Biochimica et Biophysica Acta, 1070 (1991) 27–32

BBAMEM 75392

The cesium-induced delay in myoblast membrane fusion is accompanied by changes in cellular subfraction lipid composition

Maria T. Santini [1], Pietro L. Indovina [2] and Alfredo Cantafora [3]

[1] *Laboratorio di Ultrastrutture, Istituto Superiore di Sanità and INFN-Sezione Sanità, Roma (Italy),* [2] *Dipartimento di Scienze Fisiche, Università di Napoli and INFN-Sezione Sanità, Roma (Italy) and* [3] *Laboratorio di Metabolismo e Biochimica Patologica, Istituto Superiore di Sanità, Roma (Italy)*

(Received 7 March 1991)

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

We have recently demonstrated that the delay in myoblast membrane fusion induced by cesium is accompanied by changes in isolated membrane lipids (Santini, M.T., Indovina, P.L., Cantafora, A. and Blotta, I. (1990) Biochim. Biophys. Acta 1023, 298–304). In the present study, we have investigated changes in the lipid profile of total cell homogenates and microsomal membrane fractions during myoblast membrane fusion as well as the effects that addition of cesium may have on these lipid variations in order to try to understand the production and translocation of lipids during this myogenic process. The data presented here indicate that the lipid composition of cell homogenates and microsomes varies in a different manner from isolated plasma membranes during myogenic fusion. In addition, cesium affects, in a different manner, the normally-occurring lipid production and distribution which takes place in each subcellular fraction.

Introduction

The fusion of individual mononucleated myoblasts to form multinucleated muscle fibers is an extremely important event in myogenic differentiation. The complex series of biological events leading to myoblast fusion include a net calcium influx prior to fusion [1,2] and alterations in surface proteins [3,4]. In addition, membrane lipids have been shown to play a central role in myoblast fusion from both a structural perspective [5,6] as well as in signal transduction [7,8]. In fact, we have recently demonstrated, using primary chick embryo aggregate muscle cultures, that myoblast differentiation is accompanied by changes in isolated membrane lipids [9]. Specifically, we showed a decrease in bilayer-stabilizing lipids (phosphatidylcholine) and phosphatidylinositol and an increase in bilayer-destabilizing ones (phosphatidylethanolamine and phosphatidic acid) and cholesterol in isolated myoblast cell membranes during fusion. Cultures to which 20 mM CsCl was added and in which fusion was greatly delayed did not show the same lipid changes and those that were observed seemed to reflect the cesium-induced fusion delay [9].

Lipid metabolism in cells is a highly complex process. Briefly, lipids (phospholipids and cholesterol) are produced in the endoplasmic reticulum and then are passed on to other parts of the cell, mostly the cell membrane. Phosphatidic acid, which is formed first, is subsequently modified and the different types of phospholipid molecules are then synthesized. Sphingomyelin is produced later from phosphatidylcholine and ceramide, both previously transferred from the endoplasmic reticulum to the Golgi apparatus. From this brief outline of lipid synthesis, it is apparent that a truly complete comprehension of the membrane lipid changes occurring during myoblast membrane fusion is not possible without also understanding the entire process of lipid metabolism and translocation taking place in the myoblasts during the fusion process. With this in mind, it was the purpose of the present report to continue where our previous study ended, that is, to extend examination of the lipid changes taking place during myoblast membrane fusion to include analysis not only of isolated membrane lipids, as was conducted earlier, but also to study the endoplasmic reticulum

Correspondence: A. Cantafora, Laboratorio di Metabolismo e Biochimica Patologica, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy.

microsomal fraction as well as the total myoblast cell homogenate. In this present paper, control cultures and cultures to which 20 mM CsCl was added were again examined. The results presented in this report demonstrate that the lipid composition of total cell homogenate and microsomal fractions varies in a different manner during myogenic differentiation and that these variations can be related to the production and translocation of the lipids necessary for myogenic fusion. In addition, cesium, which greatly delays fusion, also affects the normally-occurring lipid production and distribution which takes place in each subcellular fraction.

