Anionic phospholipids and the regulation of cell functions

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Introduction

For over 25 years [1] it has been realized that a considerable phospholipid asymmetry exists across the bilayer of the biological membrane, particularly the plasma membrane, and this asymmetry plays a major role in cell function [2–6]. The asymmetry is best illustrated by the fact that the external monolayer of the mammalian cell is made up almost exclusively of neutral zwitterionic phospholipids such as phosphatidylcholine (PC) and sphingomyelin together with some phosphatidylethanolamine. In contrast, the monolayer facing the cytoplasm of the cell contains anionic phospholipids as a major component together with the majority of the phosphatidylethanolamine. Intracellular anionic phospholipids include phosphatidylinerine (PS), phosphatidylglycerol (PG) and cardiolipin (CL). A number of specific physiological roles have emerged for these anionic

Abbreviations used: PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylinerine; PG, phosphatidylglycerol; PL, phosphatidylinositol; PL-A2, phospholipase A2; FABP, fatty acid binding protein; DAUDA, 1-(dansylamino)undecanoic acid; sPLA2, secreted phospholipase A2; PLD, phospholipase D; CL, cardiolipin.
phospholipids that affect intracellular functions and, in the case of PS, the interaction of the cell with extracellular proteins. These biological functions are normally initiated by the binding of specific proteins to the phospholipid interface resulting in a conformational change linked to protein activation and the biological response.

The overall phenomenon can be represented as follows:

\[ P + \text{phospholipid}_{\text{in}} \rightleftharpoons P^* + L \rightleftharpoons P*L \rightarrow \text{biological response} \]

where \( P \) is a protein molecule, \( \text{phospholipid}_{\text{in}} \) is the phospholipid interface, \( P^* \) is the activated protein bound to the interface and \( P*L \) represents specific binding of a ligand linked to the biological response such as catalysis.

The interaction of proteins with anionic phospholipids as part of protein function can be broadly divided into two categories.

**Interfacial binding**

Interfacial binding (\( P \rightleftharpoons P^* \)) is relatively non-specific and requires the presence of a significant mol\% (normally >5 mol\%) of the anionic phospholipid at the membrane surface. The presence of the anionic phospholipid will facilitate the binding of the protein by initial electrostatic interactions. However, subsequent penetration of parts of the protein into the bilayer may occur as a result of perturbation of the membrane surface. Such perturbations of the interface will be facilitated by the presence of the anionic phospholipids [7].

**Specific binding**

This type of interaction (\( P^* + L \rightleftharpoons P*L \)) would occur between specific phospholipid molecules and a binding site or sites on the protein and would follow initial interfacial binding. Specific (substrate) binding is seen in the case of phospholipases and anticipated where the protein contains domains that recognize specific anionic phospholipids such as the pleckstrin homology domain for binding of structurally related phosphoinositides [8]. The same specificity argument applies to phospholipid transfer proteins such as PI transfer protein [9].

There is considerable interest in the role of anionic phospholipids in the interfacial binding and activation of proteins. The participation of anionic phospholipids in membrane–protein interactions has been reviewed in detail [3–7,10,11] with a particular focus on the ability of certain lipids to form non-lamellar phases [7]. Although such non-lamellar phases have yet to be observed in biological, as opposed to artificial membranes, it may be the propensity of such lipids to form such structures that may have critical effects on the quality of the interface and thus be the basis of membrane perturbation.

The driving force for an inverted phase is the effective shape or molecular geometry of the phospholipid. A smaller head group relative to the hydrophobic part of the molecule would provide the crucial shape to form inverted non-lamellar phases. Anionic phospholipids with relatively small headgroups, such as PS, phosphatidic acid (PA) and CL, together with calcium provide the necessary geometry for non-lamellar phases and a resultant perturbation of the interface. Following initial electrostatic interactions between the anionic interface and cationic regions of the protein surface, a number of steps linked to conformational changes in the protein may occur.

Interfacial events can involve membrane penetration by the protein, phospholipid extraction by the protein to specific binding sites or a combination of both. Membrane penetration may involve amphipathic helices, surface tryptophan residues, amino acids modified with acyl or prenyl groups, or effects linked to a high local concentration of \( \text{H}^+ \) associated with negatively charged phospholipid headgroups [10]. Phospholipid extraction and protein binding normally involves the presence of a non-polar cavity on the interfacial surface of the protein. Kinnunen [7] has argued that such phospholipid binding may involve a single acyl chain, the phospholipid thus acting as a bridge between protein and bilayer. The phenomenon of protein translocation to the interface can play a major regulatory role in protein function. Important enzymes that are regulated by translocation to the phospholipid surface include cytosolic phospholipase A\(_2\) (PLA\(_2\)) [12], protein kinase C [13] and CTP phosphocholine cytidyl transferase [14].

