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DIFFERENTIATION-RELATED DIFFERENCES IN THE PLASMA MEMBRANE PHOSPHOLIPID ASYMMETRY OF MYOGENIC AND FIBROGENIC CELLS

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We have determined the asymmetric distribution of two aminophospholipids phosphatidylethanolamine and phosphatidylserine in the plasma membrane of chick embryo fibroblast and myoblasts. Right-side-out membrane preparations were incubated with two different amidating reagents, trinitrobenzenesulfonate and isethionylacetimidate, under nonpenetrating conditions. Inside-out membranes were incubated with trinitrobenzenesulfonate. In fibroblasts, the similar plateau values suggested that 35% of the phosphatidylethanolamine and 20% of the phosphatidylserine is externally disposed. These values agree with previous measurements on fibroblast plasma membranes. In myoblasts, however, labelling plateaux were achieved which suggested that 65% of the phosphatidylethanolamine and 45% of the phosphatidylserine is externally disposed. This represents a 2–3-fold increase in potentially fusogenic lipids on the external leaflet of the plasma membrane. This unique distribution of aminophospholipids in myoblasts extends through the stage of development during which myoblasts become competent to fuse and form myotubes in culture. Two inferences may be drawn from these results. First, the external concentration of aminophospholipids in myoblasts is enriched significantly over that of fibroblasts or erythrocytes. This orientation may contribute to its fusion competence. Second, although large amounts of externally disposed aminophospholipid may be necessary for myoblast fusion, they do not confer fusion competence.

Abbreviations:

PE:	phosphatidylethanolamine
PS:	phosphatidylserine
Growth medium:	Dulbecco's minimal essential medium containing 20 mg $CaCl_2$/ml, 10% horse serum, 2.5% chick embryo extract and 1% penicillin/streptomycin
CF-Growth medium:	growth medium with no $CaCl_2$ added
TNBS:	trinitrobenzenesulfonate, sodium salt
EDTA:	ethylenediaminetetraacetic acid
AraC:	cytosine arabinoside
Buffer I:	Hank's balanced salt solution with 20 mM Hepes (pH 7.4)
Buffer II:	120 mM sodium bicarbonate and 40 mM sodium chloride (pH 8.15)
EGTA:	ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid
Hepes:	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
BUdR:	5'-bromodeoxyuridine

Introduction

The fusion of myoblasts into multinucleate myotubes is a key event in the production of skeletal muscle fibers. Several observations support a role for lipid as a mediator of fusion in these cells: putative fusion sites display IMP-free regions [1], perturbations of the membrane lipids modulate the rate of fusion [2], and the physical properties of the membrane lipids change during fusion [3,4].

Several investigators have attempted to correlate changes in membrane lipid composition with fusion. Kent et al. [5] have analyzed the plasma membrane phospholipid, cholesterol, and fatty acids during myogenesis in culture and were unable to detect changes that correlate with fusion.

Similarily, McKay et al. [6] were unable to correlate glycolipid changes with fusion. And finally, Nakornchai et al. [7] and Reporter and Raveed [8] have found that the addition of lipids that promote fusion in nonfusing cell types inhibited the fusion of myoblasts.

In contrast to this unchanging lipid composition, we have reported [9], in a preliminary communication, significant differences in the organization of the aminophospholipids. We now more fully document and substantiate these findings. In addition, we report on measurements of myoblast asymmetry during the stage of differentiation in which fusion competence is established of related myoblast cell types and in the presence of differing calcium concentrations. Taken together these observations suggest that the external enrichment of aminophospholipid, while consistent with their involvement in fusion, does not alone confer fusion competence.

Materials and Methods

Cells

Fibroblasts were obtained from 11-day-old chick embryos using the following procedure. Embryos were decapitated, eviscerated and minced in calcium and magnesium-free phosphate-buffered saline. Trypsin was added at a final concentration of 0.05%, and the cells stirred for 15 min at room temperature. The action of the trypsin was stopped by the addition of an equal volume of growth medium, and the cells were centrifuged at $800 \times g$ for 3 min. The cell pellet was resuspended in a small volume of growth medium and passed through two layers of lens paper. The resulting single cells were plated at a concentration of approximately $1 \cdot 10^6$ cells/ml. Cultures were maintained for 7–10 days and were subcultured at least twice during that time.