Materials and Methods

Materials

Trypsin was purchased from Difco, soybean trypsin inhibitor and DNAase from Sigma, Dulbecco's modified Eagle's medium and penicillin/streptomycin from Gibco Europe and fetal bovine serum from Flow. The reference compounds dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine, 1-palmitoylphosphatidylcholine, 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 Biochimica 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 [10,11]. For the cesium experiments, 20 mM CsCl dissolved in deionized water was added under sterile conditions at 24 h of culture. The flasks were then regassed before returning to incubate. The suspended myoblast aggregates of both control and cesium cultures were then collected for total cell homogenate preparation, microsomal fraction isolation and subsequent assays at the following culture times: 19, 24, 41, 48, 65, 89 and 96 h. All experiments were repeated four times.

Cell homogenate preparation and isolation of microsomes

Total cell homogenate was prepared by harvesting myoblast cells ($4 \cdot 10^6$) by low-speed centrifugation (5 min, $1100 \times g$) at the fixed time intervals listed above. The supernatant was accurately removed and the pellet was weighed and resuspended in a small volume of phosphate-buffered saline (pH 7.4). This solution was then homogenized by a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) with the tight (B) pestle. Aliquots of the suspension were assayed for protein, lipid and enzyme determination as described below.

Isolation of microsomes was carried out by harvesting cells ($25 \cdot 10^7$) by low-speed centrifugation (5 min, $1100 \times g$), again at the times listed above. The supernatant was accurately removed and the pellet was resuspended in 15 ml of sucrose-TEA (0.25 M sucrose, 1 mM triethanolamine (TEA-HCl, buffered at pH 7.4) and homogenized by the Dounce homogenizer described above. The homogenate was centrifuged at low speed (10 min, $1700 \times g$). The supernatant was then removed and centrifuged for 60 min at $33\,000 \times g$. The resulting pellet was resuspended in 1 ml sucrose-TEA and layered over a discontinuous sucrose gradient (0.5 ml of 55% (w/w) sucrose, then 2.5 ml each of 40%, 32%, 27% and 20% sucrose) as described by Schimmel et al. [12] for the isolation of various cellular membrane fractions. After centrifugation in an SW 41 rotor at 41 000 rpm for 1.5 h, five bands formed at the interfaces of the gradient. The interface between 32% and 40%, which is enriched in microsomal membranes, was collected, diluted to about 9 ml with sucrose-TEA and centrifuged for 60 min at $105\,000 \times g$. The microsomes were further purified by resuspending the resulting pellet in 1 ml of sucrose-TEA and repeating the fractionation procedure described above on the discontinuous sucrose gradient. The pellet obtained from this second purification was used for protein, lipid and enzyme determination.

Protein, lipid and enzyme determination

Protein determination was conducted following the method of Bradford [13] with the Bio-Rad protein assay (Bio-Rad Laboratories). The quantitative determination of the phospholipids was made by lipid phosphorous determination according to Bartlett [14] after extraction of the lipids with chloroform/methanol (2:1, v/v) according to Folch et al. [15]. The determination of the phospholipid classes was performed by a thin-layer chromatographic/densitometric method as previously described [16]. 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). Therefore, 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 modification of the CHOD-PAP Kit enzymatic procedure [17]. The purity of the isolated microsomal fraction was checked by assay of the following enzyme activities: glucose-6-phosphatase [18], acid

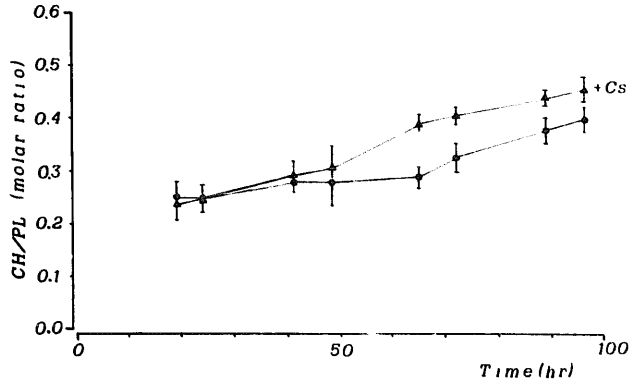

Fig. 1. Time dependence of the cholesterol (CH) to phospholipid (PL) molar ratio in control (bottom curve) and cesium (top curve) total cell homogenates. The means and standard deviations of four separate experiments are shown.

phosphatases [19], 5'-nucleotidase [20], TPNH-dependent cytochrome-*c* reductase [21] and succinate-dependent cytochrome-*c* reductase [21].