**Specific anionic phospholipids and their role in the regulation of cell functions**

**PS exposure, blood coagulation and apoptosis**

The classic physiological example of the bulk exposure of an ionic phospholipid on the external surface of the cell is that of PS. This phospholipid is normally located exclusively on the inner
monolayer surface of the plasma membrane and other internal cell membranes. The PS is actively transported from the outer to the inner leaflet of the plasma membrane by the amino-phospholipid translocase [3,4]. The exposure of PS to the external cell surface is a characteristic of platelet activation [6] and apoptosis [15]. This PS-containing interface is recognized by proteins involved both in blood clotting and macrophage phagocytosis. In addition annexin V, a calcium and phospholipid binding protein with a high affinity for PS, was first identified as a vascular anti-coagulant protein that bound with high affinity to such surfaces and inhibited blood coagulation [16,17]. Labelled annexin V now provides a standard marker for PS exposure in apoptotic cells [18].

PA exposure and the regulation of human (group IIa) secreted PLA₂

Perhaps one of the most intriguing aspects of externalized anionic phospholipids is their possible role in promoting the action of the human (Group IIa) secreted PLA₂ (human sPLA₂). The human enzyme was first identified at high concentration in the synovial fluid of patients with rheumatoid arthritis and is now known to be associated with a wide variety of inflammatory disorders where its concentration in extracellular fluids such as serum may rise in excess of 100-fold [19]. Despite its overall structural similarity to the destructive venom PLAs, we and others have demonstrated the virtual inability of this highly basic (pl > 10.5) enzyme to hydrolyze zwitterionic PC interfaces seen either in phospholipid vesicles or cell membranes [20–24]. Therefore the precise molecular mechanism by which this enzyme might become pro-inflammatory remains elusive especially since elevated levels of the human enzyme in transgenic mice does not appear to be associated with significant inflammatory pathology [25]. Indeed the primary role of human sPLA₂ is believed to be that of an acute-phase protein involved in the removal of infectious micro-organisms and damaged cells [26].

The possibility that the enzyme might be capable of attacking the perturbed membrane of normal cells, thus becoming pro-inflammatory, has been considered [27]. The extreme cationic nature of this enzyme and, in particular, the cationic nature of the interfacial surface of the enzyme point toward a dominant role for electrostatic interaction in interfacial binding while the presence of negative charge in the surface monolayer of the plasma membrane should both enhance binding of the enzyme and facilitate hydrolysis [22].

In a search for natural anionic phospholipids that might promote interfacial binding and hydrolysis of the plasma membrane by recombinant human sPLA₂ [28] we have investigated the potential role of PA in this process [21]. Not only does the addition of low concentrations of PA to vesicles prepared from PC enhance hydrolysis by human sPLA₂ but also pretreatment of pure PC-containing vesicles with phospholipase D (PLD) produce both a time- and dose-dependent increase in hydrolysis [21].

Of particular interest is the fact that pretreatment of whole cells in suspension with bacterial PLD dramatically enhances their susceptibility to human sPLA₂ [21]. This enhanced hydrolysis of cell suspensions can be measured directly using a continuous fluorescence displacement assay [29] in which released long-chain fatty acid displaces the fluorescent fatty acid 11-(dansylamino)-undecanoic acid (DAUDA) from liver fatty acid binding protein (FABP) that is present in the medium with a resulting loss in fluorescence. We observe that pretreatment of a cell suspension with bacterial PLD produces a time- and dose-dependent response such that the cell membrane becomes sensitive to hydrolysis by added human sPLA₂. Thus, hydrolysis is observed at a concentration as low as 100 ng/ml of human sPLA₂ in the medium whereas, prior to PLD treatment, in excess of 10 μg/ml of enzyme is often required to detect significant cell hydrolysis under these assay conditions. Blood levels of human sPLA₂ in septic shock can be in excess of 1 μg/ml.

In a separate study (A. D. Postle and S. M. Wright, personal communication) electrospray ionization mass spectrometry has been used to detect the formation of PA in HL 60 cell suspensions after pretreatment with added bacterial PLD. The spectra before and after treatment with PLD are shown in Figure 1. Part (b) shows the profile of molecular species of PA in the normal cell while part (a) shows the dramatic increase of PA resulting from PLD treatment, the molecular species reflecting the abundance of the equivalent PC species in untreated cells. This result directly confirms the capacity of
externally added PLD to hydrolyse cell surface PC.

