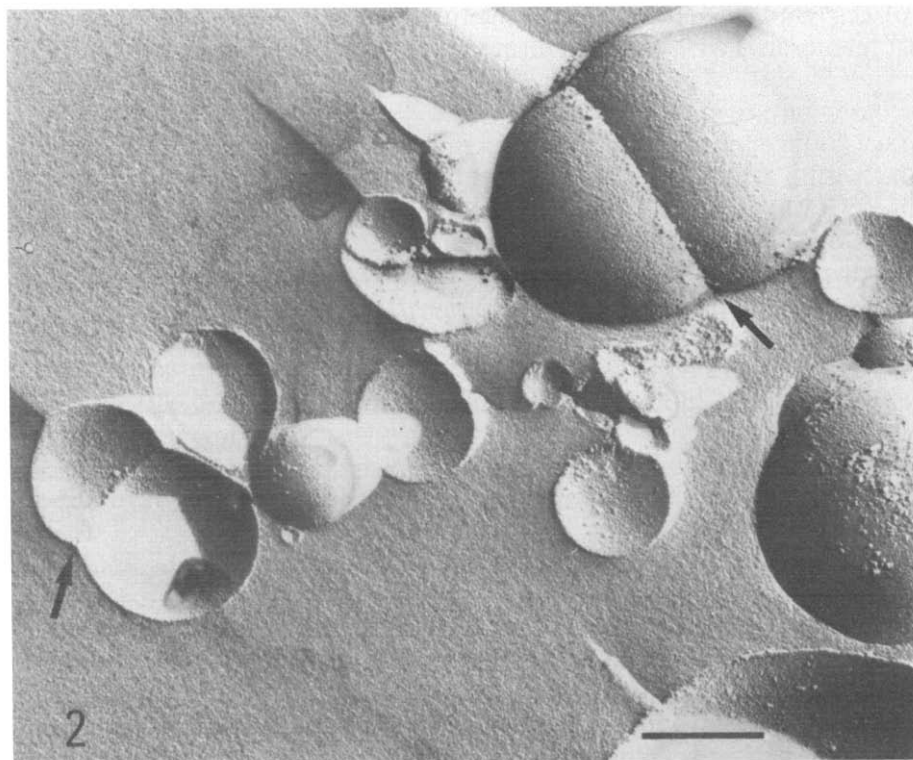


Fig. 2. Plasma membrane vesicles in response to 1.4 mM  $\text{Ca}^{2+}$  cluster, and contact each other; the twinned vesicles indicate fusion (arrows). Magnification: 80 000 $\times$ , bar = 0.2  $\mu\text{m}$ .

Plasma membranes can be characterized by the distribution of the intramembranous particles in EF- and PF-faces. The origin and the orientation of isolated cell membranes has been determined by analyzing the distribution of intramembranous particles in isolated membranes and in membranes of intact cells.

As shown in Table II the particle distribution was similar in plasma mem-

TABLE II

NUMBER OF INTRAMEMBRANOUS PARTICLES IN PLASMA MEMBRANES OF INTACT MYOBLASTS OF DIFFERENT STATE OF MATURATION AND IN ISOLATED MYOBLAST PLASMA MEMBRANES

Values are mean  $\pm$  S.E. The number of areas of 0.04  $\mu\text{m}^2$  for intact myoblasts or of 0.01  $\mu\text{m}^2$  for isolated vesicles evaluated is given in parentheses.

Time in culture	Intact myoblasts (particles per $\mu\text{m}^2$ )		Isolated vesicles (particles per $\mu\text{m}^2$ )	
	PF-face	EF-face	PF-face (convex)	EF-face (concave)
24 h	422 $\pm$ 25 (71)	134 $\pm$ 10 (35)	480 $\pm$ 220 (59)	170 $\pm$ 28 (29)
30 h	470 $\pm$ 16 (50)	151 $\pm$ 11 (62)	542 $\pm$ 270 (84)	180 $\pm$ 36 (15)
50 h	889 $\pm$ 39 (64)	186 $\pm$ 15 (38)	965 $\pm$ 305 (67)	220 $\pm$ 24 (21)

branes of cells and in isolated plasma membranes. Particle density increased in myoblast membranes with time in culture.

