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Lysolipids reversibly inhibit Ca^{2+}-, GTP- and pH-dependent fusion of biological membranes

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Membrane fusion in exocytosis, intracellular trafficking, and enveloped viral infection is thought to be mediated by specialized proteins acting to merge membrane lipid bilayers. We now show that one class of naturally-occurring phospholipids, lysolipids, inhibits fusion between cell membranes, organelles, and between organelles and plasma membrane. Inhibition was reversible, did not correlate with lysis, and could be attributed to the molecular shape of lysolipids rather than to any specific chemical moiety. Fusion was arrested at a stage preceding fusion pore formation. Our results are consistent with the hypothesis that biological fusion, irrespective of trigger, involves the formation of a highly bent intermediate between membranes, the fusion stalk.

Membrane fusion; Lysolipid; Exocytosis; Viral fusion; Mast cell; Baculovirus; Sea urchin; Microsome

1. INTRODUCTION

Because disparate biological fusion systems share structural, antigenic and functional traits, it has been proposed that they might also share a common mechanism and key fusion intermediates [1–4]. If these hypothetical common intermediates involve lipid molecules, the composition of membrane lipid bilayers may modulate fusion reactions. In spite of an extensive literature on the role of lipid composition in the fusion of purely lipid bilayers [5], little is known about the lipid requirements for biologically relevant fusion [6–8].

Addition of lysophosphatidylcholine to the contacting monolayers of artificial planar lipid membranes was shown to inhibit their monolayer fusion [9]. Lysolipids, which have a molecular shape of an inverted cone, were hypothesized to increase the elastic energy of a highly bent stalk between membranes [9]. In the present paper we will show that lysolipids inhibit four diverse biological fusion processes: sea-urchin cortical granule exocytosis [10,11], mast cell degranulation [12], rat liver microsome–microsome fusion [13] and syncytia formation of baculovirus infected insect cells [14] triggered by Ca^{2+}, GTP-γ-S, GTP and H^+, respectively. Our results reverse the long-held view that lysolipids promote biologically relevant fusion [15] and suggest the formation of stalk-type fusion intermediates as a possible common step in the different fusion processes.

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Abbreviations: GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate); CSC, cell surface complex; R-18, octadecyl rhodamine B; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PEG, polyethylenglycol.

2. MATERIALS AND METHODS

2.1. *Lipids and detergents*

Lipids were purchased from Avanti Polar Lipids (Birmingham, AL), except for palmitic acid and glycerol monopalmitate which were purchased from Nucheck (Elysian, MN), and [^{14}C]palmitoyl LPC from Amersham (Arlington Heights, IL). Stock solutions were prepared as a 0.5% (w/w) aqueous dispersion. In some experiments lipid stock solutions were prepared as a sonicated dispersion or in ethanol at 50 mg/ml. All three procedures gave similar results. Detergents were purchased from Pierce (Rockford, IL) and prepared as an aqueous dispersion.

2.2. *Calcium induced fusion of sea urchin cortical granules*

Granule exocytosis was followed using a light scattering assay in planar isolated sea urchin cortices [16], in cortical granule–cortical granule fusion [17], and in cell surface complex (CSC), prepared as described [18,19] from *Lytechinus pictus* eggs in PKME buffer (50 mM PIPES, pH 6.7, 425 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA). CSC's were mixed with various concentrations of added lipids in a final volume of 100 μl. One hundred microliters of either PKME buffer or PKME + calcium (final free calcium concentration was 330 μM) was added to each sample and the absorbance at 405 nm was measured by a microtiter-dish reader. Absorbance values were plotted as $100 \times (A_{405nm}$ with lipid + calcium) − (A_{405nm} with lipid − calcium)/(A_{405nm} no lipid + calcium) − (A_{405nm} no lipid − calcium). Absorbance of CSC's in PKME with no lipids added was typically 0.4 OD.

2.3. *pH-induced fusion of insect cells infected by baculovirus*

SF 9 insect cells (10^5 per well) were grown and infected with 10^6 pfu/ml wild-type baculovirus AcNPV as described [14]. Fusion was triggered by incubating the cells for 10 min in medium at pH 5.0.

