The breakdown of phosphatidylinositol in myoblasts stimulated to fuse by the addition of Ca$$^{2+}$$

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(Received 8 October 1981/Accepted 25 November 1981)

1. The fusion of chick-embryo myoblasts to produce myotubes was studied. The myoblasts were grown for 50 h in medium containing 10–20$$\mu$$M-Ca$$^{2+}$$; during this period they achieve fusion competence. 2. A rapid breakdown of phosphatidylinositol is observed on addition of 1.4$$\mu$$M-Ca$$^{2+}$$ to these cells. This Ca$$^{2+}$$ concentration also stimulates rapid myoblast fusion. 3. The breakdown is complete within 15 min and shows the same dependence on Ca$$^{2+}$$ concentration as the fusion process. 4. Fusion-incompetent myoblasts and cells where fusion is inhibited by sodium butyrate exhibit no phosphatidylinositol breakdown on Ca$$^{2+}$$ addition. 5. The Ca$$^{2+}$$ ionophore A23187 inhibits the Ca$$^{2+}$$-stimulated breakdown by about 50%, but has no effect on fusion. 6. A concomitant increase in 1,2-diacylglycerol labelled and fall in phosphatidylinositol labelling was observed when the lipids were labelling with [14C]glycerol on increasing the Ca$$^{2+}$$ concentration in the medium to 1.4$$\mu$$M. 7. We propose that the breakdown of phosphatidylinositol with a resultant increase in 1,2-diacylglycerol content of the cell membrane promotes myoblast fusion.

Differentiated skeletal muscle is derived from multinucleated myotubes, which are formed by the cytoplasmic fusion of post-mitotic myoblasts. Myoblast fusion can be achieved in vitro; freshly isolated myoblasts go through at least one round of DNA replication (Stockdale & Holtzer, 1961; Yaffe, 1971), withdraw from the cell cycle, acquire the typical spindle shape of myoblasts, establish contacts and fuse.

The fusion of myoblasts has been much studied without any clear picture of the process emerging. What is clear is that this fusion has an essential requirement for Ca$$^{2+}$$ (Shainberg et al., 1971). The involvement of Ca$$^{2+}$$ in the fusion of biological membranes appears to be universal (see Papahadjopoulos, 1978). Schudt et al. (1973) have shown that other cations (Mg$$^{2+}$$, Mn$$^{2+}$$, Ba$$^{2+}$$, Zn$$^{2+}$$, Cd$$^{2+}$$, Cu$$^{2+}$$, Li$$^{2+}$$, K$$^{+}$$) inhibit the Ca$$^{2+}$$-promoted fusion, whereas Sr$$^{2+}$$ was non-inhibitory and at higher concentrations (2.4$$\mu$$M) could replace Ca$$^{2+}$$ in vitro.

The role of Ca$$^{2+}$$ in the fusion process is unclear. Schudt & Pette (1975) showed that the Ca$$^{2+}$$ ionophore A23187 when added to 50h cultured myoblasts had no effect on fusion; they therefore suggested that the entry of Ca$$^{2+}$$ into the myoblasts is unnecessary. David et al. (1981) have shown, however, that entry of Ca$$^{2+}$$ into the myoblasts occurs before fusion. Michell (1975) proposed that processes which involve an increase in intracellular Ca$$^{2+}$$ concentration, e.g. stimulation of rat parotid gland by$$\alpha$$-adrenergic stimuli, are associated with the breakdown of the anionic membrane phospholipid phosphatidylinositol. It has also been proposed that breakdown of inositol phospholipids is central to membrane fusion in stimulated secretory cells (Allan & Michell, 1979).

We therefore decided to study the metabolism of this lipid during myoblast fusion. This study has made use of myoblasts that have been grown for 50 h in a medium containing less than 20$$\mu$$M-Ca$$^{2+}$$. Myoblasts grow normally in such a medium and after 50 h are fusion competent. They rapidly fuse when the Ca$$^{2+}$$ concentration in the medium is raised to 1.4$$\mu$$M (van der Bosch et al., 1972), and this system has the advantage that fusion is synchronous throughout the culture. Some of these results have been presented in a preliminary form (Wakelam & Pette, 1981).

