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Lysophosphatidylcholines activate G2A inducing $G_{\alpha i-1}$ -/ $G_{\alpha q/11}$ - Ca^{2+} flux, $G_{\beta\gamma}$ -Hck activation and clathrin/ β -arrestin-1/GRK6 recruitment in PMNs

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Abstract

Lyso-PCs (lysophosphatidylcholines) are a mixture of lipids that accumulate during storage of cellular blood components, have been implicated in TRALI (transfusion-related acute lung injury) and directly affect the physiology of neutrophils [PMNs (polymorphonuclear leucocytes)]. Because the G2A receptor, expressed on PMNs, has been reported to recognize lyso-PCs, we hypothesize that lyso-PC activation of G2A causes the increases in cytosolic Ca^{2+} via release of G_{α} and $G_{\beta\gamma}$ subunits, kinase activation, and the recruitment of clathrin, β -arrestin-1 and GRK6 (G-protein receptor kinase 6) to G2A for signal transduction. PMNs were isolated by standard techniques, primed with lyso-PCs for 5–180 s, and lysed for Western blot analysis, immunoprecipitation or subcellular fractionation, or fixed and smeared on to slides for digital microscopy. The results demonstrated that lyso-PCs cause rapid activation of the G2A receptor through S-phosphorylation and internalization resulting in $G_{\alpha i-1}$ and $G_{\alpha q/11}$ release leading to increases in cytosolic Ca^{2+} , which was inhibited by an antibody to G2A or intracellular neutralization of these subunits. Lyso-PCs also caused the release of the $G_{\beta\gamma}$ subunit which demonstrated a physical interaction (FRET+) with activated Hck (haemopoietic cell kinase; Tyr⁴¹¹). Moreover, G2A recruited clathrin, β -arrestin-1 and GRK6: clathrin is important for signal transduction, GRK6 for receptor de-sensitization, and β -arrestin-1 both propagates and terminates signals. We conclude that lyso-PC activation of G2A caused release of $G_{\alpha i-1}$, $G_{\alpha q/11}$ and $G_{\beta\gamma}$, resulting in cytosolic Ca^{2+} flux, Hck activation, and recruitment of clathrin, β -arrestin-1 and GRK6.

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AUTHOR CONTRIBUTION

Samina Khan carried out the majority of the experiments, prepared the Figures and primarily wrote the article. Nathan McLaughlin helped with the majority of the experiments. Marguerite Kelher helped with the majority of the experiments, with writing the paper and in preparing the Figures. Phillip Eckels helped in some of the experiments, especially the calcium measurement. Fabia Gamboni-Robertson helped with experiments involving microscopy. Anirban Banerjee was involved in data analyses, critical thought and review of the microscopy work. Christopher Silliman designed all experiments, aided in writing this article, and provided laboratory space and materials for the experiments.

Keywords

fluorescent resonance energy transfer (FRET); G-protein-coupled receptor; intracellular neutralization; neutrophil

INTRODUCTION

Granulocytes express numerous G-protein-linked chemoattractant receptors that are vital for directed chemotaxis and allow neutrophils [PMNs (polymorphonuclear leucocytes)] to marginate to the tissues and localize to areas of infection/inflammation to eradicate pathogens and to promote tissue healing [1–5]. These GPCRs (G-protein-coupled receptors) are linked to a number of regulatory proteins that allow for ligand activation resulting in signal transduction and receptor desensitization [2,4,5]. Important to these processes are GRKs (G-protein receptor kinases) that phosphorylate the receptor, an important step in internalization and signalling through the recruitment of heterotrimeric G-proteins and β -arrestins [6–9]. The β -arrestins serve a dual role in both receptor-mediated signalling and receptor de-sensitization, whereas the heterotrimeric G-proteins comprises α , β and γ subunits, and are responsible for primary receptor signalling through regulation of ion channels or effector enzymes that lead to changes in cellular physiology [2,10–12].

Ligand activation of GPCRs causes recruitment and release of the G_α subunit, through binding of GTP, and the $G_{\beta\gamma}$ subunit, and both regulate a variety of specific second messengers in the cytoplasm [2,13]. In PMNs, the G_α subunits induce increases in cytosolic Ca^{2+} through the regulation of ion channels which allows release from both intracellular stores and influx from the extracellular milieu, as well as activating non-receptor Src family tyrosine kinases that may affect the Ras-linked pathways including the MAPKs (mitogen-activated protein kinases), especially p42/44 MAPK, and PI3K (phosphoinositide 3-kinase) [2,14–16]. Such increases in cytosolic Ca^{2+} are required for chemotaxis and the production and release of the components of the microbicidal arsenal; conversely G_α -mediated activation of adenylate cyclase increases cAMP which appears to inhibit PMN microbicidal function [17,18]. The $G_{\beta\gamma}$ subunit is known to directly activate phospholipase $C\beta$, leading to activation of PI3K in leucocytes, as well as affecting cytosolic Ca^{2+} concentrations and regulating other second messengers [13,19].

Lyso-PCs (lysophosphatidylcholines) are bioactive lipids that accumulate during routine storage of cellular blood components and induce numerous changes in PMN physiology including directed chemotaxis, priming of the oxidase, adherence to RGD (arginine-glycine-aspartate) ligands and degranulation [20]. In addition, lyso-PCs cause ALI (acute lung injury) and cytotoxicity in *in vivo* and *in vitro* models of PMN-mediated ALI and PMN-mediated endothelial damage respectively [21,22]. Lyso-PCs have also been implicated in TRALI (transfusion-related ALI) in human patients [23], are structurally similar to PAF (platelet-activating factor) and are the ligand for the G2A receptor, which is disparate from the PAFr (PAF receptor) [24–27]. Lyso-PCs cause rapid increases in cytosolic Ca^{2+} and, unlike PAF, this cytosolic Ca^{2+} flux may be partially inhibited ($58 \pm 12\%$) by pre-treatment with pertussis toxin [20]. We hypothesize that lyso-PC activation of the G2A receptor on PMNs releases two disparate G_α subunits resulting in increases in cytosolic Ca^{2+} , the released $G_{\beta\gamma}$ subunit directly activates the Src family kinase Hck (haemopoietic cell kinase), and signalling is modulated by the recruitment of GRK6 and β -arrestin-1 to G2A.

