MYOBLAST AMINOPHOSPHOLIPID ASYMMETRY DIFFERS FROM THAT OF FIBROBLASTS

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1. Introduction

Contemporary theories of fusion processes have focused on the lipids of cell membranes. The dominant rationale for this view is the absence of freeze-fracture particles at putative fusion sites [1,2], the action of lipophilic reagents as promotors of fusion [3], and the observation that suitably composed lipid vesicles fuse [3,4,5].

Studies of model lipid bilayers specifically implicate two lipids as potentially key molecules in fusion. One of these is PS. Vesicles containing this lipid fuse in the presence of calcium [5,6]. The other major aminophospholipid, PE, forms a hexagonal II-type phase that is reported to be present in fusing cells and is postulated to be an intermediate in that process [7]. The hypothesized role in fusion of these two lipids poses an interesting problem since asymmetry studies on erythrocytes [8,9] and a cultured mammalian cell line [10] indicate a predominantly internal location for these two classes of lipids.

The fusion of mononucleate myoblasts into multinucleate myotubes, a key step in the production of muscle fibers provides a convenient system for studying a biologically significant fusion [11]. Here, we present the unexpected finding that when compared to fibroblasts, the myoblast exterior is markedly enriched in both PS and PE. This result is consistent with theories implicating their participation in fusion.

Abbreviations: complete DMEM, calcium-free Dulbecco's minimal essential medium containing glutamine, 20 μg $CaCl_2$/ml, 1% penicillin streptomycin, 10% horse serum and 2.5% chick embryo extract; calcium-free complete DMEM, as above, except that calcium chloride is omitted; EGTA, ethyleneglycol(bis-β-amino-ethyl ether) *N,N'*-tetra acetic acid; BN, 120 mM sodium bicarbonate and 40 mM sodium chloride (pH 8.15); TNBS, 2,4,6-trinitrobenzene sulfonic acid; IAI, isethionyl acetimidate; Hepes–Hanks, Hanks balanced salt solution with 20 mM Hepes (pH 7.4); PS, phosphatidylserine; PE, phosphatidylethanolamine; borate buffer, 0.43 M sodium borate and 0.013 M potassium phosphate, monobasic

2. Materials and methods

2.1. *Cells*

Fibroblasts were obtained from 11-day chick embryos as in [13]. The cells 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 in [13,14]. After digestion for 10 min in 0.1% dispase II (neutral protease from *Bacillus polymyxa*, Boehringer Mannheim Biochemicals) cells were plated on gelatin-coated 100 mm tissue culture dishes in calcium-free DMEM. Cytosine arabinoside at 0.6 μM final conc. was added 30 h after plating to kill proliferating cells such as fibroblasts. Occasionally EGTA at 200 μM final conc. was added 24 h after plating to produce fusion competent myoblasts: this had no effect on PS or PE asymmetry results. The myoblasts were harvested 48–50 h after plating.

2.2. *Membrane preparations*

The blebbing procedure is a modification of that in [15] in which the vesiculation solution was prepared in Hepes–Hanks and the cells were incubated for 1–2 h with shaking at 100 rev./min. Vesicles were used immediately for asymmetry experiments.

The isolation of phagocytic vesicles is essentially

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that in [10] in which phagocytosis of 1.1 μM polystyrene spheres is followed by cell lysis and centrifugation through a discontinuous sucrose gradient. The phagocytic vesicles were washed twice with BN by centrifugation at 12 000 × *g* and used immediately.

2.3. *Membrane labelling*

When blebs were to be labelled with TNBS, the lipids from an aliquot of the final bleb preparation were extracted, and the total phospholipid concentration was determined as in [16]. A freshly prepared solution of TNBS (Aldrich, recrystallized 5 times from 3 M HCl) at 10 mg/ml in BN was added to the vesicles at final conc. 1:5 or 1:2.5 (mol PE:mol TNBS). Unless indicated, the blebs were incubated at 4°C in the dark. Aliquots were removed immediately after the addition of the TNBS (0 min) and at varying times afterwards and placed in 4 drops 1 M HCl at 0–4°C to terminate the reaction. The lipids were extracted as in [17].