Materials and methods

Isolation and use of myoblasts

Cells were prepared by a trypsin digestion of 12-day chick-embryo breast muscles as described previously (van der Bosch et al., 1972). They were seeded in 12.5 cm-diameter gelatin-coated dishes in
Dulbecco’s minimum essential medium (Seromed) containing vitamins and essential amino acids. This was supplemented with 10% (v/v) essentially Ca²⁺-free dialysed horse serum (GIBCO) and 5% (v/v) essentially Ca²⁺-free dialysed embryo extract prepared from 12-day chicken embryos; the Ca²⁺ concentration was between 10 and 20 μM. The dishes were maintained in a sterile incubator at 37°C under air/CO₂ (23:2).

In experiments where [2-³H]myo-inositol or [2-¹⁴C]glycerol was used, the medium was half-changed after 24 h and medium containing label added to give 1 μCi of [2-³H]myo-inositol (5 Ci/mmol) per plate or 1.5 μCi of [2-¹⁴C]glycerol (5 mCi/mmol) per plate. At 25 h later, a time that was shown to permit equilibrium labelling, this medium was removed and the cells were incubated in a fresh medium for 1 h containing no label. Then 1 h later the Ca²⁺ concentration was raised (where specified) by the addition of CaCl₂. Incubations then proceeded for the stated times. Where [³²P]P₁ incorporation was studied, the prelabelling was omitted and approx. 50 μCi of [³²P]P₁ (carrier-free) per plate was added after 50 h in culture; at the same time as the Ca²⁺ concentration was increased where appropriate.

At the end of incubations, plates were removed from the incubator, the medium was rapidly removed, the plates were washed with ice-cold saline and then 'stopped' with ice-cold 10% (w/v) trichloroacetic acid.

**Extraction and measurement of lipids**

The trichloroacetic acid/cell mixtures were transferred to tubes containing 3.75 vol. of chloroform/methanol (1:2, v/v) and the lipids were extracted by the method of Lapetina & Michell (1972). Phospholipids were separated by two-dimensional t.l.c. on Na₂CO₃-impregnated silica-gel plates (Abramson & Blecher, 1964). The developing solvents were chloroform/methanol/acetic acid/water (250:74:19:3, by vol.) followed by chloroform/methanol/7M-NH₃ (46:18:3, by vol.). The plates were dried in a stream of air between solvents. Phospholipids were identified with the help of standards run on parallel plates. The standards were first purified by unidimensional preparative t.l.c. on silica-gel plates with chloroform/methanol/acetic acid/water (130:50:16:7, by vol.) as a solvent. The separated phospholipids were detected with I₂ vapour and their positions marked on the plate.

Where 1,2-diacylglycerol was measured, the lipid mixture was first separated by t.l.c. by using two solvents successively in the same direction (Freeman & West, 1966). The first solvent was diethyl ether/benzene/ethanol/acetic acid (200:250:10:1, by vol.) and the second diethyl ether/hexane (3:47, v/v). The phospholipids, which do not migrate from the origin in this system, were eluted (as below) and then run as above.

Spots were scraped from plates after the I₂ had resublimed. The gel was scraped directly into scintillation vials, when lipids had been labelled with ³²P or ¹⁴C, and radioactivity was measured by liquid-scintillation spectrophotometry (Kirk et al., 1981). Radioactivity in ³H-labelled phospholipids was determined by the same means after elution from the silica gel with chloroform/methanol/conc. NH₃ (sp.gr. 0.88) (50:50:1, by vol.) (Kirk et al., 1981). Initial experiments indicated that approx. 98% of the radioactivity in phospholipids labelled with [2-³H]-myo-inositol was located in phosphatidylinositol. Therefore the loss of radioactivity from the total cell lipid fraction was taken as a measure of phosphatidylinositol degradation.

**Measurement of protein**

All results are related to total cellular protein. This was determined in the trichloroacetic acid precipitate, after lipid extraction, by the method of Bradford (1976), with bovine serum albumin fraction V (Sigma) as a standard.