EXPERIMENTAL

Materials

All reagents, unless specified otherwise, were purchased from the Sigma Chemical Company. Solutions were made from sterile water for injection, USP (United States Pharmacopeia), from Baxter Healthcare. All buffers were made from the following stock USP solutions: 10% CaCl_2 , 23.4 % NaCl , 50 % MgSO_4 (American Reagent Laboratories), sodium phosphates (278 mg/ml monobasic and 142 mg/ml dibasic) and 50% dextrose (Abbott Laboratories). Furthermore, all solutions were sterile-filtered with Nalgene™ MF75 series disposable sterilization filter units purchased from Fisher Scientific. Ficoll–Paque was purchased from Amersham Biosciences. Antibodies against the G2A receptors A-20 and N-20, $G_{\alpha q/11}$, $G_{\alpha i-1}$, G_{β} and β -arrestin-1 were purchased from Santa Cruz Biotechnology. The individual lyso-PCs that comprise the mix were obtained from Sigma, Cayman and Avanti Polar Lipids to ensure that all forms bound to albumin contained identical activity.

Changes in cytosolic Ca^{2+} concentration

Isolated PMNs (2.5×10^7 cells) were loaded with Indo-1/AM (Indo-1 acetoxymethyl ester; Invitrogen) and washed, and changes in cytosolic Ca^{2+} for 2×10^6 PMNs were measured at 37°C, as described previously [20]. In selected experiments, PMNs were incubated for 30 min at 4°C with the A-20 antibody specific for the G2A receptor at a concentration of 1 $\mu\text{g}/10^6$ PMNs or isotype-control to determine whether the changes in cytosolic Ca^{2+} were directly linked to lyso-PC engagement of the receptor.

PMN isolation

Heparinized whole blood was drawn from healthy human donors after obtaining informed consent employing a protocol approved by the Colorado Multiple Institutional Review Board. PMNs were isolated by standard techniques, including dextran sedimentation, Ficoll–hypaque gradient centrifugation and hypotonic lysis of contaminating red blood cells [28]. Cells were resuspended to a concentration of 2.5×10^7 cells/ml in KRPD (Krebs–Ringers–phosphate buffer with 2% dextrose; pH 7.35) and used immediately for all subsequent experiments.

Whole-cell lysates, immunoprecipitations and discontinuous subcellular fractionations

PMNs (1.25×10^7 cells for whole-cell lysates and immunoprecipitations) or 1×10^8 cells (subcellular fractionation) were incubated with 1.25% albumin or 4.5 μM lyso-PCs for 5–600 s. Reactions were stopped with the addition of ice-cold relaxation buffer [10 mM Pipes (pH 7.4), 3 mM NaCl , 100 mM KCl , 3.5 mM MgCl_2 , 1.2 mM EGTA, 10 $\mu\text{g}/\text{ml}$ leupeptin, 40 mM sodium orthovanadate, 1 M nitrophenylphosphate and 50 $\mu\text{g}/\text{ml}$ PMSF] and immediately sonicated (3×30 s). Lysates were cleared and used for Western blot analysis, immunoprecipitation with specific antibodies attached to agarose beads overnight at 4°C or subjected to subcellular fractionation, as described previously [29,30]. Proteins were separated by SDS/PAGE (10% gels) and transferred on to a nitrocellulose membrane; these membranes were then probed with the specific antibodies indicated in the text or Figure legends.

Digital fluorescent microscopy

PMNs (5×10^5 cells) were warmed to 37°C and then incubated with either albumin (1.25%), 4.5 μM lyso-PCs or 2 μM PAF for 1 min and prepared as described previously [29]. Images were acquired with a Zeiss Axiovert fitted with a Cooke CCD SensiCam using a Chroma Sedat Multiple Bandpass filter wheel and Sutter filter control [29,30]. Images were acquired using Intelligent Imaging Innovations Slidebook™ software. All images compared within a

single Figure were acquired as Z-stacks at 0.2 μm intervals, and were deconvolved by applying constrained iterative deconvolution and Gaussian noise smoothing from system specific point spread functions [29,30]. Following deconvolution, images were cropped to represent the middle most planes (centre ± 10 planes) and the proteins in question masked to represent zero fluorescence in IgG negative controls [29]. In selected experiments the nucleus was masked so that only the plasma membrane and cytosol were visualized. Quantification of cellular co-localization of G2A with other proteins of interest was completed as described previously [29,30].

FRET (fluorescent resonance energy transfer) microscopy

FRET determinations were obtained using the three-cube method to account for possible bleed-through of light [29,30]. Images were acquired sequentially through three filter settings: donor filter, acceptor filter and FRET transfer filter, which is excitation through the donor filter and emitted light collected through the acceptor filter. To account for bleed-through from the donor to the acceptor or vice versa, images of samples with a single antibody (either donor or acceptor) were acquired under identical conditions as the experimental group, correction coefficients were calculated using the 'fit data' operation within the Slidebook Imaging SoftwareTM. FRET indices were calculated using:

