Biochimica et Biophysica Acta, 1023 (1990) 298–304
Elsevier

BBAMEM 74800

The cesium-induced delay in myoblast membrane fusion is accompanied by changes in isolated membrane lipids

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(Received 16 October 1989)

Key words: Cesium ion; Myogenesis; Myoblast fusion; Cholesterol; Phosphatidylethanolamine; Phosphatidylcholine; Phosphatidylinositol; Membrane lipid

We have recently demonstrated that cesium ions delay the sharp decrease in both membrane conductivity and membrane permittivity of chick embryo myoblasts seen at fusion (Santini, M.T., Bonincontro, A., Cametti, C. and Indovina, P.L. (1988) Biochim. Biophys. Acta 945, 56–64). Analysis of the conductivity dispersion data (obtained in the radiowave frequency range) indicated that cesium delays fusion by about 30 h. We suggested that cesium is affecting both active ionic transport by blocking potassium channels as well as interfering with membrane lipid and/or protein charges. In the present study, we have investigated both the possible role of membrane lipids in myoblast fusion and the possible effects of cesium on these lipids. Our data indicate that lipid changes do occur in the isolated myoblast plasma membrane of controls during myogenic differentiation especially prior to fusion and that in cesium cultures these variations do not occur. These variations are in accordance with current membrane fusion theory. Specifically, there is a decrease in bilayer-stabilizing lipids (phosphatidylcholine) and an increase in bilayer-destabilizing ones (phosphatidylethanolamine and phosphatidic acid) and cholesterol during the fusion process. In addition, although slight, during fusion there appears to be a decrease in phosphatidylinositol which is believed to be involved in the inositol phosphate second messenger system. In cesium cultures, in which fusion is greatly delayed, the same lipid changes do not take place and those that are observed seem to reflect the fusion delay.

Introduction

Membrane fusion is an extremely important biological event. Although much is known about the specific properties of membrane fusion in various systems, the molecular dynamics of the phenomena still remain unclear. Studies involving artificial membranes have led to the hypothesis that membrane lipids leave the bilayer configuration temporarily and locally and form more unstable, non-bilayer configurations during fusion [1]. In this hypothesis, membrane lipids play a central role in fusion since the types, quantities and distributions of these lipids can greatly influence membrane structure [2,3]. In addition, membrane lipids may also play a vital role in signal transduction during cellular growth and development. In recent years, it has become apparent that the phosphoinositides may represent an important second messenger signalling mechanism [4,5]. Thus, not only do membrane lipids have a structural role during fusion, but they also may serve a physiological one as well.

The fusion of individual myoblasts to form multinucleated myotubes is an extremely important event in myogenic differentiation [6]. This event as well as the many other aspects of myogenesis may be studied using myoblast cultures [7]. Particularly useful are aggregate muscle cultures which serve as homogeneous and synchronous model systems [8]. Many of the differentiation events during muscle development take place at the plasma membrane. In fact, we have previously observed changes in membrane order prior to fusion [9,10] and a sharp fall in both membrane conductivity and membrane permittivity at fusion [11]. The changes in membrane order were attributed to prostaglandin receptor activity in preparation for fusion [9,10] while the abrupt decrease in membrane conductivity and membrane permittivity was linked to alterations in ionic transport

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properties as well as changes in membrane lipid and/or protein composition, respectively [11].

Cesium is not normally present in living systems, but, when present, may cause a wide variety of effects on biological systems. For instance, cesium is known to become fixed in muscle as was demonstrated with radioactive cesium [12]. In addition, it has been previously demonstrated that non-radioactive cesium alters potassium transport in various cell types [13–16]. We have recently shown with measurements of membrane conductivity and membrane permittivity that cesium delays fusion of myoblast aggregates by 30 h during in vitro myogenesis [17]. The membrane permittivity results suggest that cesium is also affecting the changes in protein structure and/or lipid composition previously reported. In this paper, we have concentrated our efforts in trying to determine the possible role of membrane lipids in myoblast fusion and the possible influence of cesium on these lipids. The present data demonstrate that lipid composition in the isolated myoblast plasma membrane varies during myogenesis, particularly in preparation for fusion, and that cesium, which greatly delays this process, also affects the normally occurring lipid profile.