**Measurement of cell fusion**

Cultures were treated in their dishes with Giemsa’s stain after methanol fixation at the time stated. Fusion percentages were determined by counting at least 800 nuclei per dish and equal: (number of nuclei in myotubes/total number of nuclei) × 100. Only cells containing at least three nuclei were deemed myotubes.

**Expression of results**

All results are expressed as means ± S.E.M. Statistical analysis was performed by Student’s t-test. Significance of results from control values are expressed: *P < 0.01; **P < 0.001.

**Materials**

Culture media were from Seromed G.m.b.H., 8000 München 71, Germany, except for horse serum, which was from GIBCO G.m.b.H., 7500 Karlsruhe 21, Germany. Trypsin was from Flow Laboratories G.m.b.H., 5300 Bonn 1, Germany. Phospholipid and lipid standards were from Sigma G.m.b.H., München, Germany. Radiochemicals were from Amersham Buchler G.m.b.H., 3300 Braunschweig, Germany. All other chemicals were of the highest grade available from Merck G.m.b.H., 6100 Darmstadt 1, Germany.

**Results**

Fig. 1 shows the incorporation of [³²P]P₁ into five myoblast phospholipids over a 60 min period. In cells cultured in a low-Ca²⁺-containing medium,
Fig. 1. Effect of Ca$^{2+}$ on the incorporation of [$^{32}$P]P$_i$ into five myoblast phospholipids
Myoblasts were cultured for 50h. [$^{32}$P]P$_i$ was added to all plates and the Ca$^{2+}$ concentration was increased to 1.4 mM where stated. At the stated time cells were washed, 'stopped' by the addition of 10% (v/v) trichloroacetic acid, lipids were extracted, separated and radioactivity was determined as described in the Materials and methods section.

○, No Ca$^{2+}$ added ($n = 4$); ○, Ca$^{2+}$ concn. raised to 1.4 mM ($n = 6$). Bars represent ± S.E.M.

incorporation of [$^{32}$P]P$_i$ was observed for all five lipids. A high degree of incorporation was observed only for phosphatidylethanolamine; raising the Ca$^{2+}$ concentration in the medium to 1.4 mM had no effect on this incorporation. Unlike the other phospholipids examined, a large increase in labelling was observed for phosphatidylinositol when Ca$^{2+}$ was added to the medium. This increase was not significant until after 15min, but from then on continued rapidly. This increased labelling is suggestive of an enhanced synthesis of phosphatidylinositol, which in turn is probably due to increased turnover of the molecule.

Raising the Ca$^{2+}$ concentration of the culture medium also results in a loss of radioactivity from the phosphatidylinositol of 50h myoblasts prelabelled with [2-$^3$H]myo-inositol. Fig. 2 shows a time course of this effect. The breakdown is statistically significant after 10min and is complete after about 15min. The percentage breakdown varied from one preparation to another, normally being 20–30%, so a standard control of 1.4 mM-Ca$^{2+}$ for 30min was
included in all subsequent experiments. This variation is due to slight differences in cell concentration because of different numbers of cell divisions undergone by the cultures after plating and a consequent variation in the number of myoblasts that are aligned and therefore fusion competent.

The breakdown of phosphatidylinositol in these cells is either dependent on or promoted by Ca$^{2+}$. The concentration curve for Ca$^{2+}$ against phosphatidylinositol breakdown is very similar to that against fusion (Fig. 3). Schudt et al. (1973) showed that Sr$^{2+}$ was the only cation that could replace Ca$^{2+}$ in the fusion process and that others, e.g. Mg$^{2+}$, were inhibitory; we therefore examined the effect of these two bivalent cations on phosphatidylinositol breakdown and fusion. Table 1 shows that Sr$^{2+}$ can replace Ca$^{2+}$ both in stimulating fusion and in stimulating the breakdown of phosphatidylinositol; Mg$^{2+}$ fails to produce either effect and inhibits both fusion and breakdown of the lipid stimulated by Ca$^{2+}$. The effect of another inhibitor of myoblast fusion, sodium butyrate (Fiszman et al., 1980), was also examined. Table 2 shows again that when fusion is inhibited no breakdown of phosphatidylinositol is observed.