### *Kinetics, temperature and cation dependence*

The  $\text{Ca}^{2+}$ -induced fusion of plasma membrane vesicles at  $37^\circ\text{C}$  reached its maximum within 1 min (Fig. 3). At  $9^\circ\text{C}$  60% relative percentage of fusion (see Materials and Methods) was observed after 1 min of incubation. After a slow increase within 2 h the same maximum percentage of fusion observed at  $37^\circ\text{C}$  was reached.

The effect of temperature on  $\text{Ca}^{2+}$ -induced fusion is shown in Fig. 4. At various incubation times a consistent decrease of relative fusion percentages was observed by lowering the temperature.

$\text{Ca}^{2+}$ -specificity for induction of fusion is shown in Table III. Divalent cations were applied in concentrations, which have been shown for  $\text{Ca}^{2+}$  to be most effective for myoblast fusion [1–4].  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$  or  $\text{Ba}^{2+}$  at the same concentrations (1.4 mM) were unable to enhance the percentage of fused vesicles above control.

Fusion of isolated myoblast plasma membranes is enhanced by increasing the  $\text{Ca}^{2+}$  concentration up to 1.4 mM (Fig. 5). In contrast to myotube formation, fusion of myoblast membranes was further enhanced by  $\text{Ca}^{2+}$  concentrations exceeding 5 mM.  $\text{Mg}^{2+}$  or  $\text{Sr}^{2+}$ , which could not trigger fusion at 1.4 mM, were found to induce an initial fusion in parallel to the excess fusion caused by  $\text{Ca}^{2+}$ , with concentrations of each of these three cations above 5 mM (Fig. 5).

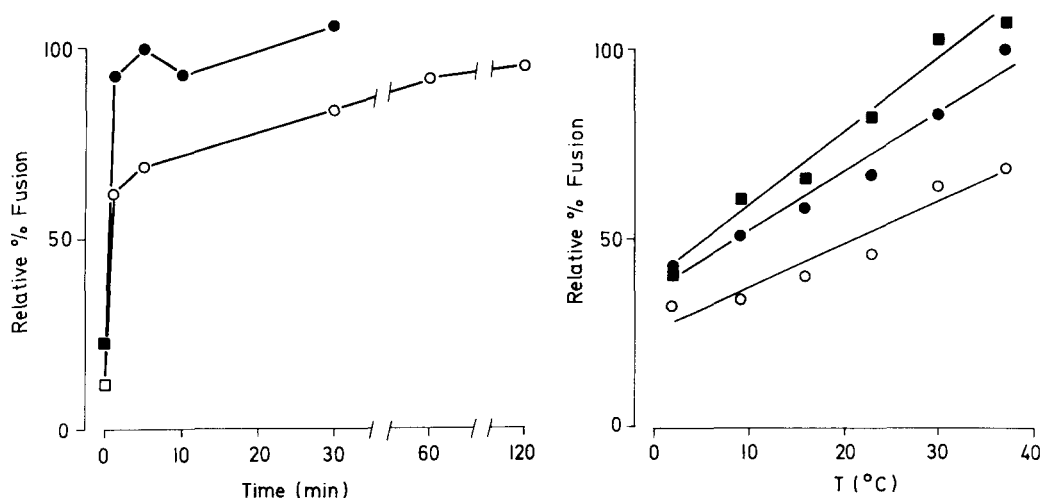


Fig. 3. Kinetics of vesicle fusion at  $9^\circ\text{C}$  (○) and  $37^\circ\text{C}$  (●), □ and ■ represent control prior to addition of  $\text{Ca}^{2+}$  at  $9^\circ\text{C}$ , and at  $37^\circ\text{C}$ . The values given are obtained from one membrane preparation. Another membrane preparation gave similar results.

Fig. 4. Vesicle fusion after 1 (○), 5 (●) and 30 min (■) of incubation as a function of temperature. Slopes are derived from regression analysis. The values given are obtained from one membrane preparation.