TNBS labelling of the phagocytic vesicles was accomplished by adding a freshly-prepared solution of TNBS to 1 mM final conc. Incubation and sampling were carried out as above. The lipids were extracted by stirring the aliquots overnight at 4°C in 95% ethanol. The beads were then centrifuged at 12 000 × *g* for 10 min and the lipid-containing supernatant was dried under a vacuum. The lipids were then extracted as in [17].

When the blebs were to be labelled with IAI, the phospholipids concentration was determined as above and a freshly-prepared solution of IAI (Sigma) at 100 mg BN/ml (pH 8.3) was added to the blebs at final conc. 1:100 (mol PE:mol IAI). Incubation, sampling and extraction of lipids were carried out as above.

2.4. *Lipid analysis*

The extracted lipids were spotted onto a heat-activated Eastman Chromagram TLC plate and chromatographed in either chloroform:methanol:water (65:25:4) when labelled with TNBS or chloroform:methanol:29% ammonium hydroxide (65:35:5) when labelled with IAI. Standards were chromatographed at the same time to locate the PS and PE. The lipids were visualized with iodine vapor, and the spots containing the PS and PE were cut out. The lipids were extracted as in [17] and the chloroform phase was dried under a stream of nitrogen. The extracted lipids were resuspended in 1.5 ml borate buffer by vortexing and 0.5 ml freshly prepared 0.02% fluorescamine (Roche) in acetone was added while vortexing. The fluorescence was read at 390 nm excitation wavelength and 475 nm emission wavelength.

3. Results and discussion

We have chosen two membrane preparations for these studies. One is based on the procedure [15] in which the plasma membrane is induced to slough off membrane blebs producing rightside-out plasma membrane. The other preparation derives from endocytosis (phagocytosis) of latex spheres, yielding inside out membrane vesicles. Analysis of the membrane lipids (in preparation) indicates that the phospholipid composition using either technique and for both types of cells are similar to results using another membrane preparation [7,19]. EM analysis [15] and assays of marker enzymes in [5,20] and ourselves suggest that the blebs are free of internal organelles. The phagocytic vesicles have been shown to be free of contaminating lipids, sealed, and of uniform sidedness [10]. As we discuss below, the labelling studies affirm the conjuncture that these membranes are in fact closed and display the expected sidedness. In addition, we have demonstrated that concanavalin A agglutinates blebs but not phagocytic vesicles (in preparation).

The kinetics of TNBS labelling of fibroblast blebs and phagocytic vesicles is presented in fig.1. As shown for PE, the labelling reaction which is performed at

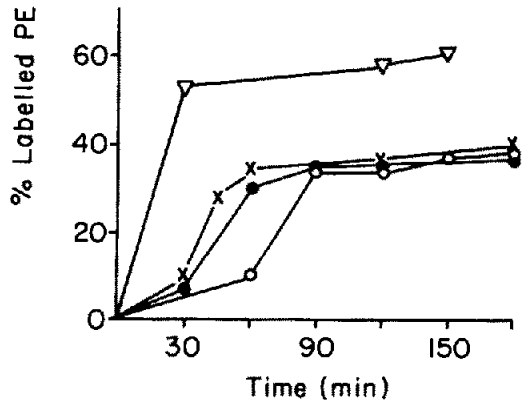

Fig.1. Percent labelled PE in fibroblast blebs reacted with TNBS at mol PE:mol TNBS = 1:2.5 (•——•) or 1:5 (x——x) and fibroblast blebs labelled with IAI (o——o) and fibroblast phagocytic vesicles labelled with TNBS (▽——▽). Values are calculated as [1-(fluorescence at given time point/fluorescence at 0 min)] × 100. See section 2 for isolation of the plasma membranes and labelling techniques.

Table 1
Aminophospholipid asymmetry in fibroblasts and myoblasts

Cell type	Membrane prep.	Label	% PE		% PS	
			out	in	out	in
Fibroblasts	Blebs	TNBS	34.2 ± 1.9	65.8 ± 1.9	17.2 ± 0.6	82.8 ± 0.6
		IAI	36.8 ± 0.7	63.2 ± 0.7	14.2 ± 0.2	85.5 ± 0.2
	Phagocytic vesicles	TNBS	40.7 ± 2.7	59.3 ± 2.7	22.9 ± 3.4	77.1 ± 3.4
Myoblasts	Blebs	TNBS	62.4 ± 3.3	37.6 ± 3.3	45.0 ± 0.4	55.0 ± 0.4
		IAI	60.8 ± 2.5	39.2 ± 2.5	45.6 ± 1.4	54.4 ± 1.4
	Phagocytic vesicles	TNBS	64.5 ± 2.3	35.5 ± 2.3	37.0 ± 3.4	63.0 ± 3.4