In view of the role for Ca$^{2+}$ in these processes, the effect of the Ca$^{2+}$ ionophore A23187 was examined. Table 3 shows that incubation in the presence of 5$\mu$g of ionophore A23187/ml has no effect on fusion, but about halves the breakdown of phosphatidylinositol in response to 1.4 mM-Ca$^{2+}$.

Myoblasts derived from 12-day chick embryos do not fuse immediately when cultured in a medium containing 1.4 mM-Ca$^{2+}$. We therefore examined the effect of raising the Ca$^{2+}$ concentration in the medium, before the cells are capable of fusing, on phosphatidylinositol. Table 4 shows that no breakdown of phosphatidylinositol and no fusion are observed when 1.4 mM-Ca$^{2+}$ is added to plates containing 24 h-old myoblasts; normal fusion and breakdown are observed when 1.4 mM-Ca$^{2+}$ is added to 50 h myoblasts from the same preparation.

The breakdown of phosphatidylinositol, in eukaryotic cells, in response to stimuli such as hormones and neurotransmitters is catalysed by a phosphatidylinositol-specific phospholipase C (EC
Phosphatidylinositol and myoblast fusion

Table 1. Effect of Sr$^{2+}$ and Mg$^{2+}$ on Ca$^{2+}$-stimulated myoblast fusion and phosphatidylinositol breakdown
For this study, 50h-cultured, pre-labelled myoblasts (see the Materials and methods section) were incubated with the stated additions for 30min. They were then washed and 'stopped' by the addition of 10% (v/v) trichloroacetic acid. Lipids were extracted and phosphatidylinositol breakdown was determined as described in the Materials and methods section. Parallel dishes were cultured under the same conditions for a further 24h and the fusion percentage was determined. Results are expressed as means ± s.e.m.; n = 5 in each case. Significance of difference from control: *P < 0.01; **P < 0.001.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Fusion</th>
<th>Phosphatidylinositol breakdown (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>1.0 ± 0.8</td>
</tr>
<tr>
<td>1.4 mM-Ca$^{2+}$</td>
<td>Normal</td>
<td>12.8 ± 1.1**</td>
</tr>
<tr>
<td>20 mM-Mg$^{2+}$</td>
<td>None</td>
<td>0.5 ± 1.5</td>
</tr>
<tr>
<td>2.4 mM-Sr$^{2+}$</td>
<td>Normal</td>
<td>12.5 ± 2.6*</td>
</tr>
<tr>
<td>1.4 mM-Ca$^{2+}$ + 20 mM-Mg$^{2+}$</td>
<td>None</td>
<td>1.4 ± 1.8</td>
</tr>
</tbody>
</table>

Table 2. Effect of sodium butyrate on myoblast fusion and phosphatidylinositol breakdown
Where stated, 5mM-sodium butyrate and 1.4 mM-Ca$^{2+}$ were added to pre-labelled myoblast cultures. Incubations were 'stopped' after 30min. All other details were as in the legend to Table 1; n = 6 in each case.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Fusion</th>
<th>Phosphatidylinositol breakdown (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>1.6 ± 1.4</td>
</tr>
<tr>
<td>1.4 mM-Ca$^{2+}$</td>
<td>Normal</td>
<td>17.2 ± 2.7**</td>
</tr>
<tr>
<td>1.4 mM-Ca$^{2+}$ + 5 mM-sodium butyrate</td>
<td>None</td>
<td>2.3 ± 2.4</td>
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</table>

Table 3. Effect of ionophore A23187 on myoblast fusion and phosphatidylinositol breakdown
Where stated, 1.4 mM-Ca$^{2+}$, ionophore A23187 (in ethanol) or ethanol were added at the same time to pre-labelled myoblasts; incubations were 'stopped' after 30min with 10% (v/v) trichloroacetic acid. All other details were as in the legend to Table 1; n = 15 in each case.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Fusion</th>
<th>Phosphatidylinositol breakdown (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td>1.4 mM-Ca$^{2+}$</td>
<td>Normal</td>
<td>25.3 ± 2.7**</td>
</tr>
<tr>
<td>1.4 mM-Ca$^{2+}$ + A23187 (5 µg/ml)</td>
<td>Normal</td>
<td>13.9 ± 3.5**</td>
</tr>
<tr>
<td>1.4 mM-Ca$^{2+}$ + 0.1% (v/v) ethanol</td>
<td>Normal</td>
<td>26.4 ± 2.9**</td>
</tr>
<tr>
<td>0.1% (v/v) Ethanol</td>
<td>None</td>
<td>1.8 ± 1.9</td>
</tr>
<tr>
<td>A23187 (5 µg/ml)</td>
<td>None</td>
<td>0.9 ± 1.2</td>
</tr>
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</table>