TABLE III

## CATION SPECIFICITY OF THE FUSION OF PLASMA MEMBRANE VESICLES ISOLATED FROM MYOBLASTS

Values are mean ( $\pm$ S.D. where  $n$  equals 3 or more). The number of membrane preparations studied is given in parentheses. The absolute percentage of fused vesicles with 1.4 mM  $\text{Ca}^{2+}$  (at  $37^\circ\text{C}$ , 5 min) counted in scanned replicas was  $7.0 \pm 2.0$  ( $n = 10$ ) and with no  $\text{Ca}^{2+}$  added  $1.0 \pm 0.3$  ( $n = 10$ ). Relative percentage fusion were obtained by setting the absolute percentage of fusion with 1.4 mM  $\text{Ca}^{2+}$  (at  $37^\circ\text{C}$ , 5 min) of each membrane preparation = 100%.

Cation	Relative % fusion
—	$14.3 \pm 4.3$ (10)
1.4 mM $\text{Ca}^{2+}$	100
1.4 mM $\text{Mg}^{2+}$	$16.0 \pm 2.7$ (3)
1.4 mM $\text{Sr}^{2+}$	$23.3 \pm 6.8$ (3)
1.4 mM $\text{Ba}^{2+}$	24.9 (2)

*Various agents interfering with vesicle fusion*

It has been suggested that contractile elements which are affected by cytochalasin B, are involved in fusion processes [27].  $\text{Ca}^{2+}$ -induced fusion of isolated plasma membranes was not affected by cytochalasin B at concentrations which have been shown to inhibit myotube formation in the monolayer [28,29]. In a control experiment the solvent  $\text{Me}_2\text{SO}$  was found to be slightly inhibitory (Table IV).

Dinitrophenol has often been used to test whether cellular activities require

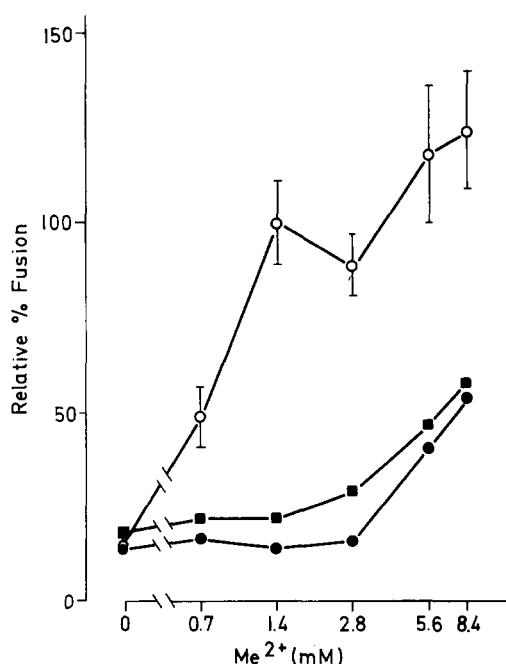


Fig. 5. Vesicle fusion as a function of  $\text{Ca}^{2+}$  (○),  $\text{Sr}^{2+}$  (■) or  $\text{Mg}^{2+}$  (●). The values given for  $\text{Ca}^{2+}$  are means obtained from three membrane preparations (mean  $\pm$  S.D.), those for  $\text{Sr}^{2+}$  and  $\text{Mg}^{2+}$  are from one membrane preparation.

TABLE IV

## EFFECT OF VARIOUS AGENTS ON FUSION OF ISOLATED MYOBLAST PLASMA MEMBRANES

Values are mean ( $\pm$ S.D. where  $n$  equals 3 or more). The number of membrane preparations studied is given in parentheses. Vesicles were preincubated with ionophore A23187, cytochalasin B,  $\text{Me}_2\text{SO}$  or dinitrophenol for 10 min at  $37^\circ\text{C}$ . Cholesterol (1 mg/ml) was added to the culture for 5 h as described [2].