$$\text{FRET}^C = F_f - D_f(F_d/D_d) - A_f(F_a/A_a)$$

where F_f , D_f and A_f represent the transfer channel, donor and acceptor channels in the presence of the all three fluorophores, and (F_d/D_d) and (F_a/A_a) represent the bleed-through corrections [29,30]. Because the method of data processing is paramount to its interpretation, each image is acquired as a Z-stack of ≥ 25 planes [29]. Bleed-through is corrected by a constrained deconvolution iterative in which the imaging properties of the optical system are employed as a measured point spread function such that one mathematically 'puts light back where it came from' [29,30]. This point spread function can be used for a calculation of a likely model of the object from the recorded data set in an iterative process [29,30]. Deconvolution eliminates effective blur caused by distortion, and by assuming a Poisson distribution of stray light it suppresses background light to very low levels [29,30]. Not only is bleed-through eliminated, but resolution is increased, which is of paramount importance in cases involving proteins of objects near resolution size [29,30]. FRET efficiencies (E_i) were calculated using previously published techniques [29], and images are displayed in pseudocolour where blue is 'cold' (no FRET) and red is 'hot' (most FRET) [29]. In selected experiments the FRET 'positivity' is illustrated in black and white, in which case the white colour represents a positive FRET.

Intracellular neutralization of specific proteins

BioPorterTM (Sigma) was reconstituted according to the manufacturer's instructions [29,30]. Briefly, individual tubes were reconstituted to a total volume of 40 μl with KRPD $\pm 4 \mu\text{g}$ of the antibody for 5 min at room temperature (25°C). PMNs (5×10^6 cells) were incubated with buffer, vehicle-only or vehicle with the antibody for 2 h at 37°C [29,30]. Following incubation, PMNs were centrifuged for 3 min at 400 g at 4°C, and resuspended [29,30]. To control for IgG introduction, a FITC-IgG was introduced, and Z-stack images acquired as described above. Antibodies against $G_{\alpha q/11}$, $G_{\alpha i-1}$ and G_{β} were sufficient for native protein as demonstrated by use in immunoprecipitation. Furthermore, for intracellular neutralization two different antibodies to disparate epitopes were used to neutralize the G_{α} subunits.

Lyso-PC preparation

The individual lyso-PCs and those used in the mixture were solubilized in 1.25% essentially fatty-acid-free, globulin-free, human albumin with 3×30 s pulses using a bath sonicator, model W-220F (Heat Systems-Ultrasonics) set at 30% maximal voltage. The lyso-PC mixture contained purified individual lyso-PCs in the following molar ratios as previously published: 1-*O*-palmitoyl (16:0/OH):24; 1-*O*-oleoyl (18:1/OH):10; 1-*O*-stearoyl (18:0/OH):10; 10:1-*O*-hexadecyl(C_{16}) lyso-PAF:0.65; and 1-*O*-octadecyl (C_{18}) lyso-PAF:0.35 [28]. The lyso-PC mixture was employed at $4.5 \mu\text{M}$ because this concentration could be achieved following the transfusion of 4 units of packed red blood cells, the average transfusion of an adult male at University Hospital, University of Colorado Denver, Aurora, CO, U.S.A. [20,21,23,28]. To determine that there was no PAF contamination in the lyso-PCs the following experiments were completed: (i) the ability of these lipids to activate p38 MAPK; (ii) inhibition of priming activity by WEB 2347, which is specific for PAFr and does not affect lyso-PC priming of the PMN oxidase [20]; and (iii) lyso-PCs activation of FRET+ co-localization of the G2A receptor with β -arrestin-1. These experiments ensured that the described signalling events were secondary to only lyso-PC priming of PMNs [20].

RESULTS

Lyso-PCs induce rapid internalization and activation of the G2A receptor

As a necessary preliminary experiment, the individual lyso-PCs, 16:0/OH, 18:0/OH, 18:1/OH and lyso-PAF, which comprise the mixture, and the mixture itself were assessed for their ability to cause internalization of the G2A receptor. The individual lyso-PCs and the mixture caused internalization of the G2A receptor as documented by subcellular fractionation (Figure 1). As compared with the albumin-treated controls, the individual lyso-PCs and the lyso-PC mix (60 s) caused a decrease in the membrane G2A immunoreactivity with a concomitant increase in the cytosol compared with the albumin-treated controls (Figure 1).

Lyso-PCs ($4.5 \mu\text{M}$) induced rapid S-phosphorylation of the G2A receptor beginning at 15 s and persisting at 30 and 60 s without an increase in G2A immunoreactivity (Figures 2A and 2B). Importantly, incubations of PMNs with lyso-PCs (4.5 and $14.5 \mu\text{M}$) did not cause appreciable cell death, as determined by Trypan Blue uptake, such that buffer controls exhibited $1 \pm 1\%$ Trypan Blue positivity, and lyso-PCs demonstrated $2 \pm 1\%$ and $3 \pm 2\%$ Trypan Blue positivity after 60 min incubation respectively, and as previously reported [20].

To further investigate the time course of lyso-PC-mediated internalization and translocation of the G2A receptor into different subcellular fractions, PMNs were fractionated into membrane, cytosol and granule fractions. The G2A receptor was then immunoprecipitated from each of these subcellular fractions with the A-20 antibody linked to agarose beads, and then immunoblotted with the N-20 antibody, which recognizes a different epitope in the N-terminus of this protein. These results demonstrated that in control cells the G2A receptor is on the membrane and there is no appreciable activity in the cytosolic or granular fractions (Figure 2C). With lyso-PC activation the receptor translocated from the membrane beginning at 15 s of ligation, appeared in the cytosol at 15 s reaching a relative maximum at 60 s, and also translocated to the granules beginning at 30 s and reached a relative maximum at 60 s (Figure 2C). The immunoreactivity was still present at 3 min and began to decrease in the cytosol and granules at 5 min (results not shown).