Myoblasts were obtained from the pectoral muscle of 11-day chick embryos as described previously [2,10] and cultured in CF-growth medium. Cytosine arabinoside at a final concentration of 0.6 μM was added 30 h after plating to kill proliferating cells such as fibroblasts. Myoblasts were harvested 48–50 h after plating except where indicated otherwise.

Myoblasts from the breast muscle of 11-day-old Japanese quail were prepared like those from the chick. The cells were cultured in CF-growth medium containing an additional 5% horse serum and 2.5% embryo extract. The cells were harvested 48–52 h after plating.

L_6 a rat myogenic cell line, was cultured as described previously [2].

Membrane preparations

The blebbing procedure is a modification of that published by Scott [11]. Cells on 100 mm dishes were washed two times in Buffer I, and 5 ml freshly prepared blebbing medium (2 mM dithiothreitol and 35 mM formaldehyde in Buffer I) was added. The cells were incubated at 37°C for 2 h with shaking at 100 rpm. The vesiculation solution containing the blebs was decanted and spun at $800 \times g$ for 10 min to remove cells. The blebs were pelleted by centrifuging at $30\,000 \times g$ for 30 min, and washed in Buffer II by two more cycles of centrifugation and resuspension. The final pellet of blebs was resuspended in 1 ml Buffer II and used immediately for asymmetry studies.

Phagocytic vesicles were isolated using the procedure of Sandra and Pagano [12]. Briefly, myoblasts or fibroblasts were mechanically removed from the dish and washed five times with phosphate-buffered saline. Latex spheres (1.1 μM) were added at a concentration of 3000 beads/cell, and the cells diluted with phosphate-buffered saline to a final concentration of 10^7 cells/ml. Phagocytosis was accomplished by shaking the cells in a reciprocal shaker at 75 rpm for 1 h at 37°C. Excess beads were removed by 4–6 cycles of resuspension and centrifugation at $500 \times g$ for 5 min. The phagocytised membrane enclosed beads were released from the cells by homogenization with a Dounce homogenizer. The homogenate, brought to 40% sucrose in 10 mM Tris-HCl (pH 7.4) was placed in the bottom of a centrifuge tube and overlaid with a discontinuous gradient consisting of equal volumes of 27% sucrose in 10 mM Tris-HCl (pH 7.4), 10% sucrose in 10 mM Tris-HCl (pH 7.4) and 5% sucrose in 10 mM Tris-HCl (pH 7.4). After centrifugation at $100\,000 \times g$ for 90 min the purified membrane coated beads were collected from the 10–27% interface and washed three times with Buffer II by centrifugation at $12\,000 \times g$ for 10

min. The phagocytic vesicles were used immediately for asymmetry studies.

Labelling analysis of labelled lipids

The lipids were extracted from an aliquot of the membrane blebs [13] or phagocytic vesicles [9] and their total phosphorus content determined by the method of Rouser [14]. Using the resulting value the membrane preparations were diluted in Buffer II and freshly prepared stock of TNBS at 10 mg/ml in Buffer II (pH 8.3), or isethionylacetimidate at 100 mg/ml Buffer II (pH 8.3) was added to the membranes to yield a final mole ratio of PE: amidating reagent = 1 : 5. The mixture was incubated at 4°C in the dark. At zero time and at increasing times thereafter an aliquot (usually 0.5 ml) was removed and placed in 4 drops of HCl on ice. The lipids were extracted immediately as above. The samples may then be stored at −20°C under nitrogen for periods up to one month.

The bleb lipids were separated on Eastman Silica Gel Chromagram TLC plates (without fluorescent indicator) in chloroform/methanol/water (65 : 25 : 4, v/v). Lipids from phagocytic vesicles were separated in chloroform/methanol 29% sodium hydroxide (65 : 35 : 5, v/v). The unreacted PS and PE spots identified by co-chromatographed standards were cut out, the lipids extracted and the final lipid phase dried under nitrogen. The lipids were then resuspended in 1.5 ml 0.43 M sodium borate and +0.13 M potassium phosphate (pH 9.0) and 0.5 ml of 0.02% fluorescamine (Roche) in acetone added while vortexing. The samples were read immediately in a fluorimeter and an excitation wavelength of 390 nm and an emmission wavelength of 475 nm. The percent aminophospholipid remaining was calculated by dividing the fluorescence intensity at the given time point by the fluorescence intensity at time zero and multiplying the result by 100. Membranes from $(7.5–10) \cdot 10^7$ cells were used for each time point.

