Diacylglycerol effects on phosphatidylinositol-specific phospholipase C activity and vesicle fusion

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Received 16 February 2001; accepted 6 March 2001

First published online 23 March 2001

Edited by Marco Baggiolini

Abstract Diacylglycerol increased the hydrolytic activity of phosphatidylinositol-specific phospholipase C on large unilamellar vesicles containing 5–40% phosphatidylinositol. Moreover, diacylglycerol increased the rate and extent of vesicle fusion (contents mixing) induced by the enzyme. Kinetic studies of intervesicular lipid mixing revealed that fusion was limited by the frequency of contacts involving two diacylglycerol-rich domains.

Key words: Phosphatidylinositol-dependent phospholipase C; Diacylglycerol; Membrane fusion; Membrane hemifusion; Liposome aggregation

1. Introduction

Diacylglycerol (DAG) is a potent regulator of cell membrane physiology (see [1] for a review). Apart from its well-known role in cell signalling [2], it may modify the bilayer properties thus modulating the activities of a number of membrane-related enzymes [1,3,4], and even facilitate bilayer-to-non-bilayer transitions, and subsequent membrane fusion/fission events [1,5,6].

In previous studies from this laboratory we have dealt with the structural effects of a phosphatidylcholine-preferring phospholipase C (PC-PLC) on phospholipid vesicles (see [5] for a review). PC-PLC induces fusion (of electrically neutral vesicles) through formation of DAG [5,7]. Independently, DAG enhances the hydrolytic activity of the enzyme [3]. More recently, we have extended our observations to phosphatidylinositol-specific phospholipase C (PI-PLC). The latter enzyme, whose mechanism of action is rather different from that of PC-PLC, can also promote fusion of PI-containing vesicles through its hydrolytic activity, in spite of the net negative charge of the bilayers. In our recent study [8] we have shown that PI cleavage by PI-PLC is followed by vesicle aggregation, intervesicular lipid mixing, and mixing of vesicular aqueous contents, i.e. vesicle fusion [9]. It was also found that varying proportions of PI in the liposomal formulations lead to different physical effects of PI-PLC. Specifically, fusion was detected only with vesicles containing more than 20 mol percent PI [8].

The present study is devoted to the effects of DAG on the hydrolytic and fusogenic effects of PI-PLC. Presumably because of its propensity to induce inverted lipid phases [10], DAG activates PI-PLC as it does with a number of other enzymes [1]. Moreover, because of its actual implication in the formation of non-bilayer intermediates [7] the presence of DAG facilitates fusion of otherwise non-fusogenic liposomes. Finally, kinetic studies of intervesicle lipid mixing under fusogenic and non-fusogenic conditions indicate that fusion takes place through vesicle collisions involving DAG-rich domains.

2. Materials and methods

2.1. Materials

PI-PLC (EC 3.1.4.10) from *Bacillus cereus*, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS) *p*-xylene-bis(pyridinium bromide) (DPX), and octadecylrhodamine B (R_{18}) were supplied by Molecular Probes Inc. (Eugene, OR, USA). Dioleoylphosphatidylcholine (DOPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphorylethanolamine-*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-PE), and L-α-phosphatidylethanolamine-*N*-(lissamine-rhodamine-B-sulfonyl) (egg) (Rh-PE), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Egg PC, egg phosphatidylethanolamine (PE), PI, and DAG derived from egg PC were grade I from Lipid Products (South Nutfield, UK). Cholesterol (Ch), Triton X-100, bovine serum albumin (BSA) (essentially free from fatty acids), and DL-dithiothreitol (DTT) were from Sigma (St. Louis, MO, USA). All other materials (salts and organic solvents) were of analytical grade or better.

2.2. Methods

2.2.1. Vesicle preparation and characterization. The appropriate lipids were mixed in organic solution and the solvent evaporated to dryness under N_2. Solvent traces were removed by evacuating the lipids for at least 2 h. The lipids were then swollen in 10 mM HEPES, 150 mM NaCl, pH 7.5 buffer. Large unilamellar vesicles (LUV) were prepared from the swollen lipids by extrusion, and sized by using 0.1 μm pore-size Nuclepore filters, as described by Mayer et al. [11]. The average size of LUV was measured by quasi-elastic light scattering, using a Malvern Zeta-sizer instrument. Lipid concentration, determined by phosphate analysis [12], was 0.3 mM in all experiments.