Lyso-PCs cause the release of both $G_{\alpha i-1}$ and $G_{\alpha q/11}$ which co-localize with G2A

As compared with albumin-treated controls, lyso-PCs caused the release of $G_{\alpha i-1}$ which co-localized with the G2A receptor at 30 and 60 s, as demonstrated by immunoprecipitating

G2A and immunoblotting for $G_{\alpha i-1}$ (Figure 3A). These data were further reinforced by digital microscopy, in which the nucleus was masked, so that only the membrane and cytosol were visualized. These images show that the G2A receptor (green) was internalized, as demonstrated by the cytosolic immunoreactivity of the G2A antibody in lyso-PC-stimulated PMNs (Figure 3B, G2A column, second row) which was not present in the albumin controls (Figure 3B, G2A column, first row). Lyso-PC ligation of the G2A receptor also caused co-localization of the G2A receptor with $G_{\alpha i-1}$, as shown by the yellow colour (Figure 3B, arrows, Merge column, second row). In addition, these data were confirmed through quantification of the G2A/ $G_{\alpha i-1}$ co-localization of lyso-PC-stimulated PMNs compared with the controls (Figure 3C). The control displayed the least amount of G2A/ $G_{\alpha i-1}$ co-localization, and the cells stimulated with lyso-PCs at 60 s demonstrated a significant increase of G2A/ $G_{\alpha i-1}$ co-localization between G2A and $G_{\alpha i-1}$. Moreover, because our previous data demonstrated that pertussis toxin was able to only partially inhibit lyso-PC-induced increases in cytosolic Ca^{2+} , a number of other G_{α} proteins were explored for possible co-precipitations with G2A [20]. Lyso-PCs also elicited the co-precipitation of the $G_{\alpha q/11}$ with the G2A receptor, beginning at 30 s, which was also present at 60 s, both when the G2A receptor was immunoprecipitated and probed for $G_{\alpha q/11}$ (Figure 4A) and the reverse, when $G_{\alpha q/11}$ was immunoprecipitated and probed for G2A (Figure 4B). These data were confirmed by digital microscopy, which also showed lyso-PC-mediated co-localization of the $G_{\alpha q/11}$ subunit with the G2A receptor at 60 s (Figure 4C, arrows), as well as internalization of the G2A receptor as demonstrated by the immunoreactivity of the G2A antibody present in the cytoplasm in PMNs primed with lyso-PCs (Figure 4C, G2A column, bottom row) compared with the albumin-treated controls, which did not demonstrate such immunoreactivity in the cytoplasm (Figure 4C, G2A column, top row). Please note that the nucleus is masked so that it is not visualized in these images. The co-localization of G2A with $G_{\alpha q/11}$ was quantified for both the control and lyso-PC-treated PMNs. There was minimal G2A/ $G_{\alpha q/11}$ co-localization in control PMNs as compared with cells stimulated with lyso-PCs for 60 s, which displayed a significant amount of G2A/ $G_{\alpha q/11}$ co-localization.

Lyso-PCs require G2A, and both $G_{\alpha i-1}$ and $G_{\alpha q/11}$ for increases in cytosolic Ca^{2+}

To determine whether blockade of the G2A receptor inhibited lyso-PC-mediated increases in cytosolic Ca^{2+} , PMNs were treated with the A-20 antibody against G2A ($1 \mu\text{g}/10^6$ PMNs) or isotypic controls for 30 min at 4°C . Blockade of G2A inhibited the lyso-PC mediated increase in cytosolic Ca^{2+} (Figure 5A).

To evaluate whether $G_{\alpha i-1}$, $G_{\alpha q/11}$ or both of these G_{α} subunits were important for lyso-PC-mediated increases in cytosolic Ca^{2+} , intracellular antibody neutralization of $G_{\alpha i-1}$, $G_{\alpha q/11}$ or both were completed, compared with PMNs loaded with isotypic IgG controls, and the lyso-PC-mediated increases in cytosolic Ca^{2+} were measured in real time. Intracellular neutralization of either $G_{\alpha i-1}$ or $G_{\alpha q/11}$ diminished the lyso-PC-induced cytosolic Ca^{2+} flux by approx. 50% (Figure 5B). Moreover, intracellular neutralization of both of these G_{α} subunits abrogated the lyso-PC-mediated increases in cytosolic Ca^{2+} altogether (Figure 5B).

Lyso-PCs cause activation, and release of $G_{\beta\gamma}$ induces a physical FRET+ interaction of $G_{\beta\gamma}$ with Hck

GPCR activation causes the release of the $G_{\beta\gamma}$ subunits, which bind and regulate a number of effector proteins within PMNs; moreover, the Src family tyrosine kinase Hck has been implicated in a number of early signalling events in PMNs [31]. To determine whether such events occur with lyso-PC signalling through the G2A receptor, PMNs were primed with lyso-PCs or albumin controls. Lyso-PCs caused the co-precipitation of G_{β} with the G2A receptor with β -subunit immunoreactivity first present at 5 s and persisting for 60 s (Figure 6A). These data were confirmed employing digital microscopy such that lyso-PCs, as