Table 4. Effect of age of myoblasts on cell fusion and phosphatidylinositol breakdown
Myoblasts from the same preparation were labelled for 24h before use with [2-3H]myo-inositol as described in the Materials and methods section; 1.4 mM-Ca$^{2+}$ was added where stated and incubations were 'stopped' after 30min. All other details were as in the legend to Table 1; n = 10 in each case.

<table>
<thead>
<tr>
<th>Cell age</th>
<th>25h</th>
<th>50h</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Fusion</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>(b) Phosphatidylinositol breakdown (%)</td>
<td>2.0 ± 1.9</td>
<td>1.1 ± 1.4</td>
</tr>
<tr>
<td>+1.4 Ca$^{2+}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Fusion</td>
<td>None</td>
<td>Normal</td>
</tr>
<tr>
<td>(b) Phosphatidylinositol breakdown (%)</td>
<td>1.3 ± 1.4</td>
<td>27.1 ± 4.7**</td>
</tr>
</tbody>
</table>

3.1.4.10) (see Michell & Kirk, 1981). The product of this reaction is 1,2-diacylglycerol, which is then phosphorylated to yield phosphatidic acid. Table 5 shows the results of an experiment which examined the loss of radioactivity from [2-14C]glycerol-labelled phosphatidylinositol and the change in [2-14C]-glycerol-labelling of 1,2-diacylglycerol. A Ca$^{2+}$-stimulation time of 30min was chosen since it is only after this time that significant increases in myoblast fusion are observed. Table 5 shows that breakdown of phosphatidylinositol is also observed when the molecule is labelled with [2-14C]-glycerol, and that a
small but significant increase in the labelling of 1,2-diacylglycerol is observed after incubation of myoblasts at 1.4 mM-Ca\(^{2+}\) for 30 min.

**Discussion**

The results reported in the present paper show that the initiation of fusion, in fusion-competent myoblasts, by raising the Ca\(^{2+}\) concentration in the medium to 1.4 mM is accompanied by the breakdown of phosphatidylinositol. This breakdown appears to be complete after 15 min (Fig. 2), which is before significant myoblast fusion has occurred; after 30 min fusion is 5% complete, but increases rapidly thereafter (van der Bosch *et al.*, 1972).

The breakdown of phosphatidylinositol is followed by what appears to be compensatory resynthesis (Fig. 1). Fig. 1 also shows that the incorporation of \[^{32}P\]\(P_1\) into other myoblast phospholipids is unaffected by raising the Ca\(^{2+}\) concentration in the medium. This would suggest that the only phospholipid affected by Ca\(^{2+}\) is phosphatidylinositol.

We suggest that this breakdown is causally related to fusion for the following reasons. (1) Breakdown is not observed in Ca\(^{2+}\)-stimulated fusion-incompetent myoblasts (Table 4). (2) It is not observed in myoblasts where fusion is inhibited by sodium butyrate (Table 2). (3) Sr\(^{2+}\), which can replace Ca\(^{2+}\) in stimulating fusion, also stimulates phosphatidylinositol breakdown. Mg\(^{2+}\), which cannot stimulate fusion, produces no breakdown and inhibits both Ca\(^{2+}\)-stimulated fusion and phosphatidylinositol breakdown (Table 1). (4) The Ca\(^{2+}\)-concentration-dependence of fusion and phosphatidylinositol breakdown are essentially the same (Fig. 3).