2.2.2. Hydrolysis and aggregation assays. All assays were carried out at 39°C with continuous stirring, in 10 mM HEPES, 150 mM NaCl, pH 7.5 buffer, in the presence of 0.1% BSA for optimum catalytic activity. Enzyme concentration was 0.16 unit/ml and liposomal concentration was 0.3 mM. Enzyme activity was assayed by determination of water-soluble phosphorous contents in 50 μl aliquots re-

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Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt; Ch, cholesterol; DAG, diacylglycerol; DPX, *p*-xylene-bis(pyridinium bromide); LUV, large unilamellar vesicles; NBD-PE, 1,2-dimyristoyl-*sn*-glycero-3-phosphorylethanolamine-*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC-PLC, phosphatidylcholine-preferring phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C; Rh-PE, L-α-phosphatidylethanolamine-*N*-(lissamine-rhodamine-B-sulfonyl) (egg)

0014-5793/01/$20.00
PII: S0014-5793(01)02333-X

moved from the reaction mixture at defined intervals. Extraction with 250 μl chloroform/methanol/hydrochloric acid (200:100:3, by volume) stops the reaction. Two phases separate that can be assayed for phosphorus. Lipid aggregation was monitored in a Cary Varian UV-vesicle spectrometer as an increase in turbidity (absorbance at 450 nm) of the sample.

2.2.3. Lipid mixing assays. For total lipid mixing 0.6% NBD-PE, 0.6% Rh-PE vesicles were mixed with probe-free liposomes at a 1:4 ratio [13]. NBD-PE emission was followed at 530 nm (excitation wavelength at 465 nm) with a cutoff filter at 515 nm. 100% mixing was set after addition of 1 mM Triton X-100.

2.2.4. Fusion assays. Intervesicular mixing of aqueous contents was assayed according to Ellens et al. [14], with the ANTS/DPX system. Liposomes contained either (i) 25 mM ANTS and 100 mM NaCl, (ii) 90 mM DPX and 60 mM NaCl, or (iii) 12.5 mM ANTS, 45 mM DPX, and 85 mM NaCl. A Sephadex G-75 (Pharmacia) chromatography column was used to separate liposomes from non-encapsulated probes, using 10 mM HEPES, 150 mM NaCl, pH 7.5 buffer as eluent. Osmolality was checked using an Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany). Solutions were corrected for perfect isotonicity inside and outside the vesicles by adding small volumes of concentrated NaCl up to 0.310 (Osm/kg). 100 and 0% contents mixing levels were set according to Nieva et al. [15]. The 100% leakage signal was obtained by adding Triton X-100 to mark liposome lysis. Two correction factors were applied to contents mixing curves: light scattering by the fused vesicles and dissociation factor. Fusion scattering was monitored using a 1:1 mixture of vesicles containing 25 mM ANTS, 100 mM NaCl, and probe-free liposomes, at a 0.3 mM final lipid concentration. The dissociation factor intends to compensate for the leakage of aqueous contents that occurs shortly after the start of fusion. It is estimated by the sum of 'F+0.5D' (F: fusion fluorescence signal, D: dissociation signal from the pre-encapsulated probes), as described by Bentz et al. [16].

3. Results and discussion

PI-PLC hydrolysis of PI in LUV of the appropriate composition induced vesicle fusion. This is shown in Fig. 1 for liposomes containing PI:PE:PC:Ch (40:30:15:15), where PI hydrolysis and intervesicular lipid mixing are plotted in parallel.

The apparent K_s of PI-PLC for LUV of pure PI was of ca. 300 μM (A.V. Villar, unpublished). In the present study we used vesicles containing 5,10 or 40 mol percent PI, corresponding to 15, 30 and 120 μM PI respectively. As expected, the hydrolysis rate was highly dependent on bilayer composition (Fig. 2). When DAG was incorporated into the liposome formulation, at either 5 or 10 additional mol percent, enzyme activity increased in a dose-dependent way. It is interesting that DAG, an end-product of the enzyme reaction, is also

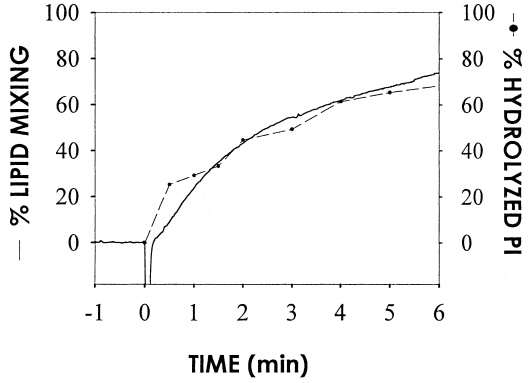

Fig. 1. PI-PLC hydrolysis and concomitant fusion of LUV consisting of PI:PE:PC:Ch (40:30:15:15). Dots: PI hydrolysis. Continuous line: intervesicular lipid mixing. Lipid and enzyme concentrations are respectively 0.3 mM and 0.16 U/ml.