Lipid compositional analysis

Lipids were extracted from the blebs by the method of Bligh and Dyer]13] and from the phagocytic vesicles as described elsewhere [9]. The lipids were chromatographed on Adsorbosil 5 Prekote TLC plates (Applied Science) using a solvent system of chloroform/methanol/water/acetic acid (75 : 45 : 8 : 2, v/v). The resulting spots were visualized with iodine vapors and compared to those of standards. The lipids were scraped from the plate and assayed for phosphorus by the method of Rouser [14].

Agglutination by concanavalin A

Fibroblast and myoblast blebs and myoblast vesicles were isolated as described above except that calcium and magnesium-free phosphate-buffered saline was used for the final washes. The blebs or vesicles were diluted to a final concentration of approx. 50 mg protein/ml calcium and magnesium-free phosphate-buffered saline and placed in 5 ml Erlenmeyer flasks. Concanavalin A, at a final concentration of 0.02 mg/ml, was added and the membranes incubated at 37°C on a reciprocal shaker at 50 rpm for 60 min. Aliquots were then removed and counted under the microscope. The percent agglutination was determined by dividing the number of aggregated blebs or vesicles by the total number of blebs or vesicles counted and multiplying the result by 100. Mannose at a final concentration of 10 mg/ml was added to parallel flasks at the start of the incubation to determine the background aggregation.

Myoblasts, after 48 h in culture, were washed three times with calcium and magnesium-free phosphate-buffered saline, harvested with 0.5 mM EDTA in calcium and magnesium-free phosphate-buffered saline, and washed three times by centrifugation for 3 min at $800 \times g$. The final cell pellet was resuspended in a small volume of calcium and magnesium-free phosphate-buffered saline and the cells counted using a hemocytometer. Approx. $3 \cdot 10^5$ cells in a total volume of 1 ml calcium and magnesium-free phosphate-buffered saline were incubated and scored as described above.

Results

Characterization of membrane preparations

Plasma membranes were derived from two sources: chemically induced membrane blebs and membrane coated latex spheres produced by phagocytosis. The phospholipid headgroup composition of blebs derived from myoblasts and fibroblasts along with those derived from myoblast

TABLE I

PHOSPHOLIPID HEADGROUP COMPOSITION IN DIFFERENT MEMBRANE PREPARATIONS

Plasma membranes as either blebs or phagocytic vesicles were prepared from 48-h cultures of chick embryonic myoblasts or from fibroblasts as described in Material and Methods. The lipids were extracted, separated by TLC, and the spots analyzed for phosphorus content. Values presented are the mean of at least two separate determinations ±S.D. The values for the myoblast membranes prepared by the sucrose gradient method are those of Kent et al. [11]. Fibroblast membranes were also prepared by the sucrose gradient method as described in Ref. 11.

Cell type	Preparation	% headgroup composition			
		PC	PE	PS + PI	Sph
Myoblasts	Blebs	51 ±2	27±2	9.0±0	14.7±0
	Vesicles	54 ±7	25±5	11.0±0	11.6±0
	Sucrose gradient	51	31	6.8	11
Fibroblasts	Blebs	54 ±3	24±3	7.0±0.5	14.6±0.8
	Sucrose gradient	56.6±0.4	26±2	6.1±0.6	16.7±0.6

phagocytic vesicles are compared to each other and to the published values obtained using membranes derived by homogenization and sucrose gradient fractionation in Table I. For both cell types, analysis of the three preparations agree within the estimated experimental error. Phosphatidylcholine (PC) and PE are the most abundant phospholipids: comprising approx. 50% and 28%, respectively, of the total phospholipid. PS plus phosphatidylinositol (PI) and sphingomyelin each contribute approx. 9% and 12%, respectively.

The orientation, or sidedness, of the blebs and phagocytic vesicles is determined by their mode of formation: the blebs are right-side-out and the phagocytic vesicles are inside-out derivatives of the plasma membrane. We have confirmed these orientations by assuming that the plasma membrane carbohydrates face externally and measuring the concanavalin A mediated agglutination of both membrane preparations (Table II). Approx. 90% of the blebs derived from either myoblasts or fibroblasts participate in a mannose-inhibitable, concanavalin A-mediated agglutination. Whole cells display nearly identical values. In contrast, the phagocytic vesicles did not agglutinate above the background values although it should be noted that because they are small and very sticky these background values are high. These observations agree with the previous sidedness determinations on membrane blebs derived from other cell types [12,15].