The Ca\(^{2+}\)-stimulated breakdown of phosphatidylinositol is followed by what appears to be compensatory resynthesis of the lipid. This is shown by the stimulated incorporation of \[^{32}P\]\(P_1\) into phosphatidylinositol seen in Fig. 1. This process shows a short time lag, and it is therefore possible that the first product of phosphatidylinositol breakdown initially accumulates. The data in Table 5 support this view. Although not totally convincing, the loss of radioactivity from \[^{14}C\]glycerol-labelled phosphatidylinositol and the observed gain in radioactivity in 1,2-diacylglycerol, when taken with the data in Fig. 1, which show slow turnover by lipids other than phosphatidylinositol, would suggest that the increase in 1,2-diacylglycerol labelling is due to phosphatidylinositol breakdown.

The breakdown of phosphatidylinositol and an increase in 1,2-diacylglycerol concentration of myoblasts would correspond, if located at the plasma membrane, to a change from a highly polar anionic environment to a hydrophobic uncharged area. We suggest that this change is important for fusion. Papahadjopoulos *et al.* (1976) have shown that vesicle fusion induced by bivalent cations requires the lipids of the interacting membranes to be in a 'fluid' state. Such a change appears to be essential for myoblast fusion also. Prives & Shinitzky (1977) showed a sharp fall in membrane viscosity just before, and a regeneration of membrane rigidity after, fusion. Herman & Fernandez (1977) also showed an increase in membrane fluidity and provided some evidence suggesting that areas of cell contact between fusing cells exhibit higher fluidity than do non-fusing regions. Kalderon & Gilula (1979), using ultrastructural techniques, found that fusion appears to take place primarily within particle-free domains and that cytoplasmic unilamellar vesicles are occasionally associated with these regions; these vesicles were never found in fusion-arrested cells. Most relevant to the work in the present paper are the results of Weidekamm *et al.* (1976) who studied myoblast fusion in cell preparations cultured as in the present work. Addition of 1.4 mM-Ca\(^{2+}\) to these cells resulted in a rapid increase in fluorescence polarization which reached a maximum after 5–10 min; this was followed by a
Phosphatidylinositol and myoblast fusion

slow (2–3h) fall. Such a time course would correspond to the breakdown of phosphatidylinositol and corresponding increase in 1,2-diacylglycerol. It must be noted, however, that we are studying whole cells, and we do not know if this breakdown is indeed occurring at the plasma membrane.

The breakdown of phosphatidylinositol in this system appears to be different from receptor-occupation-stimulated breakdown, where Ca2+ is not an essential regulatory component and no phosphatidylinositol breakdown is observed in response to ionophore A23187 (see Michell & Kirk, 1981). Table 3 shows that phosphatidylinositol breakdown still occurs in this system when ionophore A23187 is added; however, it is about 50% inhibited. The involvement of phosphatidylinositol breakdown in response to receptor-occupation stimulation is thought to be in Ca2+ entry to the cells (Michell & Kirk, 1981). David et al. (1981) have shown that Ca2+ entry into myoblasts occurs just before fusion. The present work (Table 3) and the work of Schudt & Pette (1975) show that addition of ionophore A23187, together with Ca2+, to fusion-competent myoblasts has no effect on fusion. The decrease in phosphatidylinositol breakdown by ionophore A23187 could therefore be due to the removal of the necessity for a Ca2+-gating process, with no other effect on fusion. However, since the breakdown is not completely inhibited by ionophore A23187, and in light of the discussion above about the production and role of 1,2-diacylglycerol, it is possible that this breakdown has two functions. It appears probable that two, functionally different, pools of phosphatidylinositol exist in these cells, which could indeed be broken down by different mechanisms.

The lack of fusion and phosphatidylinositol breakdown in 24h-cultured myoblasts is possibly due to a lack of some enzyme component of phosphatidylinositol metabolism, or to incomplete formation of the required membrane structure. David et al. (1981) were able to induce precociously myoblast fusion with ionophore A23187, but only in the period 1–9h before normal fusion occurred; before this no effect was observed, which would suggest that a period of development in culture is required to achieve fusion competence. It is noteworthy that Dahl et al. (1978) showed that plasma membranes derived from myoblasts cultured in the presence of cycloheximide exhibited greatly inhibited fusion compared with untreated controls.

This study was supported by a grant from the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 138. The technical assistance of Ms. R. Dieterle is gratefully acknowledged.

References