Materials and Methods

Materials

Trypsin was purchased from Difco, soybean trypsin inhibitor and DNAse from Sigma, Dulbecco's modified Eagle's medium and penicillin/streptomycin from Gibco Europe and fetal bovine serum from Flow. The reference compounds dipalmitoylphosphotidylcholine, dipalmitoylphosphotidylethanolamine, 1-palmitoyl-lysophosphatidylcholine, 1-palmitoyl-lysophosphatidylethanolamine, sphingomyelin (from bovine brain) and cholesterol were purchased from Fluka (Buchs, Switzerland). The reference compounds for phosphatidylserine, phosphatidylinositol and phosphatidic acid were purchased from Sigma. The enzymatic determination of cholesterol was conducted with the CHOD-PAP Kit purchased from Boehringer Biochemia Robin (Milano, Italy). All other chemicals were obtained from Farmitalia Carlo Erba (Milano, Italy).

Myoblast cell cultures

Primary cultures were prepared from the pectoral muscle of 11-day embryonic chicks and aggregate cultures prepared as described previously [8,9]. For the cesium experiments, 20 mM CsCl dissolved in deionized water was added under sterile conditions at 24 h of culture by which time myoblast aggregation was complete [8]. The flasks were then regassed before returning to incubate. The suspended myoblast aggregates of both control and cesium cultures were then collected for plasma membrane isolation and subsequent assays at the following culture times: 19 h, 24 h, 41 h, 48 h, 65 h, 89 h and 96 h throughout in vitro myogenic differentiation. Both control and cesium experiments were repeated four times.

Isolation of myoblast plasma membrane

Isolation of myoblast plasma membrane was conducted as described previously [18]. Cells ($25 \cdot 10^7$) were harvested by low-speed centrifugation (5 min, $1100 \times g$) at the times listed above. The supernatant was accurately removed and the pellet was weighed and kept on ice. The cell pellet was suspended in 15 ml of sucrose-TEA (0.25 M sucrose, 1 mM triethanolamine (TEA)-HCl) (pH 7.4)) and homogenized by a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) with the tight (B) pestle. The homogenate was centrifuged (10 min, $1700 \times g$), the supernatant removed and centrifuged for 60 min at $33\,000 \times g$. The resulting pellet was suspended in 1 ml sucrose-TEA and layered over a sucrose discontinuous gradient (0.5 ml of 55% (w/w) sucrose, then 2.5 ml each of 40%, 32%, 27% and 20% sucrose). After centrifugation in a SW 41 rotor at 41 000 rpm for 1.5 h, the five bands formed at the interfaces were removed and each diluted to about 9 ml with 1 mM TEA-HCl (pH 7.4). The diluted material was then centrifuged for 60 min at $105\,000 \times g$. The pellets were suspended in TEA-HCl and assayed for protein, lipid and enzyme determination.

Protein, lipid and enzyme determination

An aliquot of the suspension was used for protein determination following the method of Bradford [19] with the Bio-Rad protein assay (Bio-Rad Laboratories Gmbh). Another aliquot was extracted with chloroform/methanol (2 : 1, by vol.), according to Folch et al. [20]. The quantitative determination of the phospholipids was made on the lipid extract by lipid phosphorous determination according to Bartlett [21]. The determination of the phospholipid classes was performed by a thin-layer chromatographic/densitometric method, as previously described [22]. The monodimensional solvent system used cannot completely resolve phosphatidylserine (PS) from phosphatidylinositol (PI). For this reason, the data for PS and PI is not presented individually, but rather as the sum of the two phospholipids (PS + PI). However, from other tests conducted previously, PS remains constant throughout in vitro myogenesis (data not shown). Thus, the changes seen in the PS + PI sum may be attributed to PI alone. The determination of cholesterol was made on the lipid extract by a modification of the CHOD-PAP kit enzymatic procedure [23]. The final aliquot was used to determine the purity of the isolated plasma membrane fraction by assay of the following enzyme activities: phosphodiesterase I, 5′-nucleotide, TPNH–cytochrome-*c*-reductase and succinate–cytochrome-*c* reductase as described by Schimmel et al. [18].