Following the incubation, the buffer was replaced with normal pH 6.4 medium. To study the effects of lysolipids on fusion, cells were incubated in 0.5 ml either in the presence or absence of lysolipids for 15 min (5 min before and 10 min during low pH treatment of cells). After one hour, the ratio of nuclei within syncytia to the total number of cell nuclei in the same field was counted as described [14].

2.4. GTP-dependent fusion of rat liver microsomes

Fusion was detected in the fusion system previously described [13] by an octadecyl rhodamine B chloride (R 18) fluorescence dequenching assay [20], except that the final concentrations of microsomal protein, GTP and Mg^{2+}, were 0.22 mg/ml, 194 μM, and 0.98 mM, respectively. Microsome suspensions (204 μl) were incubated with exogenous lipids for 3 min at 37°C before GTP was added to trigger fusion. Dequenching of R 18 was not observed in the absence of GTP. Maximum dequenching was determined after solubilization of membranes with 2% Triton X-100. The rates of fusion were obtained by a linear regression analysis of the initial portions of the dequenching kinetic curves.

2.5. GTP-γ-S-triggered exocytosis in Beige mouse mast cells

Mast cells were obtained from female Beige mice (C57BL/6N-bg) by peritoneal lavage, and cell degranulation triggered by 10 μM of GTP-γ-S was studied as described [21]. To measure membrane capacitance we applied sinusoidally varying voltage with 1 kHz frequency and 50 mV peak-to-peak amplitude.

3. RESULTS AND DISCUSSION

The addition of exogenous lysolipids to the medium resulted in fast (within 5 min) and dose-dependent inhibition of cortical granule exocytosis, baculovirus infected cell–cell fusion, and microsome–microsome fusion (Fig. 1a). This inhibition did not depend upon the choice of experimental assay or preparation: we measured similar dose-response curves of inhibition by LPC, in cell surface complex exocytosis (Fig. 1a), isolated planar cortex exocytosis (see below), and isolated cortical granule co-fusion ([17] (n = 2, data not shown).

Inhibition was also observed in patch clamp studies of GTP-γ-S triggered exocytosis in mast cell. Inclusion of LPC in the pipette internal solution inhibited the usual step-wise changes in capacitance which correspond to single degranulation events (Fig. 1b). In 13 out of 15 experiments, in the absence of LPC, the first fusion event occurred in less than 5 min with an average delay time of 2 min. In the presence of LPC there was no fusion in 13 out of 16 experiments (cells were observed for at least 5–10 min). In the remaining 3 cells, the first fusion event occurred on average 4.5 min after establishment of whole-cell configuration.

The concentrations of LPC needed for inhibition varied significantly between experimental systems studied. This variation, and, in particular, the significantly higher concentrations required for inhibition of cell–cell fusion can be explained by differences in the amount of biological material present, rate of lipid metabolism, and/or composition of membranes (including the difference in the composition of the inner and outer leaflets of membranes).