compared with albumin-treated controls, elicited a co-localization of G_{β} with G2A at 60 s (Figure 6B). Furthermore, lyso-PCs induced a FRET+ interaction of the G_{β} subunits with the G subunits, indicating that lyso-PC stimulation of G2A caused specific $G_{\beta\gamma}$ subunit release as compared with quiescent PMNs in which there was no $G_{\beta\gamma}$ co-localization (Figure 7A, third column). This result may be surprising; however, one must consider the following details: (i) the antibody employed against the G_{γ} subunit recognizes amino acids 1–72 (Santa Cruz Biotechnology), (ii) the active site of G_{γ} which binds to GRK2, involves amino acids 52–68 and (iii) this site is blocked by the presence of G_{α} subunit in resting cells and would inhibit the binding of the G_{γ} antibody used [13]. Therefore one would expect very small amounts of G_{γ} immunoreactivity in the vehicle-treated control PMNs, which was demonstrated in Figure 7(A) [13]. Moreover, one would also expect that the immunoreactivity of the antibody against G_{γ} would increase upon ligand activation of the GPCR, and in accordance the G_{γ} immunoreactivity increases with lyso-PC activation and release of $G_{\beta\gamma}$ from G_{α} resulting in a FRET+ interaction between G_{β} and G_{γ} (Figure 7A). The FRET efficiency was 58% between the labelled primaries, indicating a distance of <5 nm between the antibodies implying a physical relationship between G_{β} and G_{γ} , as expected [29,30]. Lyso-PCs also elicited a FRET+ interaction between the $G_{\beta\gamma}$ subunits and Hck (FRET efficiency of 54%), indicating that the distance between the fluorophores was <5 nm (Figure 7A, third column, third row). Furthermore, the G_{β} subunit further demonstrated a FRET+ interaction with activated Hck Tyr⁴¹¹, the active form, indicating that G_{β} may have a direct role in the activation of Hck (Figure 7B). In addition, pre-incubation with PP2 (protein phosphatase 2), a selective Hck antagonist, and genistein, a tyrosine kinase antagonist, inhibited the lyso-PC priming of the PMA activation of the respiratory burst by $91 \pm 8\%$ and $94 \pm 4\%$ respectively, demonstrating that tyrosine kinases, and more specifically Src family tyrosine kinases, are required for lyso-PC-mediated signalling in PMNs.

Lyso-PCs cause the recruitment of GRK6 and β -arrestin-1 to G2A and require clathrin for internalization

Implicit to GPCR signalling are CME (clathrin-mediated endocytosis) and the activation of the GRKs, which phosphorylate receptors, and recruitment of the β -arrestins which both transduce signals and terminate continued signalling through activated GPCRs [4,12,13,29,32–34]. To this end, the requirement of clathrin for internalization of G2a and the recruitment of GRK6 and β -arrestin-1 to the G2A receptor were investigated in response to lyso-PC priming compared with albumin-treated controls. Lyso-PCs induced internalization of the G2A receptor, as compared with albumin-treated controls, as visualized by the decrease in G2A membrane immunoreactivity (Figure 8). Intracellular neutralization of α -adaptin and the clathrin heavy chain abrogated the lyso-PC-mediated internalization of G2A (Figure 8). Moreover, lyso-PCs elicited the recruitment of clathrin to β -arrestin-1 at 60 s as compared with albumin-treated control PMNs (Figure 9). In addition, lyso-PCs caused the recruitment of GRK6 to the G2A receptor at 30 and 60 s, as documented by immunoprecipitation of G2A with the presence of GRK6 immunoreactivity in lyso-PC-primed PMNs which was not present in the albumin-treated controls (Figure 10A). Lyso-PCs also caused co-precipitation of β -arrestin-1 with GRK6 (Figure 10B). Lastly, lyso-PCs caused a FRET+ interaction between β -arrestin-1 and G2A, which was not demonstrable in the controls (Figures 11B and 11D) and, importantly did not cause co-localization of PAFr with β -arrestin-1 (Figure 11C). Conversely, PAF caused a FRET+ co-localization of the PAFr with β -arrestin-1, but did not demonstrate a co-localization of G2A with β -arrestin-1, indicating that lyso-PCs specifically activated the G2A receptor and not the PAFr, and PAF activated the PAFr and not G2A (Figures 11E and 11F).

DISCUSSION

The presented data have demonstrated that both the individual lyso-PC moieties and the mixture, when bound to albumin, elicited rapid intracellular internalization and activation of the G2A receptor through S-phosphorylation. Blocking of the receptor with the A-20 antibody resulted in abrogation of the lyso-PC-elicited increase in cytosolic Ca^{2+} in accordance with previous data [25]. Activation also caused the rapid release of two disparate G_α subunits: $G_{\alpha i-1}$ and $G_{\alpha q/11}$, which together are responsible for the observed rapid changes in cytosolic Ca^{2+} . Moreover, lyso-PCs induced release of the $G_{\beta\gamma}$ subunit which then rapidly co-localizes (FRET+) with activated Hck. Ligand activation of the G2A receptor also caused the recruitment of GRK6 and β -arrestin-1 and required clathrin and α -adaptin for internalization, as demonstrated by specific intracellular neutralization of clathrin heavy chain and α -adaptin, which inhibited CME. Clathrin is important for signal transduction through CME, and GRK6 aids in the desensitization of G2A to decrease further receptor activation [4,9,13,29,34]. In addition, β -arrestin-1 may cause both propagation of signalling by providing the recruitment of kinases, as well as serving as a scaffold; however, β -arrestins also effectively decrease further signalling through activated GPCRs [4,8,11,12,29,32,33,35]. Thus these data provide direct support that lyso-PCs from stored blood activate G2A, which is specific for lyso-PCs, and that CME is vital for G2A signalling.