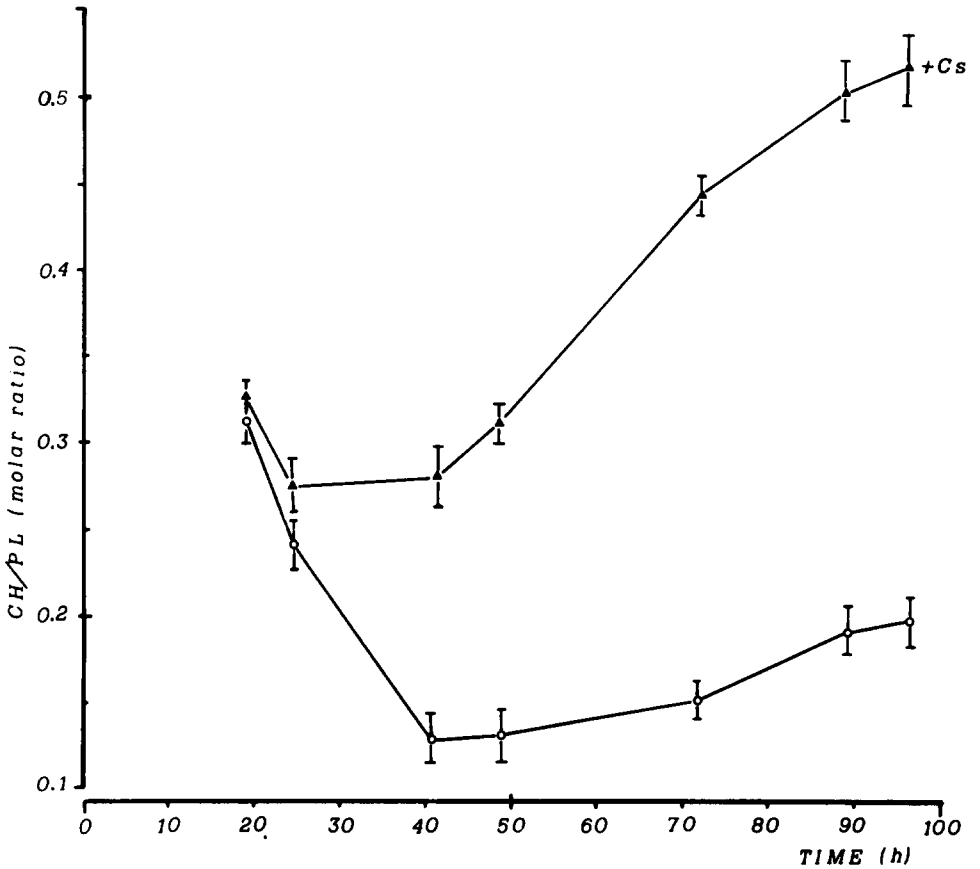


Fig. 1. Time dependence of the cholesterol (CH) to phospholipid (PL) molar ratio in control cultures (bottom curve) and in cesium cultures (top curve). The means and standard deviations of four separate experiments are shown.

Results

In this paper, we report on changes in the lipid composition of isolated plasma membranes of both control and cesium cultures examined at regular intervals during myogenic differentiation. Fig. 1 shows the relationship between the total concentration of cholesterol (CH) and phospholipid (PL) in the isolated myoblast plasma membranes expressed as the CH/PL molar ratio plotted as a function of culture time. In control cultures (bottom curve), the CH/PL ratio decreases abruptly reaching a minimum value at 41 h. As can be seen from Table I, this effect appears to be due to both an increase in the PL concentration as well as to a decrease in the CH concentration. In fact, the PL concentration increases from 1.35 to 2.44 μmol/mg