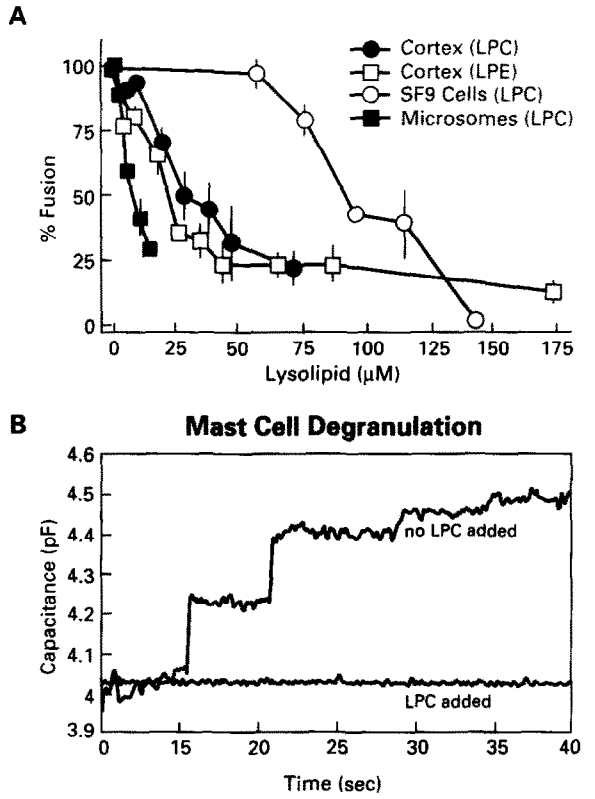


Fig. 1. Inhibition of biological membrane fusion by lysolipids. Panel A: lysolipid-induced inhibition of calcium-triggered cortical exocytosis (oleoyl LPC (●), oleoyl lysophosphatidylethanolamine, LPE (□)); pH-induced fusion of insect cells infected by baculovirus (oleoyl LPC (○)); GTP-dependent fusion of rat liver microsomes (oleoyl LPC (■)). Membrane fusion was assayed as described below. Each point is mean ± S.E., n = 3, normalized to fusion response in the absence of exogenous lipid. Added LPC did not change the buffer free calcium or pH (not shown). Panel B: capacitance traces of mast cells in the presence and absence of 9.6 μM oleoyl LPC in the pipette solution. The time at which the whole-cell configuration was established is taken as zero.

Since transient changes in membrane capacitance were not detected in LPC-treated mast cells, and dequenching of membrane dye was not detected with LPC-treated microsomes, we conclude that lysolipids arrest fusion at a stage preceding the formation of small pores which allow aqueous and lipid transfer.

For inhibition, LPC had to be added to the aqueous space between fusing membranes; when added to media external to mast cells, but not in the patch pipette, inhibition was not observed (n = 10).

LPC was previously thought to cause membrane fusion, but this was observed only at higher, lytic concentrations [15]. Inhibition of fusion did not correlate with lysis in our systems. For example, we measured a decrease in turbidity due to microsome lysis only above 200 μM palmitoyl LPC, while 9.3 μM LPC gave 50% inhibition of fusion (Fig. 2a). For different lysolipids and experimental systems the ratios between the lysol-