Results

In this paper, we report on quantitative as well as qualitative changes in the lipid composition of myoblast cell homogenates and of isolated myoblast microsomes of both control and cesium chick embryo myoblast cultures examined at regular intervals during myogenic differentiation. Figs. 1 and 2 show the relationship between the total concentration of cholesterol (CH) and phospholipid (PL) expressed as the CH/PL molar ratio plotted as a function of culture time in both total cell homogenates and microsomal fractions, respectively. Curves for controls as well as cesium cultures are shown. Total cell homogenates (Fig. 1) show a steady increase in the CH/PL ratio up to 48 h of culture. After this time, although both control and cesium cultures continue to show an increase in this ratio, in cesium cultures this increase is more pronounced. As can be seen from Table I, the effect in

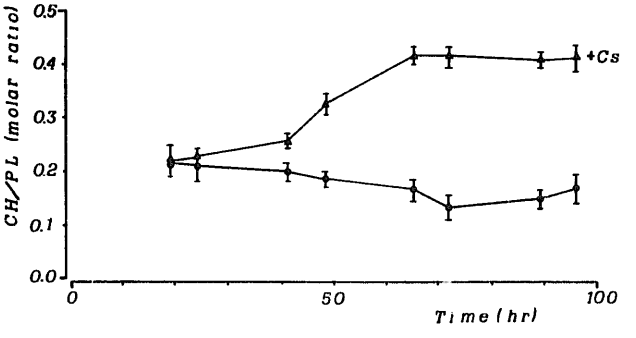

Fig. 2. Time dependence of the cholesterol (CH) to phospholipid (PL) molar ratio in control (bottom curve) and cesium (top curve) microsomal fractions. The means and standard deviations of four separate experiments are shown.

TABLE I

Concentration of phospholipid (PL) and cholesterol (CH) in cell homogenates during in vitro myogenesis in the presence and absence of cesium

Means and standard deviations of four separate experiments are given.

Time (h)	Control cells		Cs-added cells	
	μmol PL/ mg protein	μmol CH/ mg protein	μmol PL/ mg protein	μmol CH/ mg protein
19	1.83±0.12	0.46±0.07	–	–
24	1.84±0.11	0.46±0.06	2.13±0.14	0.53±0.06
41	1.85±0.09	0.52±0.08	1.60±0.08	0.46±0.07
48	2.53±0.15	0.70±0.09	2.82±0.16	0.83±0.09
65	1.85±0.10	0.53±0.07	1.23±0.07	0.52±0.07
72	1.43±0.08	0.47±0.06	1.10±0.09	0.47±0.06
89	1.04±0.07	0.39±0.05	1.02±0.10	0.45±0.06
96	1.00±0.09	0.40±0.06	1.02±0.12	0.47±0.08

controls appears to be due mostly to an increase in the CH concentration during the first 48 h of culture (from 0.46 μmol/mg protein at 19 h to 0.70 μmol/mg protein at 48 h). Beyond this culture time, the rise in the CH/PL ratio appears to be due more to a decrease in the PL concentration (from 2.53 μmol/mg protein at 48 h to 1.00 μmol/mg protein at 96 h). In cesium homogenates, the observed sharper rise in the CH/PL ratio appears to be due mostly to a decrease in the PL concentration throughout the times tested (from 2.13 μmol/mg protein at 24 h to 1.02 μmol/mg protein at 96 h). In the microsomal fraction (Fig. 2), control cultures (bottom curve) show a slow decrease in this ratio rather than an increase as in homogenates, especially after 48 h. In cesium cultures (top curve), however, this ratio again shows an increase. This increase is more apparent after 48 h of culture. As can be seen from Table II, in control microsomes these changes appear to be due to an increase in the PL concentra-

TABLE II

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

Means and standard deviations of four separate experiments are given.