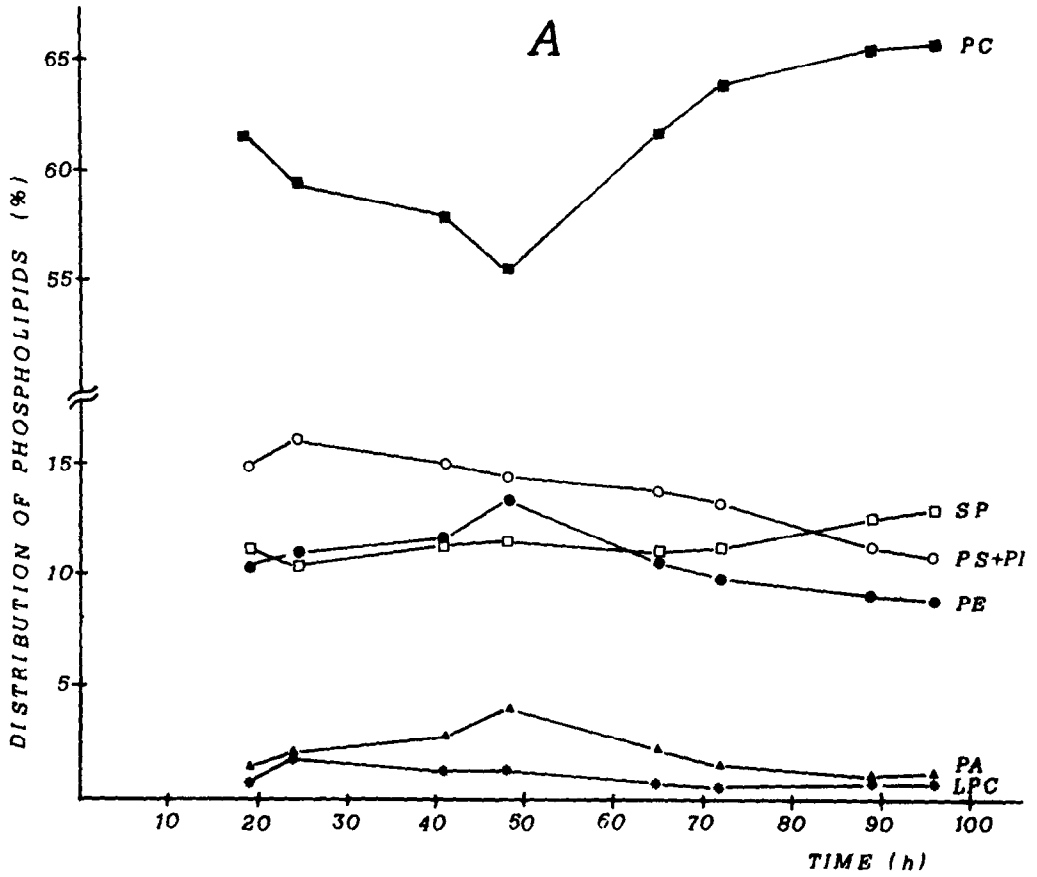
TABLE I

Concentration of phospholipid (PL) and cholesterol (CH) in isolated myoblast membranes during in vitro myogenesis in the presence and absence of cesium

Means and standard deviations of four experiments are shown.

Time (h)	Control cells		Cs-added cells	
	μmol PL/ mg protein	μmol CH/ mg protein	μmol PL/ mg protein	μmol CH/ mg protein
19	1.35±0.10	0.42±0.11	1.32±0.15	0.43±0.08
24	1.91±0.14	0.46±0.08	1.50±0.14	0.41±0.09
41	2.44±0.11	0.32±0.10	1.36±0.15	0.38±0.09
48	2.22±0.14	0.29±0.09	1.23±0.12	0.38±0.10
65	1.84±0.12	0.39±0.11	1.18±0.11	0.42±0.07
72	2.21±0.12	0.34±0.06	1.04±0.15	0.46±0.10
89	2.18±0.11	0.43±0.10	0.99±0.16	0.50±0.08
96	1.99±0.13	0.39±0.10	0.98±0.16	0.51±0.06

Fig. 2. (A) Changes in percent distribution of phospholipid classes in control cultures as a function of time in culture. Specifically, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine and phosphatidylinositol (PS+PI), sphingomyelin (SP) and lysophosphatidylcholine (LPC) are presented. The means of four separate experiments are plotted. The standard deviations of the four experiments which were calculated and found to fall between ±0.3 and ±0.5% were not plotted so as to make the figure more legible. (B) Changes in percent distribution of phospholipid classes in cesium cultures as a function of time in culture. PC, PE, PA, PS+PI, SP and LPC are again presented. Again, the means of four experiments are plotted. The standard deviations which are not shown were calculated as in Fig. 2A.