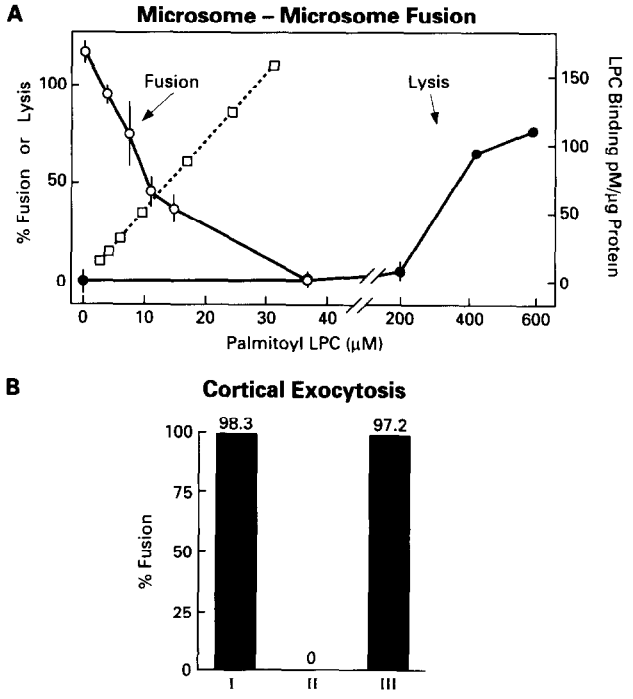


Fig. 2. LPC induced inhibition of fusion correlates with binding but not membrane lysis, and is completely reversible. A. Microsome fusion (○) was assayed as described above (n = 3). Subsequent to a 10 min incubation, palmitoyl LPC binding (□) was determined by centrifugation of microsomes at 10,000 × g for 5 min and measurement of the distribution of [^{14}C]palmitoyl LPC between microsome pellet and supernatant (n = 2). Lysis of microsomes (●) was detected by measuring the turbidity of the incubation mixture at 490 nm, normalized to the turbidity decrease after addition of 2% Triton X100 (n = 3). All points are mean ± S.E. B. Bar I shows the percent of granules in the sea urchin cortex fusing in response to perfusion with PKME buffer containing 313 µM calcium. If the cortex was first incubated with PKME containing 32 µM myristoyl LPC, there was no change in light scattering following perfusion with calcium (II). If the cortex was first treated with myristoyl LPC, then washed with 3 ml of PKME buffer to remove cortex associated LPC, and finally perfused with a buffer containing 313 µM free calcium, we saw a complete recovery of fusion activity (III). All points are mean ± S.E., n = 3. If we used 48 µM oleoyl LPC to inhibit fusion, we found that washing with even 10 ml of PKME did not reverse the inhibition (data not shown). In contrast, washing with 1 ml of PKME containing 2 mg/ml of delipidated bovine serum albumin to extract lysolipids from membranes [32] resulted in a 47 ± 20% (n = 3) recovery of fusion activity.

ipid concentration required to observe the onset of lysis and that causing more than 50% inhibition of fusion varied from 1.2 to >20 (see Table I).

LPC-induced inhibition was completely reversible. Isolated planar sea urchin cortices were perfused with solutions containing enough myristoyl LPC to inhibit fusion (Fig. 2b). When the LPC-treated cortices were washed with calcium-free solutions, subsequent perfusion with solutions containing 313 µM calcium led to complete fusion. In contrast, the fusion competence of cortices treated with oleoyl LPC could be restored only by perfusion with buffer containing delipidated bovine serum albumin. Presumably, oleoyl LPC is harder to extract from membranes because of its longer hydrocarbon chain [22]. Inhibition of pH induced syncytia formation by myristoyl LPC was also completely reversible (data not shown, n = 3). Thus, we can exclude solubilization and irreversible denaturation of cellular components as possible mechanisms of this inhibition.

We varied the chemical moieties of lysolipid to determine which were responsible for inhibition of fusion (Table I). Inhibition was observed for different combinations of hydrocarbon chains and polar heads. Since both charged and zwitterionic lysolipids suppressed fusion, the inhibition is not due to electrostatic interactions as described [23].

Different lipids have different molecular shapes in membranes and can be separated into three groups: cones, cylinders and inverted cones, on the basis of comparing the surface area of the lipid polar head with the area of a cross-section of hydrocarbon tail [24]. All the compounds in Table I which inhibited membrane fusion are micelle-forming amphiphiles and have the shape of inverted cones, i.e. they have large polar heads and small hydrophobic tails. Dioleoyl phosphatidylcholine (PC) differs from oleoyl LPC by one chain, yet it did not inhibit fusion either because of its cylindrical shape [25] or because it did not incorporate into the membrane [26]. However, even dicapryl PC (cylinder) and palmitic acid (cone), which readily incorporate into membranes [27,28] were not potent inhibitors. In any case, the lack of inhibiting activity of PC suggests that head group binding is not responsible for LPC inhibition.

Experiments with synthetic non-ionic surfactants have shown that the inhibition is not caused by any products of lysolipid biological degradation or transformation and supported the suggestion that an inverted cone shape of the amphiphile molecule rather than a specific chemical group is responsible for the inhibition. Tween 80 has the same oleate hydrophobic tail as oleoyl LPC, and a relatively large polar head whose structure is quite different from that of LPC (see Table I). At concentrations much below those which cause lysis, Tween 80 reversibly inhibited fusion in the two systems tested suggesting a common mechanism with lysolipids for inhibition. Lauroyl derivative of the same surfactant, Tween 20, also inhibited fusion. In contrast, Span 20, having a molecular shape closer to cylindrical than that of Tween 20, caused no inhibition.

There was no correlation between the lysolipid concentration required for inhibition and lysolipid critical micelle concentration (CMC). For example, lauroyl and stearoyl LPC inhibited fusion below and above their CMC's respectively (Fig. 3). These results, together with the aforementioned observation that fusion competence was hard to restore by perfusion in the case of longer hydrocarbon chain lysolipids, did not prove but suggested that inhibition was caused by the LPC incorpo-

rated into membranes. We determined that 1.2 μM of palmitoyl LPC bound/mg microsome protein resulted in 50% inhibition. The binding constant of LPC to microsomes was $\sim 10^6$ M^{-1}, close to that published [22] for erythrocytes, $\sim 3 \times 10^5$ M^{-1}, but we do not know if this value corresponds to membrane incorporated LPC or just bound.