Plasma membranes of fibroblasts and myoblasts were obtained as in section 2 and labelled with TNBS for 120 min or IAI for 150 min. Values given are av. 3–5 separate determinations ± SEM

4°C reaches a plateau after ~90 min that persists at least through 180 min. This plateau is independent of TNBS over a 2-fold concentration range and occurs after roughly 35% and 65% of the PE in the blebs and phagocytic vesicles, respectively, is labelled. When the reaction is performed at 37°C however, no plateau is observed and ~95% of the PE is labelled after 120 min incubation. Thus virtually all the aminophospholipid reacts with the TNBS at this temperature. A highly precendented and straightforward interpretation is that TNBS penetrates the membrane at 37°C but is impermeable at 4°C [10], and hence the observed plateau represents the aminophospholipid on the outer monolayer. The complementarity in the labelling when phagocytic vesicles are used supports this interpretation.

Another amino-directed reagent, IAI, that is structurally dissimilar to TNBS, displays a labelling plateau that is nearly identical to that just described for TNBS (fig.1). The plateau forms after nearly the same extent of labelling as that seen with TNBS, i.e., 36.8% of the PE has been labelled.

The PS asymmetry in fibroblasts was assayed by the different determinations above: blebs with TNBS, blebs with IAI and phagocytic vesicles with TNBS. At 37°C virtually all PS is reacted with TNBS after 120 min incubation (not shown), while at 4°C a plateau is observed (table 1). Thus ~15–20% of the PS is on the outer leaflet and 80–85% on the inner leaflet of fibroblasts. The fibroblasts therefore appear to display an asymmetry in PS and PE, two aminophospholipids. The values for PE and PS agree with those obtained for other cells [8,10].

The labelling kinetics of PE and PS using IAI or TNBS in membrane blebs or phagocytic vesicles derived from myoblasts are virtually identical, with one exception, to those just described for fibroblasts. A plateau is again reached after 90 min for TNBS and 120 min for IAI-labelled membranes at 4°C. However, the extent of labelling at the plateau in myoblasts is markedly different from that of fibroblasts. Using membrane blebs, the reaction reaches a plateau after 45% of the PS and 60–65% of the PE is reacted. The

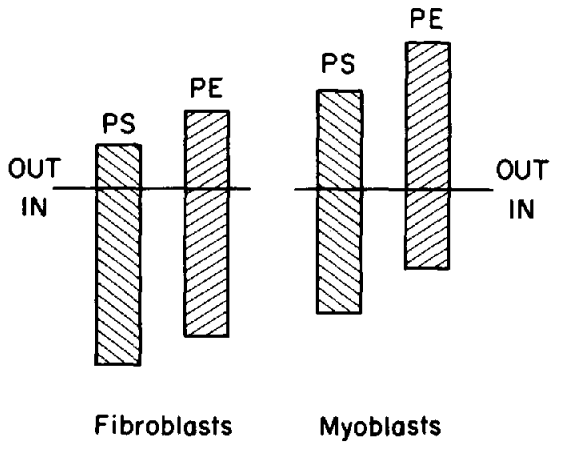

Fig.2. Schematic representation of the asymmetric distribution of PS and PE in the plasma membranes of myoblasts and fibroblasts.

extent of reaction in the phagocytic vesicles again complements these values, i.e., 65% of the PE and 37% of the PS is externally disposed (table 1).

The contrast between the aminophospholipid asymmetry of the fibroblasts and myoblasts is striking and significant. As reported for all the other animal cell plasma membranes examined [8–10] the fibroblasts have ~20% of their PS and 35% of their PE externally disposed. The myoblast data is unique in this regard being enriched ~2-fold in externally disposed PS and PE (fig.2).

At the very least, these results argue against a general preference for cytoplasmically disposed aminophospholipids. One is tempted, however, to speculate that this unusual arrangement of plasma membrane lipids in myoblasts is in fact germaine to fusion. The nature of the asymmetry is consistent with this notion and support extrapolations from model system studies implicating aminophospholipids in biological fusion.

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