Amidation of fibroblast aminophospholipids

Fibroblast derived blebs incubated with TNBS (mole PE/mole TNBS = 1 : 5) at 37°C showed a rapid decrease in percent PE remaining unlabelled.

TABLE II

CONCANAVALIN A MEDIATED AGGLUTINATION OF BLEBS, PHAGOCYTIC VESICLES, AND CELLS

Plasma membranes blebs and phagocytic vesicles at 50 mg protein/ml and cells at $3 \cdot 10^5$/ml were incubated for 60 min at 37°C, with shaking at 50 rpm. Aliquots were scored for agglutination in the microscope. The agglutination was calculated by dividing the number of agglutinated particles (membranes or cells) by the total number counted and multiplying the result by 100. The values without error bars represent single, duplicate determinations. Abbreviations: CMF-PBS, calcium and magnesium-free phosphate-buffered saline; Con A, concanavalin A.

Incubation medium	Myoblast blebs	Myoblast vesicles	Myoblast cells	Fibroblast cells
CMF-PBS	10.3±2.9	42.6±4.4	7.9±1.6	11.8±0.2
CMF-PBS + Con A	84.7±4.9	44.4±3.7	88.4±3.1	87.1±3.9
CMF-PBS + Con A + mannose	23.8	46.1±7.3	14.1	11.5±3.2

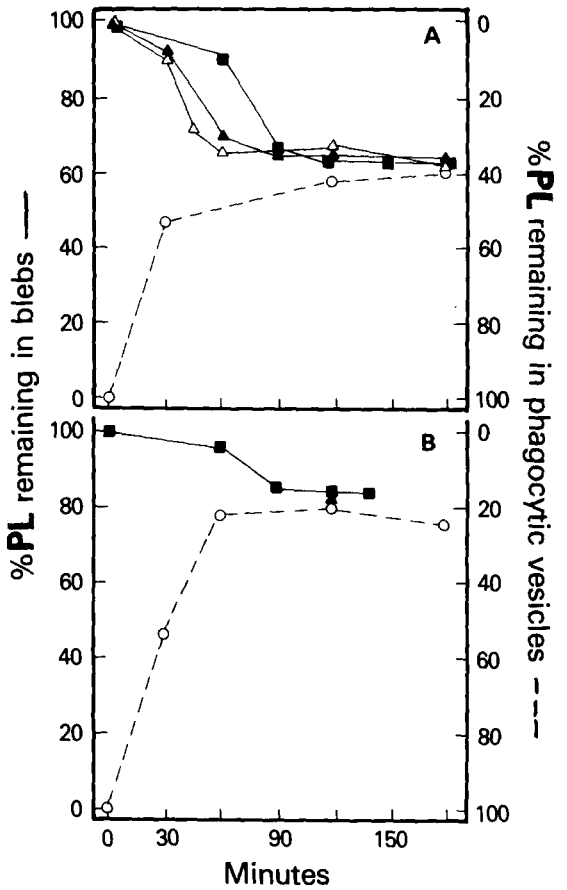

Fig. 1. The labelling kinetics of (A) PE and (B) PS in embryonic chick fibroblast blebs reacted at 4°C with TNBS at mole PE/mole TNBS = 1 : 2.5 (△———△) or 1 : 5 (▲———▲); blebs labelled with isethionylacetimidate (■———■); and phagocytic vesicles labelled with TNBS (○— — —○). All values, presented as % unreacted phospholipid, were calculated by dividing the fluorescence at the given time point by the fluorescence at zero time and multiplying the result by 100. Note the inverted scale for the phagocytic vesicles.

Within 120 min, 90–95% of the PE was labelled (data not shown). In contrast the labelling kinetics at 4°C displayed a plateau after 90 min of incubation that persisted for at least an additional 90 min (Fig. 1A). Identical results were obtained when the concentration of TNBS was halved. In both cases the plateau was observed when 65% of the PE remained unlabelled. Similar results were obtained for the kinetics of PE labelling by isethionylacetimidate in fibroblast blebs (Fig. 1A). The plateau was reached after a somewhat longer incubation, approx. 120 min, occurring when 65% of the PE remained unlabelled. The labelling kinetics of fibroblast phagocytic vesicles with TNBS at 4°C also displayed a plateau. It occurred after a similar time, however 40% of the PE remained unlabelled.