Additives	Relative % fusion		
	No $\text{Ca}^{2+}$ added	0.14 mM $\text{Ca}^{2+}$	1.4 mM $\text{Ca}^{2+}$
None	14.6 $\pm$ 4.8 (5)	30.4 (1)	100 (5)
A23187 ( $10^{-6}$ M)	21.7 (1)	34.7 (1)	—
Cytochalasin B (5 $\mu\text{g}/\text{ml}$ ) + $\text{Me}_2\text{SO}$ (0.2%, v/v)	—	—	79.5 (2)
$\text{Me}_2\text{SO}$ (0.2%, v/v)	—	—	77.8 $\pm$ 8.0 (3)
Dinitrophenol ( $10^{-4}$ M)	—	—	48.6 (2)
Dinitrophenol ( $10^{-5}$ M)	—	—	64.8 (1)
Cholesterol	—	—	24.0 (1)

ATP [13,30–33]. The effect of dinitrophenol on the level of membrane fusion was examined. Dinitrophenol was found to reduce vesicle fusion at  $10^{-5}$  M and, to a greater extent, at  $10^{-4}$  M (Table IV).

The ionophore A23187 is known to increase  $\text{Ca}^{2+}$  permeability of membranes [34]. As shown for myotube formation [4] vesicle fusion was not increased at suboptimal  $\text{Ca}^{2+}$ -concentrations in the presence of A23187 (Table IV).

Plasma membranes were also isolated from myoblasts which had been preincubated with cholesterol under otherwise normal culture conditions [3].  $\text{Ca}^{2+}$ -induced fusion was almost abolished (Table IV). This is consistent with the finding of inhibition of myotube formation by cholesterol [3].

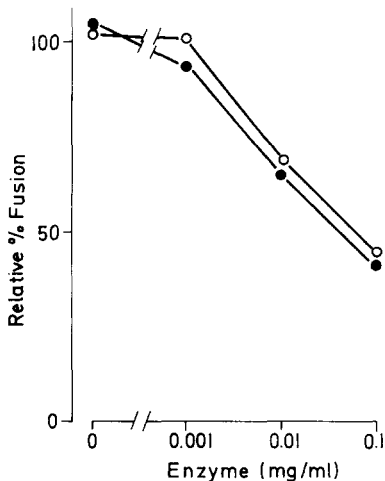


Fig. 6. Effect of neuraminidase (○) and trypsin (●) on vesicle fusion induced by 1.4 mM  $\text{Ca}^{2+}$ . Controls were carried out with heat inactivated enzymes. The values were obtained with one membrane preparation.

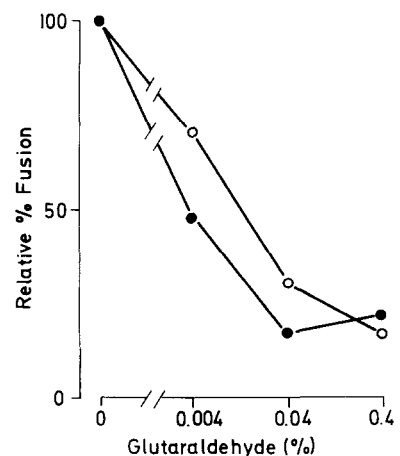


Fig. 7. Effect of glutaraldehyde (5 min, at  $37^\circ\text{C}$ ) on vesicle fusion induced by 1.4 mM  $\text{Ca}^{2+}$  with (●) and without (○) preincubation with 1.4 mM  $\text{Mg}^{2+}$ . The values were obtained with one membrane preparation.



TABLE V

EFFECT OF CYCLOHEXIMIDE ON FUSION OF ISOLATED MYOBLAST PLASMA MEMBRANES

Time of cycloheximide (10 $\mu$ g/ml) addition prior to harvest (h)	Relative % fusion	
	No $\text{Ca}^{2+}$ added	1.4 mM $\text{Ca}^{2+}$
0	16	100
3	20	54
4	22	44
5	23	43
Control *	—	$89 \pm 8.5$ (3)

\* Isolated membranes were preincubated with cycloheximide (10  $\mu$ g/ml) at 37 °C for 5 min before addition of  $\text{Ca}^{2+}$ .