Time (h)	Control cells		Cs-added cells	
	μmol PL/ mg protein	μmol CH/ mg protein	μmol PL/ mg protein	μmol CH/ mg protein
19	0.56±0.09	0.13±0.02	0.55±0.08	0.12±0.01
24	0.68±0.10	0.14±0.03	0.60±0.07	0.14±0.02
41	0.70±0.09	0.14±0.02	0.69±0.06	0.18±0.01
48	0.68±0.08	0.13±0.02	0.72±0.09	0.24±0.03
65	0.52±0.07	0.09±0.01	0.40±0.06	0.17±0.01
72	0.70±0.08	0.09±0.01	0.38±0.04	0.16±0.01
89	0.75±0.07	0.11±0.02	0.37±0.03	0.15±0.02
96	0.65±0.10	0.11±0.03	0.38±0.05	0.16±0.02

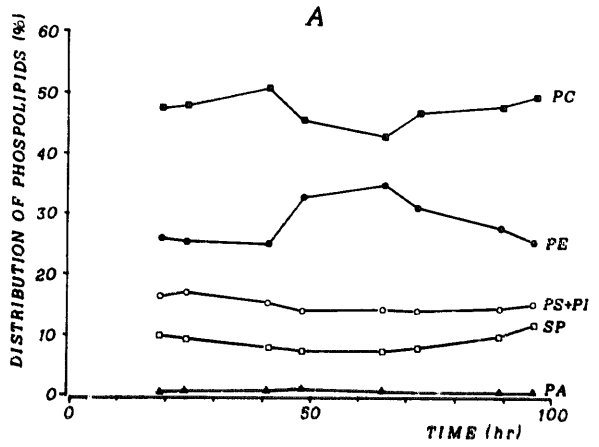

Fig. 3. (A) Changes in percent distribution of phospholipid classes in total cell homogenates as a function of time in culture. Specifically, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine and phosphatidylinositol (PS + PI) and sphingomyelin (SP) are 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.

tion up to 48 h (from 0.56 μmol/mg protein at 19 h to 0.68 μmol/mg protein at 48 h) and then to a decrease in the CH concentration after this culture time (from 0.13 μmol/mg protein at 48 h to 0.11 μmol/mg protein at 96 h). In cesium cultures, the increase in the CH/PL ratio appears to be due to both an increase in the PL concentration (from 0.55 μmol/mg protein at 19 h to 0.72 μmol/mg protein at 48 h) as well as an increase in the CH concentration (from 0.12 μmol/mg protein at 19 h to 0.24 μmol/mg protein at 48 h) up to 48 h and then due mostly to a decrease in the PL concentration after this time (from 0.72 μmol/mg protein at 48 h to 0.38 μmol/mg protein at 96 h). Thus,

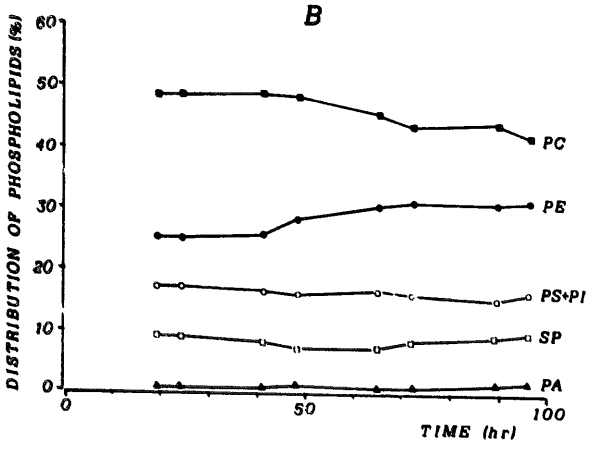

Fig. 3. (B) Changes in percent distribution of phospholipid classes in total cell homogenates to which cesium was added as a function of time in culture. PC, PE, PA, PS+PI and SP are presented. The means of four separate experiments are plotted. The standard deviations which are not shown were calculated as in Fig. 3A.

control and cesium microsomes behave in a different manner from control and treated cell homogenates.