The isethionylacetimidate or TNBS labelling kinetics of PS in fibroblasts was analogous to that just described for PE. However, the plateau occurred when 83% and 23% of the PS remained unlabelled in the blebs and phagocytic vesicles, respectively (Fig. 1B).

Amidation of myoblast aminophospholipids

Experiments identical to those described above were performed for blebs and phagocytic vesicles

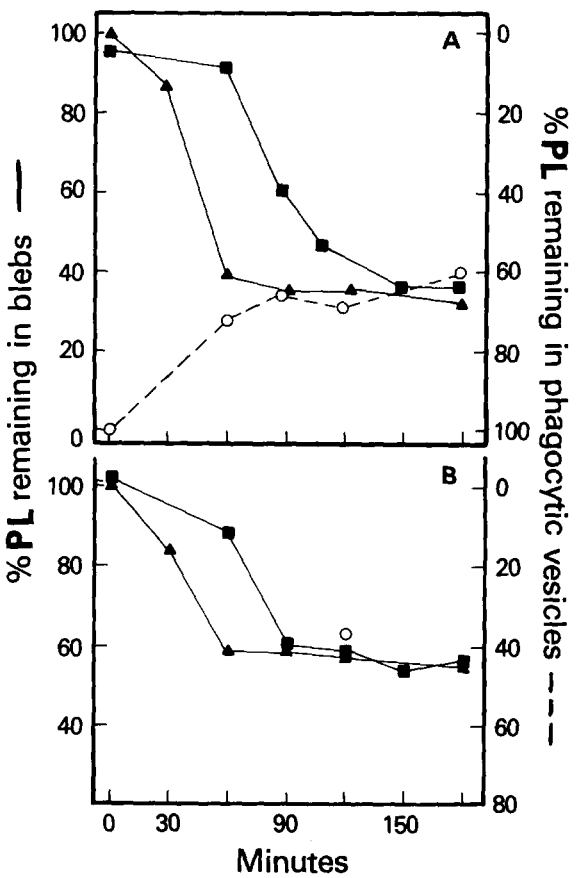

Fig. 2. The labelling kinetics of (A) PE and (B) PS in 48 h chick embryonic myoblast blebs labelled at 4°C with TNBS at mole PE/mole TNBS = 1 : 5 (▲———▲), blebs labelled with isethionylacetimidate (■———■) and phagocytic vesicles labelled with TNBS (○— — —○). Note the inverted scale for the phagocytic vesicles.

TABLE III

AMINOPHOSPHOLIPID DISTRIBUTION IN JAPANESE QUAIL MYOBLASTS AND L_6 CELLS

Plasma membrane blebs from 48-h cultures of Japanese quail myoblasts or from unfused or fusing cultures of L_6 cells were reacted with TNBS at mole PE/mole TNBS = 1.5 for 120 min at 4°C. The % PS or PE on the inner leaflet represents the amount of unreacted lipid. The data are the mean of at least two separate determinations. The standard deviations were less than 13% of the mean.

Cell type	State	%PS		%PS	
		Outer leaflet	Inner leaflet	Outer leaflet	Inner leaflet
Japanese quail myoblast	Fusing	43.9	56.1	73.2	36.8
L_6	Unfused	27.8	72.2	28.8	71.2
L_6	Fusing	21.3	78.7	30.1	69.9

derived from chick myoblasts after 48 h in culture. The labelling kinetics of PE for blebs showed plateaux after 90 min with TNBS and 120 min with isethionylacetimidate. In both cases plateaux were maintained for at least an additional 60 min. As described for the fibroblasts, the two labels yielded the same plateaux values, however in contrast to the fibroblast labeling, the plateaux were obtained when approx. 40% of the PE remained unreacted (Fig. 2A). The TNBS-labelling kinetics of PE in myoblast phagocytic vesicles again demonstrated a plateau after 90 min of incubation, and again, in contrast to the fibroblasts, 65% of the PE remained unlabelled.