A
PC
SP
PS+PI
PE
PA
LPC
DISTRIBUTION OF PHOSPHOLIPIDS (%)
65
60
55
15
10
5
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20
30
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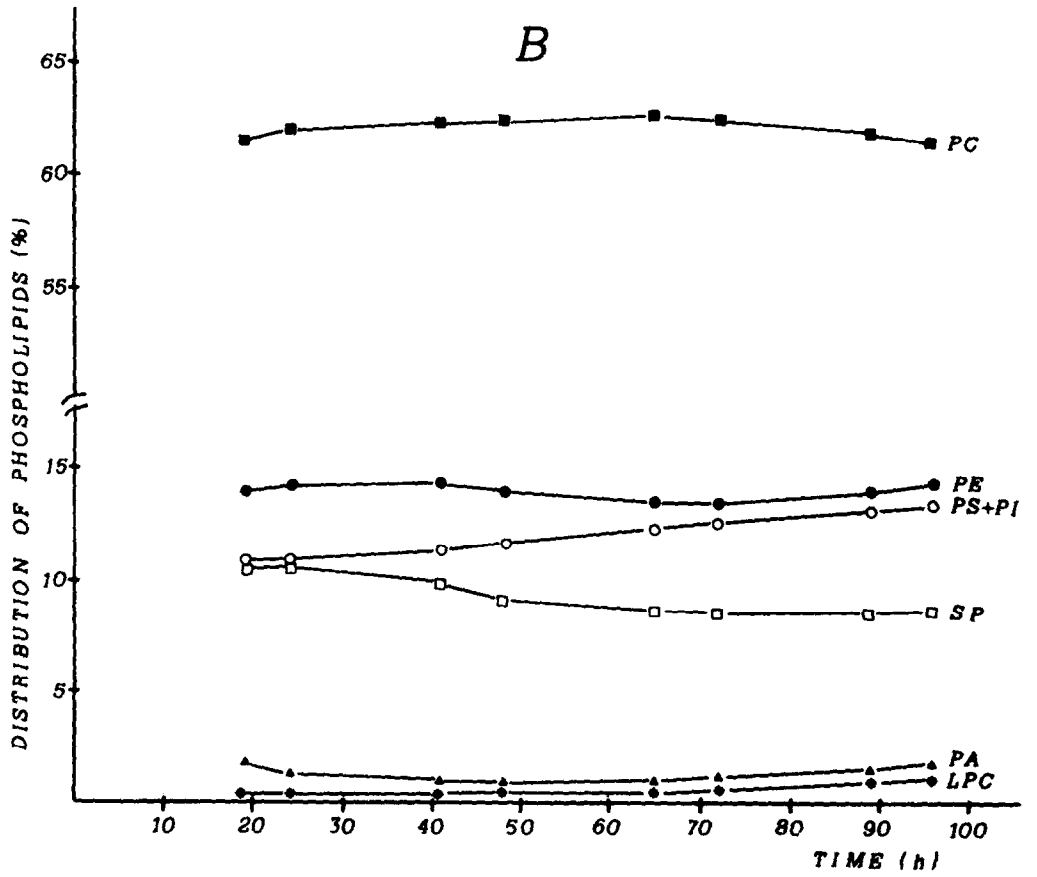
70
80
90
100
TIME (h)