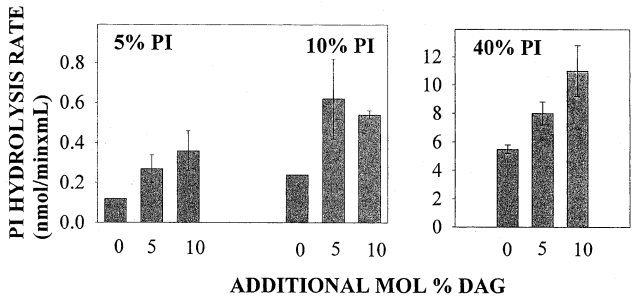

Fig. 2. Enhancement of PI-PLC hydrolytic activity by DAG. Enzyme rates were measured in LUV containing either PI:PE:PC:Ch (5:47:24:24), PI:PE:PC:Ch (10:46:22:22), or PI:PE:PC:Ch (40:30:15:15), to which 0, 5 or 10% DAG were added in the liposome preparation stage.

an activator of PI-PLC. DAG has been shown to activate a number of membrane-related enzymes, e.g. PC-PLC [3], CTP: phosphocholine cytidyltransferase [17] or monoacylglycerol acyltransferase [18] (see [1,19] for reviews). The mechanism of PI-PLC activation by DAG is presumably the same as in the above-mentioned enzymes, namely the DAG-induced in-

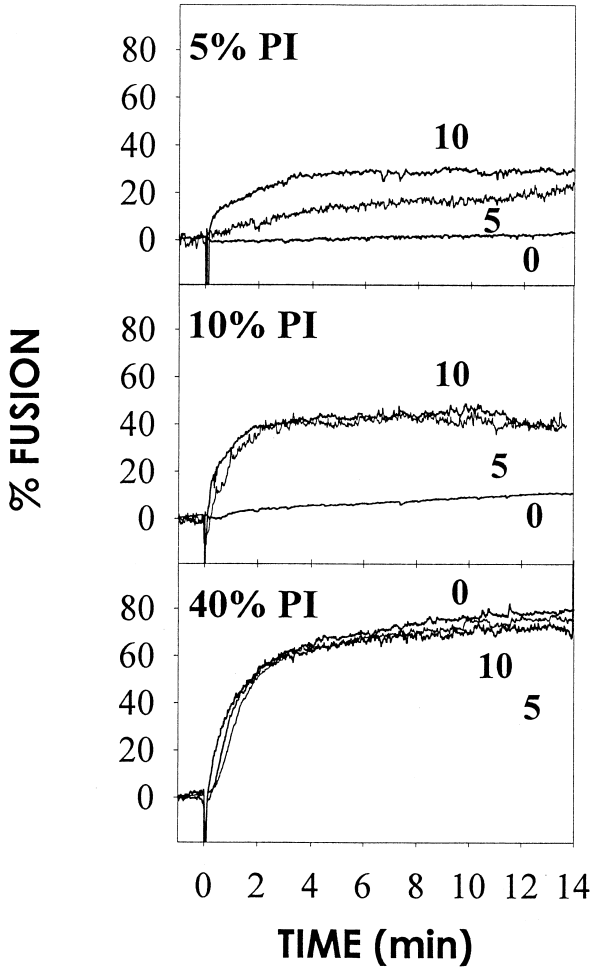

Fig. 3. Enhancement of PI-PLC-induced vesicle fusion (mixing of aqueous contents) by DAG. Lipid composition was, from top to bottom, 5, 10 and 40% PI. The compositions have been detailed in the legend to Fig. 2. In each case, curves for liposomes containing 0, 5 or 10% additional DAG are shown.

Table 1
Reaction order of vesicle aggregation and intravesicular lipid mixing processes, induced by PI-PLC

Lipid composition (PI:PE:PC:Ch mol ratios)	Reaction order[a]		
	Aggregation[b] (total lipid)	Lipid mixing[b] (total lipid)	Lipid mixing[c] (DAG)
40:30:15:15	1.8	1.2	1.8
30:36:17:17	1.8	1.3	1.7
20:40:20:20	2.1	1.6	0.9
10:46:22:22	2.1	1.4	0.9

[a]Reaction order is obtained from the slope of straight lines as shown in Fig. 4.
[b]Reaction order of vesicle aggregation, or lipid mixing, with respect to total lipid (vesicle) concentration.
[c]Reaction order of lipid mixing with respect to DAG concentration (as in Fig. 4).

crease in the bilayer propensity to form inverted phases, while remaining nevertheless in the lamellar form.