Fig. 2B shows the labelling kinetics of PS in chick myoblast blebs and phagocytic vesicles. Plateaux were observed after 45% of the bleb PS had been labelled with either TNBS or isethionylacetimidate. When phagocytic vesicles were reacted with TNBS a plateau was reached when 37% of the PS remained unlabelled.

Blebs derived from 48–50 h cultures of quail

TABLE IV

AMINO PHOSPHOLIPID DISTRIBUTION IN FIBROBLASTS AND MYOBLASTS AS A FUNCTION OF CULTURE AGE AND MEDIUM COMPONENTS

Myoblasts were grown for between 24 and 240 h in CF-growth medium. To some cultures AraC was added after 24 h in culture to a final concentration of 0.6 μM. BUdR was added at a final concentration of 3.2 μM at time of plating. When cells were maintained for longer than 48 h in BUdR, fresh BUdR was added every 3 days for 8 days. The medium was then removed and replaced with CF-growth medium. AraC was added the following day and cells used 24 h later. Fibroblasts were grown as described in Materials and Methods. Blebs made from each of these cells were reacted with TNBS (mole PE/mole TNBS = 1 : 5) for 120 min at 4°C. Lipids were extracted and analyzed as described previously [20]. The amount of lipid described as being in the inner leaflet is the amount that remained unlabelled. Values represent the mean of 2–4 separate determinations. The standard deviations were less than 15% of the mean values. Abbreviations: CFDMEM, CF-growth medium; DMEM, growth medium; AraC, cytosine arabinoside; BUdR, 5'-bromodeoxyuridine.

Cell type	Hours in culture	State	Medium	%PS		%PE	
				Outer leaflet	Inner leaflet	Outer leaflet	Inner leaflet
Myoblast	24	Unfused	CFDMEM	43.7	56.3	–	–
	48	Fusing	CFDMEM	44.7	55.3	62.3	37.7
	48	Fusing	CFDMEM + AraC	46.9	53.1	65.7	34.2
	96	Fusing	CFDMEM	42.6	57.4	66.3	33.7
	48	Unfused	CFDMEM + BUdR	46.0	54.0	68.5	31.5
	240	Unfused	CFDMEM + BUdR	51.2	48.8	–	–
	96	Unfused	CFDMEM	19.0	81.0	34.1	65.9
Fibroblast	240	Proliferating	DMEM	17.2	82.8	34.2	65.8

myoblasts were labelled with TNBS (mole PE/mole TNBS = 1 : 5) for 120 min at 4°C. The results, presented in Table III, show that 56% of PS and 37°C of PE remained unlabelled. Table III also shows the extent of TNBS labelling in blebs from fusing and unfused cultures of L_6 cells. In infused L_6 72% of the PS and 71% of the PE remains unlabelled after incubating for 120 min at 4°C similar results were obtained when blebs from fusing cultures of L_6 were used, i.e. 79% of the PS and 70% of the PE remains unlabelled.

Developmental changes in aminophospholipid amidation

Blebs were obtained from chick myoblasts at three different ages in culture: 24 h (prefusion), 48 h (fusing), and 96 h (post-fusion). They were labelled with TNBS for 120 min at 4°C. The fraction of labelled PE and PS (64% and 45%, respectively) was the same for prefusion, fusing and fused myoblasts (Table IV). In addition, the TNBS-labelling kinetics of BUdR-treated myoblasts, an agent which induces proliferation without differentiation, were identical to those seen for fusing myoblasts. Overgrowth of proliferating cells such as fibroblasts occurs when AraC was omitted from older (96 h) cultures. Blebs from these cultures were incubated with TNBS as described above. Table IV shows that 19% of the PS and 34% of the PE was labelled, a result the same as that obtained for fibroblasts.

Influence of calcium levels on aminophospholipid labelling

Myoblasts were grown for 48 h in the presence of low (200 μM), moderate (1 mM), or normal (1.8 mM) levels of calcium. Blebs were prepared from these cultures and reacted with TNBS for 120 min at 4°C (Table V). The amidation of blebs derived from cells grown under either condition displayed plateaux when 45–50% of the PE and 62–65% of the PS had reacted. Thus the calcium level does not modulate the labelling kinetics.