The qualitative changes in phospholipids during myogenic differentiation were also examined in control and cesium homogenates and microsomes. In Figs. 3 and 4 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 total cell homogenate and microsomal fractions, respectively, of both control and cesium myoblast cells. In homogenate controls (Fig. 3A), PS + PI, SP and PA show no significant changes, but PC and PE show variation. Specifically, at 24 h of culture, about 45% of the total phospholipids are PC while PE represents about 25% of the lipids. After 24 h, changes in this distribution occur: the amount of PC increases and then decreases up to about 48 h, reaching 40%, while PE remains constant up to 41 h and then increases up to 48 h of culture, reaching 35%. The amounts of these two lipids, in fact, vary inversely with each other. From about 48 to 65 h (fusion occurs at 60 h [22]), both PC and PE remain nearly constant. At 65 h, however, PC and PE again begin to vary inversely with each other. From this time up to 96 h of culture, when fusion has ended, PC increases while PE decreases. Values of PC and PE similar to the initial ones are again reached.

In cesium homogenates (Fig. 3B), the amounts of PS + PI, SP and PA again remain constant. In addition, up to 65 h, the changes in per cent distribution of PC and PE are quite similar to those of control cul-

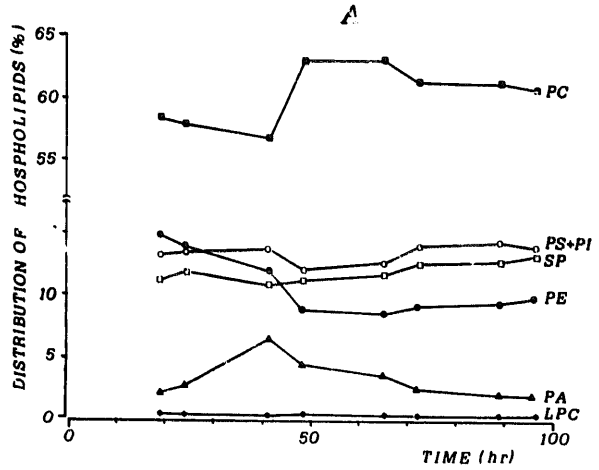

Fig. 4. (A) Changes in percent distribution of phospholipid classes in microsomal membranes as a function of time in culture. Specifically, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine and phosphatidylinositol (PS + PI), sphingomyelin (SP) and lysophosphatidylcholine (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.

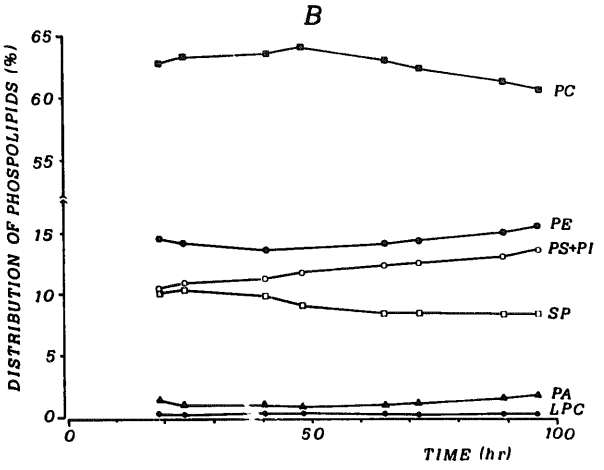

Fig. 4. (B) Changes in percent distribution of phospholipid classes in microsomal membranes to which cesium was added as a function of time in culture. PC, PE, PA, PS + PI, SP and LPC are presented. The means of four separate experiments are plotted. The standard deviations which are not shown were calculated as in Fig. 4A.

tures. In fact, there is a decrease in PC from about 45 to 40% and an increase in PE from about 25 to 35%. The same inverse relationship between the two phospholipids exists. Beyond 65 h, however, PC and PE do not vary in the same manner as in the control cultures: there is no increase in PC or decrease in PE. As can be noted in the cesium homogenates, the amounts of both phospholipids remain constant at the previous levels up to 96 h of culture.