### *Fusion after modification of plasma membrane vesicles*

In order to obtain information about membrane components involved in the fusion process, the isolated membrane vesicles have been chemically modified. Pretreatment of membrane vesicles with trypsin or neuraminidase reduced fusion with increasing enzyme concentrations (Fig. 6). Pronase and dispase were also found to diminish fusion (data not shown). Similarly, prefixation of membrane vesicles in increasing concentrations of glutaraldehyde (5 min, 37 °C) decreased  $\text{Ca}^{2+}$ -induced fusion (Fig. 7). The inhibitory effect of glutaraldehyde on  $\text{Ca}^{2+}$ -induced fusion could not be abolished by pretreatment of vesicles with 1.4 mM  $\text{Mg}^{2+}$ , which results in vesicle aggregation. In this case, the percentage of fused vesicles is further reduced (Fig. 7) which may be due to the antagonistic effect of  $\text{Mg}^{2+}$  on  $\text{Ca}^{2+}$ -induced fusion [24].

To examine the possibility that proteins are involved in the fusion process of myoblast membranes, protein synthesis was inhibited by cycloheximide. The fusion of cell membrane vesicles isolated from cultures pretreated with cycloheximide was found to be reduced (Table V). Inhibition increased with prolonged incubation with cycloheximide (Table V). Also, myotube formation is remarkably decreased if protein synthesis is inhibited by cycloheximide [35,36]. Myotube formation is also inhibited in the presence of puromycin [36].

## Discussion

Plasma membrane vesicles derived from myoblast cell cultures can be induced to fuse [24]. This model system for membrane fusion during myotube formation has been further characterized. The major findings were related to: (a) the influence of various agents, (b) the dependence of time and temperature, (c) the cation specificity, (d) the involvement of proteins in this process, and (e) the increasing fusion capacity of isolated membrane vesicles during myoblast maturation.

Cytochalasin B, a drug interacting with microfilaments, is known to inhibit myotube formation [28,29].  $\text{Ca}^{2+}$ -induced fusion of isolated plasma membranes, however, was insensitive to this drug (Table IV). Thus, the process of membrane fusion does not seem to require the participation of cytochalasin B-sensitive elements, in contrast to that of myotube formation. Dinitrophenol,

which uncouples oxidative phosphorylation, has been used to examine the ATP-requirement of endocytosis [37] and cell fusion [30,32]. Whereas the basic process of fusion occurs in the absence of ATP, dinitrophenol was found to depress fusion of isolated plasma membranes in this study. Therefore, the inhibitory effects of dinitrophenol on fusion of cells and isolated vesicles can be attributed to an effect on cellular ATP-levels and also to a direct interaction with fusing membranes.

The process of membrane fusion was so rapid at 37°C that its kinetics could not be resolved with the techniques used. At lower temperatures a two-phase velocity curve was obtained (Fig. 3). Measurements of fusion after 1, 5 and 30 min of incubation revealed linear relationships between fusion and temperature with different slopes. Abrupt changes in the slope, which might indicate thermotropic transitions linked to the fusion process, were not detected in these experiments.

The absence of thermotropic transitions between 5 and 37°C has also been observed [38] measuring fluorescence polarization of suspended myoblasts. In a previous paper [39], however, it was shown that addition of  $\text{Ca}^{2+}$  to  $\text{Ca}^{2+}$ -deprived myoblasts or myoblast membranes resulted in a transiently increased polarization of the fluorescent dye [39]. This was interpreted by the production of membrane domains of lower and higher rigidity induced by  $\text{Ca}^{2+}$ . A similar lateral phase separation has been observed to be associated with phagocytosis [40]. Apparently, these different domains are stable in the presence of  $\text{Ca}^{2+}$  at temperatures between 2 and 37°C, and the thermally-induced mixing of these different phases might therefore occur only at temperatures above 37°C. Such high transition temperatures (60–70°C) have been observed with acidic phospholipid mixtures in the presence of  $\text{Ca}^{2+}$  [41–43].