B
PC
PE
PS+PI
SP
PA
LPC
DISTRIBUTION OF PHOSPHOLIPIDS (%)
65
60
55
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10
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30
40
50
60
70
80
90
100
TIME (h)

protein at 19 h and 41 h, respectively, while the CH concentration decreases from 0.42 μmol/mg protein at 19 h to 0.32 μmol/mg protein at 41 h. After 48 h, there is a slow, steady increase of cholesterol (Table I), but this substance never appears to reach its initial level during the times observed. This increase in cholesterol is also apparent from the CH/PL curve in Fig. 1. When cesium cultures are examined (Fig. 1, top curve), however, the same behavior in the CH/PL ratio is not observed. As can be seen from Table I, there is only a slight decrease in the cholesterol concentration up to 41 h (from 0.43 μmol/mg protein at 19 h to 0.38 μmol/mg protein at 41 h). In addition, the PL concentration increases slightly from 1.32 to 1.36 μmol/mg protein at 19 and 41 h, respectively. These slight variations result in no significant change in the CH/PL molar ratio of Fig. 1. After 41 h, there is an actual increase in the cholesterol concentration. The CH/PL ratio also increases reaching a value double to the initial one at 96 h. This increase in the ratio may also be due to a decrease of the PL concentration which decreases from 1.36 μmol/mg protein at 41 h to 0.98 μmol/mg protein at 96 h (Table I).

In Fig. 2 are shown the per cent distribution of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine and phosphatidylinositol (PS + PI), sphingomyelin (SP) and lysophosphatidylcholine (LPC) of isolated plasma membranes of both control and cesium myoblast cells. In control cultures (Fig. 2a), these phospholipids seem to show maximum variation at about 48 h in preparation for fusion (fusion in these systems occurs at 60 h [11]). In fact, PC decreases up to 48 h and then increases steadily after this culture time. PE and PA, on the other hand, vary in an inverse manner with PC. That is, there is an increase in these two phospholipids up to 48 h and then a steady decrease after this culture time. Thus, PC and PE and PA vary inversely with each other. In addition, PS + PI show a slight, but steady decrease from 24 to 96 h of culture. Finally, the other phospholipids examined (SP and LPC) showed no significant variations. In cesium cultures (Fig. 2b), there is no significant variation in PC, PE and PA at all times examined. In addition, PS + PI, SP and LPC showed no significant variations at all times tested as well.

Discussion

The data presented in this paper demonstrate that changes in both phospholipid and cholesterol content occur during myogenic differentiation, particularly in preparation for fusion. Our results seem to give biochemical confirmation to previous reports [9,10,24,25] in which it was demonstrated, using fluorescence polarization and electron paramagnetic resonance (EPR), that an increase in membrane fluidity prior to myoblast membrane fusion occurs. This increased fluidity indicates possible variations in membrane lipid composition and/or membrane structure. Membrane fluidity (or membrane order) is dependent upon the types of phospholipids and the amount of cholesterol present in the membrane and their distribution. Both of these are important in helping to determine, at least in part, membrane organization. For instance, a relative increase in cholesterol is known to stabilize membranes composed of bilayer-favoring lipids such as PC, PS and SP while a relative decrease in cholesterol can induce more fluid phases in PE-rich membranes [3]. The formation of these unstable membrane configurations in PE-rich model membranes is hypothesized to be the mechanism by which these model systems fuse [1,26]. Therefore, it is reasonable to speculate that a similar mechanism may be involved in myoblast membrane fusion. In fact, the data presented in this report seem to give validity to this hypothesis. In fusing cultures, there is an abrupt decrease in cholesterol leading to a fluidization of the membrane in preparation for fusion (minimum value occurs at 41 h) while in cesium cultures in which fusion is greatly delayed the cholesterol content at first remains nearly the same and then actually increases at 41 h. In these non-fusing membranes, there is no apparent fluidization of the membrane due to lowered cholesterol levels. In control cultures, after fusion is complete (60 h), there is a slow, steady increase in cholesterol levels indicating a possible renewed rigidization of the membrane.

The phospholipid distribution data presented in this report also seem to confirm the proposed model of myoblast membrane fusion. In fusing controls, PC which is known to stabilize membrane bilayers [2], decreases in preparation for fusion (48 h) while there is a concomitant increase in the bilayer-destabilizing phospholipids PE and PA. These variations in phospholipids together with the decrease in cholesterol observed may result in a highly fluidized and destabilized fusion-competent myoblast membrane.