Examination of phospholipid class distribution in microsomes yields very different results. In microsome controls (Fig. 4A), PS + PI, SP and LPC show very little variation while PC, PE and PA vary consistently. PC decreases up to about 48 h of culture, increases abruptly at 48 h and then decreases again slightly up to 96 h. The per cent distribution varies from about 58% at 19 h to about 55% at 41 h and then from about 65% at 48 h back to about 60% at 96 h. PE decreases up to 48 h from 15 to 9% and then increases slightly to 11% through the remaining times tested. PA increases from 2% at 19 h to 7% at 48 h and then decreases, returning to its original value by 96 h. Again, there is an inverse relationship between PC and PE, but it is opposite to the one seen in cell homogenates (Fig. 3A).

In cesium microsomes (Fig. 4B), PA and LPC do not vary while the other phospholipids do, but the variations are very slight. Although an inverse relationship between PC and PE (an initial increase from 62 to 64% and then a decrease to 60% in PC and an initial decrease from 15 to 13% and then an increase back to 15% in PE) again can be noted, it is almost negligible. The increase in PS + PI from 10 to 14% and the decrease in SP from 10 to 7% is also very slight. It appears that addition of cesium has eliminated most of the variations seen in the phospholipid distribution. It should also be noted that the percentages of the two phospholipids PC and PE in microsomes are quite different from those seen in homogenates in both control and cesium samples. In fact, microsomes contain an overall higher percentage of PC and a lower amount of PE.

Discussion

The present results indicate that the time-dependent lipid modifications observed in chick embryo myoblasts during in vitro myogenesis with and without cesium do not manifest themselves evenly within the cell, but rather behave differently in various cellular subfractions. For instance, in cell homogenate controls the CH/PL ratio rises with increasing culture time while in microsomal and isolated plasma membrane controls [9] it decreases. The addition of cesium further points out the compartmentalized nature of the lipid changes. In fact, if the difference in the CH/PL ratio between control and cesium-treated cultures at the last culture time tested (96 h) is expressed in per cent variation, a difference of 15% in homogenates and 145% in microsomes is measured. In isolated plasma membranes a variation of 160% was previously observed [9]. Thus, the very small variation in lipid composition observed in the whole cell may hide dramatic changes taking place at specific cellular sites, such as the plasma membrane (where fusion actually occurs), as well as in the inner membranes (where the lipids are synthesized). In addition, the greatest variations in the CH/PL ratio take place at the plasma membrane and not at other cellular sites.

It should also be noted that the lowest value in the CH/PL ratio (which indicates loss of cholesterol and fluidization of the myoblast membrane prior to and necessary for fusion) occurs in controls at about 70 h in microsomes and at about 40 h in plasma membranes [9]. Since fusion in these cultures takes place at 60 h, the decrease in cholesterol in microsomes occurs after fusion has already taken place and thus appears not to be related directly to this event. Consequently, it may be postulated that the changes in lipid composition observed are not related to lipid biosynthesis (otherwise a decrease in cholesterol would have been observed in microsomes first and later at the plasma membrane), but rather to movement and/or transformation of these lipid molecules at the plasma membrane itself prior to fusion. This hypothesis is supported by the localized changes in cholesterol content which are known to occur in specific lipid domains [23] and by the removal of cholesterol from plasma membranes mediated by proteins and phospholipids [24].