One possible explanation for how amphipathic molecules inhibit triggered membrane fusion is that they bind to crucial protein components of the fusion machinery. While we cannot eliminate the possibilities of a direct action of lysolipids on a fusion protein, such as binding to proposed hydrophobic fusion peptides, the wide range of biological systems inhibited, including myoblast fusion [27], suggests a physicochemical mechanism of inhibition common to all the systems studied, perhaps related to properties of membrane lipids. LPC promotes the later stage of phospholipid bilayer fusion,

TABLE 1

FUSION INHIBITION AND LYSIS IN PRESENCE OF DIFFERENT AMPHIPHILES

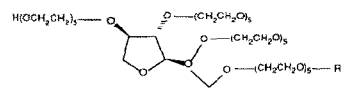
Biological Amphiphiles

Name	R1	R2	R3	System[a]	50% inhibition	Onset of lysis[b]
egg lysophosphatidylcholine	Mix	OH	PCg	1_2	10^2 mg/ml	3.75×10^2 mg/ml
platelet-activating factor	Et Mix	Ace	PCg	1_2	4×10^2 mg/ml	none up to 0.25 mg/ml
liver lysophosphatidylinositol	Mix	OH	PIg	2	3×10^2 mg/ml[c]	7.5×10^2 mg/ml
brain lysophosphatidylserine	Mix	OH	PSg	2	2.5×10^2 mg/ml	7.5×10^2 mg/ml
egg lysophosphatidylethanolamine	Mix	OH	PEg	2	2×10^2 mg/ml	3×10^2 mg/ml
lysophosphatidylethanolamine (C18:1)	Ole	OH	PEg	1_3	26 µM	none up to 522 µM
lysophosphatidylcholine (C12)	Lau	OH	PCg	1_3	67 µM	none up to 400 µM
lysophosphatidylcholine (C14)	Myr	OH	PCg	1_3	8 µM	30 µM
				1_2	16 µM	53 µM
				2	64 µM	11 µM
lysophosphatidylcholine (C16)	Pam	OH	PCg	1_3	30 µM	100 µM
				2	72 µM	160 µM
				3	9.3 M	200M
lysophosphatidylcholine (C18)	Ste	OH	PCg	1_2	26 µM	193 µM
				2	120 µM	150 µM
lysophosphatidylcholine (C18:1)	Ole	OH	PCg	1_3	29 µM	72 µM
				1_2	9.5 µM	19 µM
				2	90 µM	146 µM
				3	8.4 µM	n.d.
phosphatidylcholine (C6/C6)	Hxo	Hxo	PCg	1_3	none up to 550 µM	none up to 550 µM
phosphatidylcholine (C10/C10)	Dec	Dec	PCg	1_2	440 µM[d]	none up to 440 µM

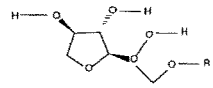
Biological Amphiphiles

Name	R1	R2	R3	System[a]	50% inhibition	Onset of lysis[b]
phosphatidylcholine (C18:1/C2)	Ole	Ac	PCg	1_2	90 µM	none up to 450 µM
phosphatidylcholine (C18:1/C18:1)	Ole	Ole	PCg	1_2	none up to 320 µM	none up to 320 µM
				2	none up to 380 µM	none up to 380 µM
				3	none up to 320 µM	none up to 320 µM
glycerolmonopalmitate	Pam	OH	OH	2	none up to 550 µM	none up to 550 µM
palmitic acid	Pam	-	-	2	none up to 1 mM	none up to 1 mM

Non-Biological Amphiphiles



R = Lauroyl for Tween 20
R = Oleoyl for Tween 80



R = Lauroyl for Span 20

Tween-80	1_3	0.0045%	none up to 1%
	1_2	0.00125%	n.d.
	2	0.15%	none up to 1.2%
Tween-20	1_3	0.0025%	none up to 0.1%
	1_2	<0.05%	n.d.
Span-20	1	none up to 1%	none up to 1%