In addition, the observed increase in PA as well as the decrease in PI may have added significance in the fusion process. As was stated earlier in the Introduction, it has become apparent that the inositol phospholipids may play an important role in signal transduction [4,5]. It is believed that phosphatidylinositol (PI) is phosphorylated to phosphatidylinositol 4-phosphate (PIP) and phosphatidyl 4,5-bisphosphate (PIP_2). An agonist, such as a hormone, acts on its receptor to stimulate hydrolysis of PIP_2 to yield two products: diacylglycerol (DG) and inositol trisphosphate (IP_3). The latter is recycled back to free inositol for PI resynthesis while a kinase phosphorylates DG to form PA. Thus, an increase in PA and a decrease in PI is consistent with inositol phospholipid breakdown. Is it possible that a mechanism similar to the one described

is present in chick embryo myoblasts during fusion? Wakelam and Pette [27–29] have hypothesized that indeed the inositol phospholipids may play a major role in myoblast fusion. These investigators postulated that the polyphosphoinositides act as a fusion block, either themselves or by their binding to membrane proteins. Myoblast fusion is initiated by the binding of a fusion-stimulating factor (a prostaglandin?) to cell surface receptors. This activates inositol phospholipid breakdown which causes calcium entry and removal of the fusion block. In addition, the inositol phospholipid breakdown could result in a more fluid membrane and the breakdown products DG and PA, two known fusogens, could stimulate fusion as well. In this study, the observed increase in PA and decrease in PI in controls seem to support the series of events believed to lead to myoblast fusion. Added support comes from cesium cultures where fusion is greatly delayed. In these cultures, there is no variation in all the phospholipids examined. There appears to be a block in phosphoinositol breakdown as evidenced by no increase in PA and no decrease in PI. There may be no subsequent initiation of signalling events leading to membrane fusion, no calcium entry and no destabilization of the myoblast membrane by an increase in PA or PE. Thus, fusion is greatly delayed. Cesium appears to be affecting membrane fusion by also influencing lipid composition.

Possible mechanisms by which cesium causes these effects may be related to potassium channels. We have previously suggested that cesium blocks potassium channels and that this block may perturb cellular ionic gradients and membrane potential [11]. Perhaps, it is these gradient variations which cause charge interference with enzymes, specifically with metalloendoproteases which are so important for fusion [30]. However, further studies examining the effects of charge on enzyme function are necessary in order to determine whether this is the case.

More direct evidence for the appearance of local bilayer destabilization during myoblast fusion comes from proton nuclear magnetic resonance (NMR) studies of the acyl chains of lipid domains (Santini et al., in preparation). These studies show higher T1 and T2 relaxation times for these chains during the process of myoblast fusion as compared to the pre- and post-fusion values.

Previous biochemical reports [31,32] demonstrated only minor variations in phospholipid and cholesterol during in vitro myogenesis. A possible explanation for these differences may be the type of myoblast cultures used. In the older investigations, stationary muscle cultures were utilized while suspension cultures were used in the present report. These latter cultures are much more homogeneous and synchronous than the others since fibroblasts are eliminated [8]. As a result, changes in myoblasts are not masked by the presence of the fibroblasts and, therefore, variations in membrane components are more clearly visible. A second possible explanation for the contrasting results may be that in one report [31] the culture conditions were manipulated in order to achieve synchronous fusion while such manipulations were not carried out in the present work. The possible effects of these manipulations are not known. Finally, both reports analyzed lipid composition at a few time points and not a short intervals of a few hours each throughout the process of membrane fusion. Examination of lipid composition only before and after fusion [31] or at intervals of days [32] may not have been sufficient to note variations. In fact, even our data show no major changes in the lipids if only a few random points are examined, whereas a more thorough analysis does demonstrate such variations during fusion.

Acknowledgements

We thank Mr. Giacomo Monteleone, Dr. Simona Ceccarelli, Mrs. Loreta Ragni, Mr. Luigi Pierangeli and Dr. Stefano Silvestrini for their technical support. This investigation was partially supported by an Italian Consiglio Nazionale delle Ricerche Grant 85.00.304.02 (to P.L.I.).

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