The qualitative changes in phospholipid class distribution also differ in the various subcellular fractions examined. Specifically, control cell homogenates and

isolated plasma membranes [9] show a similar reciprocal variation of PC and PE which is opposite to the reciprocal relationship observed in microsomes. These differences in behavior may be explained by the fact that during fusion a decrease in PC (which stabilizes bilayers) and an increase in PE (which destabilizes bilayers) occur at the plasma membrane. The loss in PC is counteracted by the increased synthesis of this phospholipid at the microsomes (as witnessed by the increase in concentration of PC) while the increase in PE at the membrane may be traced to a decrease at the microsomes of this phospholipid by migration and/or conversion into PC.

It is also interesting to note that the increase in phosphatidic PA and the proportional decrease in PI observed previously at the plasma membrane before fusion [9] also occurs at the microsomes. However, the maximum changes in these two phospholipids occur earlier in microsomes (at about 40 h) than at the membrane (48 h). These observations confirm the important role of inositol phospholipid breakdown during myoblast membrane fusion [25–28].

Finally, studies of total cell homogenates and microsomal membranes confirm our previous findings in plasma membranes that the phospholipid class distribution at the end of the experimental period examined tends to approach the values found at the beginning of the experiments. This is an important observation since it supports our view that myoblast membrane fusion cannot be studied by examining only two time-points, but must rather be studied by evaluation of a series of points throughout the entire myogenic differentiation process.

References

1 David, J.D., See, W.M. and Higginbotham, C.A. (1981) Dev. Biol. 82, 297–307.
2 David, J.D. and Higginbotham, C.A. (1981) Dev. Biol. 82, 308–316.
3 Pauw, P.G. and David, J.D. (1979) Dev. Biol. 70, 27–38.
4 Walsh, F.S. and Philips, E. (1981) Dev. Biol. 81, 229–237.
5 Cullis, P.R. and DeKruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420.
6 Cullis, P.R., DeKruijff, B., Verkleij, A.J. and Hope, M.J. (1986) Biochem. Soc. Trans. 14, 242–245.
7 Berridge, M.J. (1984) Biochem. J. 220, 345–360.
8 Hokin, L.E. (1985) Ann. Rev. Biochem. 54, 205–235.
9 Santini, M.T., Indovina, P.L., Cantafora, A. and Blotta, I. (1990) Biochim. Biophys. Acta 1023, 298–304.
10 Hausman, R.E., Dobi, E.T., Woodford, E.J., Petrides, S., Ernst, M. and Nichols, E.B. (1986) Dev. Biol. 113, 40–48.
11 Santini, M.T., Indovina, P.L. and Hausman, R.E. (1987) Biochim. Biophys. Acta 896, 19–25.
12 Schimmel, S.D., Kent, C., Bischoff, R. and Vagelos, P.R. (1973) Proc. Natl. Acad. Sci. USA 70, 3195–3199.
13 Bradford, M.M. (1976) Anal. Chem. 72, 248–254.
14 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
15 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509.
16 Masella, R. and Cantafora, A. (1988) Clin. Chim. Acta 176, 63–70.
17 Bravo, E. and Cantafora, A. (1989) Giorn. It. Chim. Clin. 14, 141–144.
18 Aronson, N.N. and Touster, O. (1974) Methods Enzymol. 31, 90–102.
19 Engstrom, L. (1961) Biochim. Biophys. Acta 52, 36–48.
20 Avruch, J. and Wallach, D.F.H. (1971) Biochim. Biophys. Acta 233, 334–347.
21 Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415–438.
22 Santini, M.T., Bonincontro, A., Cametti, S. and Indovina, P.L (1988) Biochim. Biophys. Acta 945, 56–64.
23 Karnovsky, M.J., Kleinfeld, A.M., Hoover, R.L. and Klausner, R.D. (1982) J. Cell Biol. 94, 1–6.
24 Bartholow, L.C. and Geyer, R.P. (1982) J. Biol. Chem. 257, 3126–3130.
25 Wakelam, M.J.O. (1983) Biochem. J. 214, 77–82.
26 Wakelam, M.J.O. (1985) Biochem. J. 228, 1–12.
27 Wakelam, M.J.O. and Pette, D. (1982) Biochem. J. 202, 723–729.
28 Wakelam, M.J.O. and Pette, D. (1984) Ciba Found. Symp. 103, 100–118.