Transbilayer movement of PE

When blebs derived from either myoblasts or fibroblasts were incubated with TNBS at 37°C, greater than 90% of the PE was labelled by 120 min. This result could occur either if TNBS penetrated the blebs at the higher temperature, or if rapid, transbilayer movement were occurring. To distinguish between these two possibilities, blebs from fibroblasts or myoblasts were incubated with TNBS at 4°C (non-penetrating conditions). After all available PE had reacted, i.e. the plateau was reached, the excess TNBS was removed and the blebs were incubated at 37°C for 120 min. This was followed by TNBS labelling at 4°C. 64% of the PE remained unlabelled compared to 66% PE remaining unlabelled prior to the temperature shifts. This suggests that complete labelling at the higher temperature results from the permeability of the blebs to the TNBS rather than to rapid transbilayer migration.

TABLE V

EFFECT OF CALCIUM ON MYOBLAST ASYMMETRY

Embryonic chick myoblasts were grown for 48 h in CF-growth medium, CF-growth medium containing 0.3 mM EGTA or in growth medium. Blebs were prepared and reacted with TNBS for 120 min at 4°C. Lipids were separated by TLC and quantitated. Values represent the mean of 2–4 separate determinations. The standard deviations were less than 15% of the means. Abbreviations: CFDMEM, CF-growth medium; DMEM, growth medium.

Medium	Calcium level	%PS		%PE	
		Outer leaflet	Inner leaflet	Outer leaflet	Inner leaflet
CFDMEM + EGTA	Low (< 0.2 mM)	44.7	55.3	62.3	37.7
CFDMEM	Medium (~ 1 mM)	45.9	54.1	63.5	34.5
DMEM	High (1.8 mM)	50.0	50.0	63.4	36.6

Discussion

Previous attempts to identify changes in the lipids of fusing myoblasts have focused on their composition in the plasma membrane. During development in cultures, no significant changes that correlate with fusion have been reported [5]. The phospholipid analysis reported here also reveal no significant differences between myoblasts and fibroblasts and thus are in agreement with the earlier observations.

In the absence of significant compositional changes during myogenesis, we have looked for changes in lipid organization. Since the aminophospholipids are implicated in fusion [16–18] and are believed to reside preferentially on the cytoplasmic side of the bilayer [19,20] their orientation in myogenic cells is of interest and importance.

The use of amidating reagents provides a precedented and well documented approach to measuring lipid orientation. In this paradigm, established by previous studies [12,21,22], the complete labelling observed at higher temperatures reflects the potential accessibility and reactivity of the total pool of plasma membrane aminophospholipids. The plateaus observed at lower temperatures reflect the labelling of the externally oriented phospholipid which results from the probe's inaccessibility to those oriented toward the cell interior. This interpretation appears valid for the labelling of the myogenic and fibrogenic cells reported here. First, the plasma membrane blebs and phagocytic vesicles have opposite polarity as implied both by their mechanism of formation and by concanavalin A agglutinability; the labelling plateaus for both PE and PS are likwise complementary. Second, the aminophospholipid orientation determined for the chick fibroblasts agrees very well with other determinations for fibroblast derived cells.

Continuing this paradigm, therefore, the labelling plateaux reflect relative lipid orientation and demonstrate signficant differences in the orientation of myoblast and fibroblast aminophospholipids. Thus while only 35% and 20% of the PE and PS, respectively, are externally oriented in fibroblasts, 65% and 45% of the PE and PS, respectively, are externally oriented in myoblasts. This corresponds to a 2–3-fold enrichment. Such an arrangement has not previously been observed using these reagents on plasma membranes from animal cells [12,19,22].

An obvious rationale behind the enrichment in the externally orientated aminophospholipids of myoblasts is to produce a more fusion prone lipid phase. Recently Deutsch and Kelly [21] reported that the aminophospholipid orientation of synaptic vesicles, representing another fusing system, is very similar to that of the myoblasts. Thus, the two fusing membranes studied to data exhibit similar lipid orientations.

Observations using myoblasts from 11-day-old Japanese quail embryos, show a distribution of aminophospholipids virtually identical to that observed in the chick myoblasts. In contrast our measurements of the aminophospholipid distribution in L_6, a rat myogenic cell line, yields values very close to those observed in chick fibroblasts. However L_6 fuses relatively slowly, and their aminophospholipid orientation may in fact provide a rationale for this observation.