If the PA were the preferred substrate for the human sPLA₂, then two potential types of pro-inflammatory lipid mediators would be produced, fatty acids including arachidonic acid and lysoPA. It is noteworthy that lysoPA has been observed after pretreatment of micro vesicles with human sPLA₂ [24] and this recombinant enzyme shows a clear substrate preference for PA [30]. It remains to be established if production of PA in the surface monolayer of intact cells in vivo can provide the necessary activation to initiate an inflammatory response in the presence of inflammatory levels of the human sPLA₂. Although there is no indication that PLD is

**Figure 1**

Electrospray ionization MS of PA molecular species isolated from HL60 cells treated with bacterial PLD

Phospholipids were extracted from 3 x 10⁶ HL60 cells after addition of 14:0/14:0 PA (0.5 nmoles) as internal standard. Anionic phospholipids were separated using aminopropyl solid phase extraction cartridges. Samples in chloroform were analysed in negative ionization mode using a Quattro II triple quadrupole mass spectrometer. The following PA molecular species were detected: 591, 14:0/14:0; 645, 16:0/16:1; 647, 16:0/16:0; 671, 16:0/18:2; 673, 16:0/18:1; 695, 16:0/20:4; 697, 18:1/18:2; 699, 18:1/18:1; 701, 18:0/18:1; 721, 18:1/20:4; 723, 18:0/20:4. (a) Cells treated with PLD (Genzyme, 50 Units/ml/10⁶ cells) for 30 min. At 37°C. (b) Basal levels, no PLD treatment.
observed extracellularly, an equivalent glycosylphosphatidylinositol PLD is found in serum [31] and could generate PA on the cell surface.

**PG, CL (diphosphatidyl glycerol) and the regulation of liver FABP function**

Liver FABP provides another example of a protein that can bind specifically to an anionic phospholipid interface. Liver FABP belongs to a family of small (14 kDa) cytosolic lipid binding proteins that includes FABPs from muscle, intestine and adipose tissue [32,33]. Although the precise physiological function of these abundant proteins remains obscure a buffering role is likely whereby the binding of the high concentrations of fatty acids associated with these tissues would prevent cellular damage [34]. Of particular interest is a vectorial role of FABPs in the transport and targeting of fatty acids to specific sites within the cell. Such a role has already been highlighted in the case of the intestinal, muscle and adipose proteins [35,36] where fatty acid transfer to model phospholipid membranes occurs by a collisional interaction. However, no such collisional mechanism was observed in the case of liver FABP and fatty acid transfer involving aqueous diffusion of the ligand was proposed [37].

We have observed that a collisional transfer mechanism is possible with liver FABP under appropriate conditions involving phospholipid vesicles containing specific anionic phospholipids. This collisional event could be detected because on addition of phospholipid vesicles to a highly fluorescent liver FABP/DAUDA complex, there was complete binding to the phospholipid interface and a dramatic fall in fluorescence due to release of the fluorescent ligand, DAUDA, from the FABP (Figure 2). On addition of more FABP to this assay (Figure 2) there was complete fluorescence recovery thus confirming that DAUDA was released from the phospholipid vesicle-bound FABP. The binding of the FABP to phospholipid vesicles showed phospholipid specificity with DAUDA release being most sensitive to CL and PG (A. E. A. Thumser, J. K. Davis and D. C. Wilton, unpublished work) while no ligand release was seen with PC or PE. The process was very ionic strength-dependent, demonstrating a major contribution of electrostatic binding, while complete dissociation of DAUDA from FABP required a molar ratio of phospholipid to protein consistent with the protein coating the anionic phospholipid interface. The binding process could be partially reversed, as judged by a recovery of fluorescence, by the addition of high salt (200 mM NaCl) or calcium (5 mM) and reversal was most apparent when the FABP bound to mixed vesicles contained both anionic phospholipid (PG) and PC. Under these conditions maximal reversal (>90%) also required the addition of ethanol suggesting that there were non-polar interactions between the protein and the interface. It would appear that the proportion of non-polar interactions depended on the density of anionic phospholipid in the interface. This type of binding has also been observed in the case of cytochrome c [38]. Overall the phenomenon of interfacial binding of liver FABP and ligand release provides an attractive mechanism for delivery of long-chain fatty acids to the membrane surface within the cell for further metabolism.

In conclusion, anionic phospholipids provide an environment for a wide range of protein interactions that facilitate interfacial binding. The probable underlying feature of these interactions is the propensity of anionic phospholipids to form non-lamellar structures thus perturbing the interface. This perturbation facilitates conformational changes in the protein linked to membrane penetration by the protein or, alternatively, phospholipid removal from the interface into
specific binding sites on the protein. These conformational changes in the protein may be used to modulate function.

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