G2A is expressed on the membrane surface of human PMNs and binding of lyso-PCs, specifically the 18:1/OH lyso-PC, to 1% BSA specifically activated G2A, as quantified by increases in cytosolic Ca^{2+} [24,25,27]. However, when the 18:1/OH lyso-PC was added to isolated PMNs in a 4% BSA solution, this response was inhibited; moreover, addition of the 18:1/OH lyso-PC without a protein carrier or addition of 18:1/OH lyso-PS (lysophosphatidylserine) or 18:1/OH lyso-PE (lysophosphatidylethanolamine) could all activate G2A through membrane perturbation, dimerization of the G2A receptors and $G_{\alpha i}$ and phospholipase- γ activation. These data require increases in G2A in the plasma membrane, receptor dimerization and desensitization of this area of the plasma membrane [24,25,27]. The presented data demonstrate that lyso-PC activation of G2A caused CME. Furthermore, we used purified human albumin, rather than a mammalian equivalent, to solubilize and present the lipid ligands, similar to the manner in which lipids circulate in the plasma or plasma fraction of blood components. Previous data from this laboratory demonstrated that human albumin concentrations up to 5% did not decrease the pro-inflammatory activity of lyso-PCs on human PMNs, and identified a cellular association of PMN membranes with NBD (7-nitrobenz-2-oxa-1,3-diazole)-labelled 1-*O*-lauroyl lyso-PC (albumin-bound), which could be significantly diminished by the addition of unlabelled 1-*O*-lauroyl lyso-PC without concomitant loss of PMN viability [20]. Moreover, parallel experiments employed whole blood incubated with a tritiated lyso-PC marker, and demonstrated that lyso-PCs were associated with leucocytes and platelets (2.5%), not red blood cells (<0.02%), with most of the counts (97.4%) remaining in the acellular plasma, indicating that the cellular association was not non-specific [20]. In addition, the presented data showed that ligand activation of lyso-PCs bound to human albumin caused: (i) rapid S-phosphorylation of G2A, (ii) activation and release of the heterotrimeric G-protein subunits (α and $\beta\gamma$), resulting in cytosolic Ca^{2+} flux and Hck activation, and (iii) recruitment of GRK6, β -arrestin-1 and clathrin, which provided evidence that G2A is a GPCR that undergoes CME identical with PAFr on PMNs [29,30].

The literature with respect to the role of the G2A receptor remains controversial and must be examined carefully for the different effects of lipids dissolved in alcohols, organic solvents or in aqueous solutions without solubilizing the lyso-PCs with a carrier protein on cells is very different [20,36]. For example, lyso-PCs, when not bound to albumin, activate the

NADPH oxidase in PMNs, inhibit the oxidase activity to fMLP (*N*-formylmethionyl-leucyl-phenylalanine) and PMA, including phagocyte oxidase (*phox*) protein translocation, and are not PMN chemoattractants [36,37]. However, when bound to albumin (1.25–5%), lyso-PCs induce PMN chemotaxis, prime the PMN oxidase through translocation of the p47^{phox}, and augment the bactericidal activity of PMNs *in vivo* [20,27]. In the latter study the identical G2A ligands as employed in the presented study, specifically the 16:0/OH, 18:0/OH and 18:1/OH, augmented oxidase activity, resulting in increased bactericidal activity, and inhibited the lethal effects of endotoxin, which was abrogated by antibody inhibition of the G2A receptor [27]. The presented data are in accordance with these findings and have mechanistically elucidated the early cellular events mediated by activation of the G2A receptor by these lipid ligands resulting in the transduction of outside-in signalling through CME [27]. In addition, lyso-PCs are present in plasma as a mixture of compounds and rarely appear as a single purified lipid entity, although they could be displaced by large concentrations of non-esterified fatty acids, which may occur in animal models of cardiac ischaemia [38,39]. It is also important to document the cellular effects of lyso-PCs in solution as they appear in the plasma fraction of stored blood components and in the plasma of transfused patients in which they are probably albumin-bound [21,40].

Lyso-PCs caused the release of two different G_{α} subunits, $G_{\alpha i-1}$ and $G_{\alpha q/11}$, upon activation. S-phosphorylation of G2A, and release of these two G_{α} subunits, $G_{\alpha i-1}$ and $G_{\alpha q/11}$, were responsible for the observed rapid increase in cytosolic Ca^{2+} . Importantly, the lyso-PC (4.5 μ M) -induced increase in cytosolic Ca^{2+} were blunted as compared with previous data from our laboratory; however, such a blunting of the rise in cytosolic Ca^{2+} may be expected when PMNs are loaded with intracellular antibodies [29,41]. In addition, the release of two disparate G_{α} subunits is not unique to lyso-PCs because a number of other GPCRs, including the dopamine and endothelin-1 receptors, have been shown to cause the release of multiple G_{α} subunits required for increases in cytosolic Ca^{2+} and changes in other cellular signalling [42,43]. Moreover, previous reports used pertussis toxin inhibition to implicate $G_{\alpha i-1}$ in lyso-PC signalling in human PMNs; however, only one purified lyso-PC moiety was employed, not a mixture, and the 18:1/OH lyso-PC was delivered in methanol and not bound to human albumin [25]. As stated previously, lyso-PCs rarely, if ever, appear in biological fluids as a single entity, but rather are found as a mixture of compounds [38,39].

Lyso-PC stimulation of G2A also caused the release of the $G_{\beta\gamma}$ subunit which demonstrated a FRET+ physical interaction with Hck. The $G_{\beta\gamma}$ subunits have caused the activation of a variety of signalling molecules, including: phospholipase C, MKK (MAPK kinase) 4, MKK7, adenylate cyclase and multiple Src family kinases [44–46]. Therefore activation of Hck in human PMNs by the $G_{\beta\gamma}$ subunit is novel and explains the regulatory role of Hck and other Src family protein kinases with regard to PMN adhesion and chemotaxis, as well as promoting activation of small GTPases in other cell lines [31,47].

A number of papers have attributed the biological effects of lyso-PCs to contaminating PAF and PAF-like compounds [36,48]. Contrary to these observations, lyso-PCs binding to the G2A receptor caused β -arrestin-1 recruitment and did not cause β -arrestin-1 recruitment to the PAFr. Likewise, PAF caused β -arrestin-1 recruitment to the PAFr and not to G2A. These data provide direct evidence that lyso-PCs and PAF activate disparate signalling pathways through distinct receptors and both employ CME for signal transduction [29]. In addition, lyso-PCs also caused recruitment of GRK6, as well as β -arrestin-1, to G2A and, therefore, activation of G2A is similar to many GPCRs that cause signalling as well as the recruitment of clathrin and the aforementioned 'termination proteins' which manifest receptor desensitization [13,34,49]. Lastly, lyso-PC activation of G2A also caused the recruitment of clathrin and β -arrestin-1, which provide a scaffold for signal transduction followed by internalization of G2A, all three steps which are characteristic of CME of GPCRs [4,29,35].