Although the external orientation of aminophospholipids likely contributes to fusion, it alone does not confer fusion competence. The period of active fusion occurs between approx. 35 and 72 h in culture. The external enrichment in aminophospholipid extends beyond this period; it is observed in 24 h as well as in 96 h cultures. In addition, BUdR-treated cultures, which remain in a proliferative mode an neither fuse nor exhibit other properties characteristic of terminal differentiation, display this unique distribution. These observations on 24 h and BUdR-treated cultures suggest that the external orientation appears prior to terminal differentiation. In previous publications evidence has been presented suggesting that fusion competence is more likely determined by the synthesis of proteinaceous components [10]. Thus, it remains to be demonstrated whether the relatively high external concentration of aminophospholipid is necessary but not sufficient for fusion, or alternatively, whether it simply modulates the rate.

The role of aminophospholipids in promoting the fusion of model lipid vesicles is clearly documented, and it is by implication that we assume that any alteration in the composition or orientation of this lipid class in the plasma membrane of

a fusing cell, like the myoblast, reflects its fusability. This implication, however obvious and likely, must be considered in light of (1) the small number of cell types that have been analyzed for their aminophospholipid orientation and (2) the other membrane related functions of muscle. Among terminally differentiated cells for example, it is unclear whether the asymmetry of fibroblasts and erythrocytes is in fact typical and provides a general basis for comparison. Among less diffferentiated cells, a basis for comparison has yet to be established. In addition to fusion, muscle displays other membrane phenomena, including excitability, to which the unusual aminophospholipid orientation may contribute and provide part if not of its raison d'etre.

The functional association of the membrane aminophospholipids with fusion provides potential clues concerning the rationale of membrane asymmetry in general. The high concentrations of aminophospholipids on the cytoplasmic surface of most cells and on the external surface of cytoplasmically located membrane vesicles may thus facilitate fusion mediated exocytic processes. If this view is correct, one might then query whether the orientation observed in the myoblast is optimal for one or both fusion processes or whether it represents a compromise to allow processes to occur on both membrane surfaces. Fusion related process do not provide the only purpose for lipid orientations. It has already been demonstrated, for example, that an internal location for PE would tend to allow highly curved membrane structures [23]. In any case, a further stimulus has been provided for the systematic study of model systems possessing different lipid compositions.

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References

1 Kalderon, N. and Gilula, N.B. (1979) J. Cell. Biol. 81, 411–425
2 Horwitz, A.F., Wight, A., Ludwig, P. and Cornell, R. (1978) J. Cell Biol. 77, 334–357
3 Elson, H.F. and Yguerabide, J. (1979) J. Supramol. Struct. 12, 47–61
4 Prives, J. and Shinitsky, M. (1977) Nature (Lond.) 268, 761–763
5 Kent, C., Schimmel, S.D. and Vagelos, P.R. (1974) Biochim. Biophys. Acta 360, 312–321
6 McKay, J., Hakamori, S.I. and Nameroff, M. (1979) J. Cell Biol. 83, 36a
7 Nakornchai, S., Falconer, A.R., Fisher, D., Goodall, A.H., Hallinan, T. and Lucy, J.A. (1981) Biochim. Biophys. Acta 643, 152–160
8 Reporter, M. and Raveed, D. (1978) Science 181, 863–865
9 Sessions, A. and Horwitz, A.F. (1981) FEBS Lett. 134, 75–78
10 Knudsen, K. and Horwitz, A.F. (1977) Dev. Biol. 58, 328–338
11 Scott, R.E. (1976) Science 194, 743–745
12 Sandra, A. and Pagano, R.E. (1978) Biochemistry 17, 332–338
13 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917
14 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496
15 Scott, R.E. (1978) Cell. Differ. 7, 335–343
16 Cullis, P.R. and Hope, J. (1978) Nature 271, 672–674
17 Düzgünes, N., Wilschut, J., Fraley, R. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 642, 182–195
18 Hui, S.W., Stewart, T.P. and Boni, L.T. (1981) Science 212, 921–923
19 OpdenKamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
20 Rothman, J.E. and Lenard, J. (1977) Science 195, 741–752
21 Deutsch, J.W. and Kelly, R.B. (1981) Biochemistry 20, 378–385
22 Gordesky, S.E., Marinetti, G.V. and Love, R. (1975) J. Membrane Biol. 20, 111–132
23 Michaelson, M., Horwitz, A.F. and Klein, M.P. (1974) Biochemistry 13, 2605–2612