Apart from this quantitative increase in enzyme activity, DAG was also responsible for qualitative changes in the PI-PLC effects on liposomes. When fusion was assessed as intervesicular contents mixing, vesicles containing 40% PI in the bilayers showed extensive fusion, but this was not the case for vesicles containing 5 or 10% PI (Fig. 3). However, as shown also in the figure the presence of 5 or 10 mol percent DAG in the bilayer formulations allowed the observation of vesicle fusion as mixing of contents. The presence of a minimum amount of DAG was established as a requirement for fusion in the presence of PC-PLC [5,7], and the same explanation probably applies to the case of PI-PLC.

The kinetics of vesicle aggregation and fusion (measured as intervesicular lipid mixing) was further explored as a function of vesicle concentration. PI-PLC induces vesicle leakage concomitantly with mixing of aqueous contents [8], thus quantitative measurements of lipid mixing are more reliable than those of contents mixing. In our previous paper [8] it was shown that the kinetics of aggregation for vesicles containing 40% PI were second order with respect to total lipid concentration. The kinetics of intervesicular lipid mixing for those vesicles were first order with respect to total lipid concentration, but second order with respect to DAG concentration in the bilayers. These studies have now been extended to vesicles containing 30, 20, or 10% PI (Fig. 4). Lipid mixing occurs in all cases, but contents mixing is only detected with liposomes containing 40 or 30% PI [8]. Thus the mixtures containing 20 or 10% PI give rise to hemifusion, but not to complete fusion.

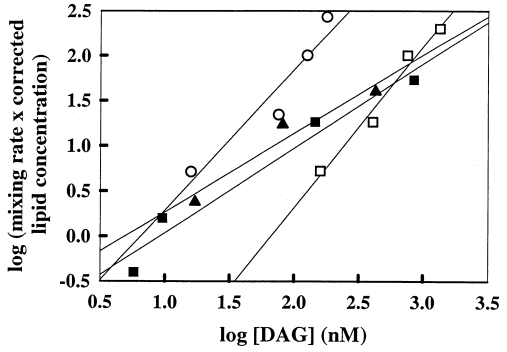

Fig. 4. Kinetics of lipid mixing as a function of DAG concentration in the bilayer, for lipid vesicles of varying lipid compositions. (□) PI:PE:PC:Ch 40:30:15:15, slope: 1.8, $r^2 = 0.98$. (○) PI:PE:PC:Ch 30:36:17:17, slope: 1.7, $r^2 = 0.91$. (■) PI:PE:PC:Ch 20:40:20:20, slope: 0.9, $r^2 = 0.96$. (▲) PI:PE:PC:Ch 10:46:22:22, slope: 0.9, $r^2 = 0.94$.

The kinetic data for vesicle aggregation and intervesicular lipid mixing are summarized in Table 1. All four mixtures show second-order dependence with respect to vesicle concentration for aggregation, but for lipid mixing, i.e. the pattern observed in [8] for 40% PI is repeated for the other lipid compositions. However, the study of the reaction order for lipid mixing with respect to DAG concentration (the latter measured 1 min after start of lipid mixing), provides the interesting result that the fusogenic, but not the non-fusogenic LUV display second-order dependence (Fig. 4). This is a novel and clear experimental indication that contact between two DAG-enriched domains is required for fusion to occur. Other intervesicular collisions, perhaps, may give rise to exchange of lipids in the outer monolayers, but not to pore formation.

The results presented in this paper support the role of DAG as a generator of 'sticky' surfaces for vesicle–vesicle docking. Previous data obtained with PC-PLC [3] indicated that DAG-rich 'patches' on the vesicle surface were involved in liposome aggregation. The in-plane segregation of DAG into domains enriched in this lipid is a well-known phenomenon [1]. Our data showing the second-order dependence of lipid mixing on DAG concentration in fusogenic, but not in non-fusogenic, vesicles highlight the importance of vesicle–vesicle collisions through DAG-enriched domains in the fusion event.

Acknowledgements: This work was supported in part by Grants from DGICYT (Spain) (PB96-0171), The Basque Government (Ex. 1999-5) and The University of the Basque Country (UPV 042.310 G03/98). A.V.V. was a pre-doctoral fellow of the Basque Government.

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