Abbreviations

(C18:1) (C12) and so on show the chain length and number of double bonds. Mix - stands for mixed chains of natural lipids. Et - ether lipid, Ole - oleoyl, Lau - lauroyl, Myr - myristoyl, Pam - palmitoyl, Ste - stearoyl, Hxo - hexanoyl, Dec - decanoyl, OH - hydroxyl, Ac - acetyl. PCg - phosphocholine group, PIg - phosphoinositol group, PSg - phosphoserine group, PEg - phosphoethanolamine group. none - not observed, n.d. - not determined.

a -Systems used: (1) Fusion triggered by calcium in (1_3) isolated sea urchin planar cortex, (1_2) sea urchin CSCs and (1_3) cortical granule/cortical granule fusion, (2) pH induced fusion of Sf 9 cells infected by baculovirus, (3) GTP-dependent fusion of rat microsomes.

b -as determined microscopically for cortex, by light scattering for CSCs and microsomes, and by testing cell viability for insect cells using trypan blue exclusion test.

c -The inhibition at this concentration was 100%.

d -The inhibition at this concentration was only 28%.

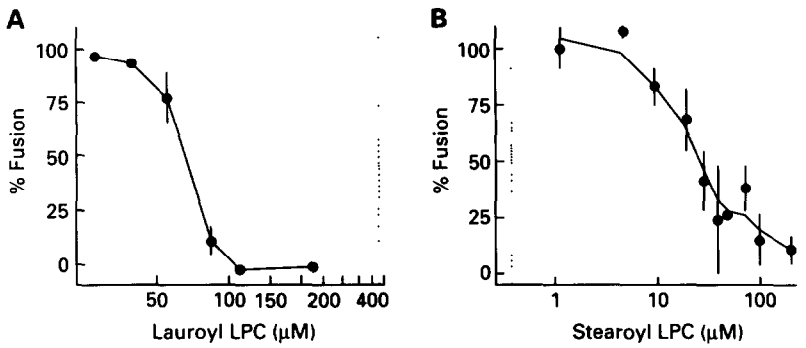


Fig. 3. Membrane fusion inhibited by lysolipids both below and above their CMC. Dose–response curves for (A) lauroyl LPC (sea urchin cortices) and (B) stearoyl LPC (sea urchin CSCs). All points are mean ± S.E., $n = 3$. The CMC values (vertical dotted lines) for lauroyl and stearoyl LPC are 430 μM and 0.4 μM, respectively [33].

the joining of aqueous compartments, when incorporated into distal leaflets of contacting planar membranes [28], and in the case of PEG-induced fusion of liposomes [29]. However, adding LPC between two planar lipid bilayers inhibits monolayer fusion, an early stage of fusion in these membranes [9]. The incorporation of micellar (inverted cone) molecules into contacting monolayers is hypothesized [9] to restrict formation of a highly curved stalk, a fusion intermediate [9,28] which connects two membranes. Biological membranes may also require local bending of monolayers to form fusion intermediates. Alternatively, an increase in surface area of contacting membrane leaflets, resulting from asymmetric intercalation of lysolipids, may cause inhibition through compression of proteins and buckling of membranes. Consistent with these physicochemical mechanisms, we have shown that many micelle-forming substances can inhibit biological membrane fusion, regardless of the specific chemical moieties they contain. Indeed, other membrane active compounds such as amphipathic peptides, local anesthetics, spermicides and surfactants may inhibit membrane fusion in a similar way.

We conclude that lysolipids, considered for a long time as putative biological fusogens [15], do not promote but, in contrast, reversibly inhibit four different fusion reactions including both viral fusion and granule exocytosis. If the introduction of lysolipids into contacting leaflets inhibits membrane fusion by preventing formation of curved fusion intermediates such as stalks [9,28], our data suggest that these lipid-involving intermediates are common between disparate processes of biological membrane fusion. Because lysolipids are tightly regulated biological compounds [30], present in nearly all biological membranes analyzed [31], it is intriguing to speculate that cells control local lysolipid concentrations to regulate fusion processes.

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