In conclusion, the mixture of lyso-PCs which accumulate during blood storage cause activation of the G2A receptor on PMNs and cause the release of G-protein subunits that have specific cell signalling roles: the two G_{α} subunits, $G_{\alpha i-1}$ and $G_{\alpha q/11}$, directly cause the increase in cytosolic Ca^{2+} and the $G_{\beta\gamma}$ subunit activates the Src family kinase Hck. Unlike in other reports, there was no decrease in cellular integrity or no membrane perturbation resulting in Trypan Blue or propidium iodide 'positivity' over 60 min incubations of isolated PMNs with this mixture of lyso-PCs bound to human albumin. These data provide direct evidence that lyso-PCs cause activation of a GPCR present on primary cells, and provide an insight into the signal transduction employed by these GPCRs, which includes activation of heterotrimeric G-proteins and CME, with many of the signalling events similar to known chemoattractants including both PAF and fMLP [2,48,50].

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Abbreviations used

ALI	acute lung injury
CME	clathrin-mediated endocytosis
fMLP	<i>N</i> -formylmethionyl-leucyl-phenylalanine
FRET	fluorescent resonance energy transfer
GPCR	G-protein-coupled receptor
GRK	G-protein receptor kinase
Hck	haemopoietic cell kinase
Indo-1/AM	Indo-1 acetoxymethyl ester
KRPD	Krebs–Ringers–phosphate buffer with 2 % dextrose
lyso-PC	lysophosphatidylcholine
MAPK	mitogen-activated protein kinase
PAF	platelet-activating factor
PAFr	PAF receptor
PI3K	phosphoinositide 3-kinase
PMN	polymorphonuclear leucocyte
USP	United States Pharmacopeia

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Figure 1. Lyso-PCs induce internalization of the G2A receptor

PMNs were treated with albumin, individual lyso-PCs or the lyso-PC mixture for 60 s followed by subcellular fractionation. Both the individual lyso-PC species and the mixture induced the appearance of G2A immunoreactivity in the cytosol of the PMNs. The immunoblot is representative of three separate experiments with PMNs from disparate donors. C, albumin-treated control PMNs.

Figure 2. Lyso-PCs cause activation, S-phosphorylation and rapid internalization of the G2A receptor

G2A immunoreactivity present in PMN whole-cell lysates incubated with albumin (C), and the lyso-PC mix for 15–60s is shown in (A) with similar immunoreactivity in each lane. (B) PMNs were incubated with albumin (C) or 4.5 μ M lyso-PCs for 15–60 s, the reactions were stopped with ice-cold relaxation buffer, the PMNs were lysed, and the G2A receptor was immunoprecipitated from whole-cell lysates using the A-20 antibody linked to agarose beads overnight at 4 °C. The agarose beads were isolated and digested, the proteins were separated by SDS/PAGE followed by immunoblotting with antibodies against phosphoserine (PS). As compared with the controls, lyso-PCs caused S-phosphorylation of G2A beginning at 15 s, which was also present at 30 s and 60 s. This Figure, which shows the entire gel, represents a sample size of three experiments using PMNs from disparate donors that yielded virtually identical results. (C) PMNs were treated with albumin or the lyso-PC mixture (4.5 μ M) for 15–60s, the reaction was stopped, and the PMNs were lysed by sonication and underwent subcellular fractionation. Each fraction was then immunoprecipitated with the A-20 antibody against G2A, the proteins were separated by SDS/PAGE, transferred on to nitrocellulose, and the immunoblots were probed with the N-20 antibody which recognizes the N-terminus of the G2A receptor. These results demonstrate that the G2A receptor is on the membrane in the controls and rapidly began to translocate from the membrane to the cytosol at 15 s which reached a relative maximum at 60 s, with increased immunoreactivity in the cytosol and granules and decreased G2A

immunoreactivity in the membrane fraction. These data shown represent a sample size of three. IP, immunoprecipitation.

Figure 3. Lyso-PCs cause recruitment of $G_{\alpha i-1}$ to the G2A receptor

(A) PMNs were treated with albumin (C) or 4.5 μ M lyso-PCs for 30–60 s, the reactions were stopped, the PMNs were lysed, and the whole-cell lysates were immunoprecipitated with the A-20 antibody. The proteins were then separated by SDS/PAGE (10 % gel), and immunoblotted with an antibody against the $G_{\alpha i-1}$ subunit. Co-precipitation of G2A with the $G_{\alpha i-1}$ subunit occurred at 30 s and increased at 60 s. (B) These PMNs underwent the same treatment protocol as in (A), except following 60 s of lyso-PC or albumin stimulation the cells were fixed and smeared on to glass slides. As compared with the albumin-treated control PMNs (top row), lyso-PCs caused the internalization of G2A, as demonstrated by the green immunoreactivity in the membrane and the cytosol (bottom row, G2A column) and the recruitment of both the $G_{\alpha i-1}$ (red) to the G2A (green) receptor as demonstrated by the appearance of the yellow colour, for the co-localization of the green and red fluorophores emit this yellow colour (arrows). Importantly, the nuclei have been masked so that only the membrane and cytosol are visible. These data for both panels represent three separate experiments on PMNs from three disparate donors; 25–30 cells were analysed in each case and, of those, >95 % of the PMNs responded to lyso-PCs. (C) The histogram illustrates the percentage of total G2A immunoreactivity (voxels) that co-localized with $G_{\alpha i-1}$ immunoreactivity in PMNs treated with albumin compared with lyso-PCs for 60 s. Lyso-PCs significantly increased the percentage of co-localization compared with control PMNs. * $P < 0.05$. IP, immunoprecipitation.