Thus, the  $\text{Ca}^{2+}$ -dependent existence of different membrane domains (which mix only above 37°C) could be relevant for membrane fusion. This hypothesis is further supported by the sensitivity of fusion against cholesterol (Table IV) and lysophosphatidylcholine [24], agents which interfere with phase transitions and phase separations [58,59].

As shown previously, [3–5,24], 1.4 mM  $\text{Ca}^{2+}$  can induce fusion of isolated cell membranes *in vitro* as well as myotube formation in culture. At the same concentration as  $\text{Ca}^{2+}$ , other divalent cations such as  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$  or  $\text{Ba}^{2+}$  failed to induce both myotube formation [5] and fusion of myoblast cell membranes. The optimum  $\text{Ca}^{2+}$  concentration for myotube formation was found to be 1.4 mM; there was inhibition at concentrations above 2.8 mM. In contrast, fusion of isolated myoblast cell membranes was enhanced by  $\text{Ca}^{2+}$  at concentrations above 2.8 mM. At these high concentrations  $\text{Sr}^{2+}$  and  $\text{Mg}^{2+}$  were also effective. Thus, two types of fusion can be distinguished: one type is specifically triggered by  $\text{Ca}^{2+}$  at physiological concentration and a second type is characterized by a non-specific requirement for divalent cations at higher concentrations. Two types of fusion have been also detected for isolated secretory vesicles: a  $\text{Ca}^{2+}$ -specific type requiring cytoplasmic concentration ( $10^{-6}$  M) and a cation-unspecific type requiring  $5 \cdot 10^{-3}$  M divalent cations [19].

The addition of  $\text{Ca}^{2+}$ -ionophore did not increase either the fusion of plasma membrane vesicles or myotube formation in culture [4]. Since the ionophore is believed to increase intravesicular  $\text{Ca}^{2+}$  concentrations, this suggests that mem-

brane components interacting specifically with  $\text{Ca}^{2+}$  are exposed to the extracellular space.

$\text{Ca}^{2+}$  has been used to trigger myotube formation as well as fusion of isolated plasma membranes derived from myoblasts grown at low  $\text{Ca}^{2+}$  concentrations. In vivo, however, the  $\text{Ca}^{2+}$  concentrations of the extracellular fluid is constant. Therefore, although  $\text{Ca}^{2+}$  is essential, this ion cannot have a regulatory function in cell fusion in contrast to its role in secretory processes [52]. Thus, intrinsic membrane components obviously are responsible for rendering myoblast membranes competent for fusion. It has been observed that competence for myotube formation increases with myoblast maturation [2]. This is consistent with the demonstration of increased fusion of plasma membrane vesicles isolated from more mature myoblasts (Table I).

The correlation between increasing fusion capacity and density of intramembranous particles, must not necessarily reflect an essential function of these particles in the fusion mechanism. The role of intramembranous particles in the process of membrane contact and fusion is a matter of debate [19–24, 45–50, 53–57].

In the present study membranes treated with glutaraldehyde or modified with proteolytic enzymes or neuraminidase exhibit reduced ability for fusion. On the contrary, liposome fusion with glutaraldehyde-fixed cells [13,14,37], liposome-liposome fusion (Ekerdt, R., Dahl, G. and Gratzl, M., in preparation) and phospholipid mobility [51] in the presence of glutaraldehyde are either unaffected or only slightly impaired. The experiments with chemically and enzymatically modified membranes indicate that proteinaceous membrane components participate in the fusion of myoblast cell membranes. The essential role of proteins in this fusion process is further supported by the reduction of fusion following inhibition of protein synthesis. The precise function played by proteins in the fusion of biological membranes remains to be elucidated.

In conclusion it is suggested that during maturation of the myogenic cell special protein(s) are synthesized and inserted into the plasma membrane, which render this membrane competent for fusion. Specific interactions on the outer cell surface with  $\text{Ca}^{2+}$  induce a heterogeneous distribution of membrane components. The ionogenic formations of such domains is possible at all temperatures between 2 and 37°C. In the areas of vesicle or cell contact which in the latter case was found to be formed and solved frequently, additional transmembrane interactions between  $\text{Ca}^{2+}$ -induced domains initiate the formation of the primary pore.

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