Figure 4. Lyso-PCs elicit a co-precipitation of $G_{\alpha q/11}$ with the activated G2A receptor (A) PMNs were treated with buffer or 4.5 μ M lyso-PCs for 30–60 s, the reaction was stopped by the addition of ice-cold relaxation buffer, and the cells were lysed and immunoprecipitated overnight with the A-20 antibody conjugated to agarose beads at 4 °C. The beads were digested and the proteins were separated by SDS/PAGE (10 % gels) and Western blotted with antibodies against $G_{\alpha q/11}$. As compared with albumin-treated controls, lyso-PCs induced a co-precipitation of $G_{\alpha q/11}$ with the G2A receptor at 30 and 60 s. In addition, when the immunoprecipitation was reversed, lyso-PCs also caused a similar co-precipitation of G2A when $G_{\alpha q/11}$ was immunoprecipitated from lyso-PC-primed PMNs as compared with controls (B). These data were confirmed by digital microscopy that demonstrated that lyso-PC elicited G2A internalization as demonstrated by the green immunoreactivity in the cytosol (C, bottom row, G2A columns) compared with albumin-treated controls (C, top row, G2A column). Lyso-PCs (bottom row) also caused co-localization of G2A (green) with $G_{\alpha q/11}$ (red), as shown by the increase in yellow colour in the ‘merged’ column the lyso-PC-primed PMNs (C, bottom row, arrows), compared with the albumin-treated controls (C, top row). The digital microscopy is from the examination of 25–30 PMNs in each treatment group. (D) Lyso-PCs at 60 s, significantly increased the

percentage of total cellular G2A/G α _{q/11} co-localization compared with the albumin-treated controls, as demonstrated in **(B)**. * $P < 0.05$. IP, immunoprecipitation.

Figure 5. G2A antibody blockade and intracellular neutralization of $\text{G}_{\alpha i-1}$ and $\text{G}_{\alpha q/11}$ inhibit lyso-PC-induced increases in cytosolic Ca^{2+} concentration

(A) Indo-1/AM-loaded PMNs were incubated with the A-20 antibody against the G2A receptor ($1 \mu\text{g}/10^6$ PMNs) or isotype controls, and then stimulated with $4.5 \mu\text{M}$ lyso-PCs and the cytosolic Ca^{2+} concentration was measured over real time. Antibody blockade inhibited the lyso-PC-mediated increase in cytosolic Ca^{2+} . (B) Antibodies were introduced into PMNs via an endosomal delivery system that were specific for $\text{G}_{\alpha i-1}$, $\text{G}_{\alpha q/11}$ or murine isotype controls to see whether such intracellular neutralization would affect the lyso-PC-mediated increase in cytosolic Ca^{2+} . Inhibition of either $\text{G}_{\alpha i-1}$ or $\text{G}_{\alpha q/11}$ partially inhibited the increase in cytosolic Ca^{2+} over real time as compared with PMNs which had isotypic IgG introduced (B). Loading PMNs with antibodies against both G_{α} subunits almost totally inhibited the lyso-PC-elicited rise in cytosolic Ca^{2+} . These data from both panels represent a sample size of three employing PMNs from three disparate donors.

Figure 6. Lyso-PCs induce a co-precipitation of G β with the G2A receptor

Isolated PMNs were stimulated with either albumin or 4.5 μ M lyso-PCs for 5–60 s. The reaction was stopped with the addition of ice-cold relaxation buffer and the PMNs were lysed and immunoprecipitated with the A-20 antibody against G2A, and the resulting proteins were separated by SDS/PAGE (10 % gel) and immunoblotted for the G β subunit. Lyso-PCs induced co-precipitation of G2A with G β , which first began at 5 s and persisted for 60 s (**A**). These data were confirmed using digital microscopy which demonstrated that lyso-PCs caused internalization of G2A (**B**, bottom row, G2A column) and co-localization of G2A (green) with G β (red) which emitted a yellow colour (**B**, bottom row, Merge column, arrows), whereas albumin-treated controls demonstrated minimal co-localization of the G2A receptor and G β . These data represent three separate experiments using PMNs from three disparate donors with 25–30 PMN analysed for each treatment group in the digital microscopy (**B**). The microscopy (40 $\times$) has masked the nucleus such that only the membranes and cytosol are present. In (**C**) the membranes were immunoblotted with an antibody against the G β subunit in PMNs treated with albumin (C) or lyso-PCs for 15–60 s. Some constitutive immunoreactivity was present in the membrane. IP, immunoprecipitation.

Figure 7. Lyso-PCs cause a FRET+ interaction between the $G\beta\gamma$ subunits and the activated form of the Src kinase Hck

Isolated PMNs were incubated with albumin or 4.5 μ M lyso-PCs for 60 s at 37 °C, fixed and smeared on to glass slides. The PMNs were porated and incubated with specific antibodies against $G\beta$, $G\gamma$, Hck or activated tyrosine-phosphorylated Hck (p-Hck). Albumin did not cause a FRET+ interaction either between $G\beta$ and Hck, or between $G\beta$ and $G\gamma$, because the epitope recognized is blocked by the presence of $G\alpha$ in the G-protein complex in resting PMNs (A, upper panels). In contrast, lyso-PCs induced a FRET+ interaction between both Hck and $G\beta$, and a FRET+ interaction between $G\beta$ and $G\gamma$, now that the $G\alpha$ subunits have been released (A, lower panels). If the FRETs between Hck and $G\beta$, and $G\beta$ and $G\gamma$ are overlayed (FRET^c overlay), there is similar cellular distribution, implying that all three proteins are in the same cellular location, as demonstrated by areas of high amounts of fluorescence intensity presented in pseudocolour [A.L.U.F.I. (arbitrary linear units of fluorescence intensity): red, the highest; blue, the lowest]. Because of this interaction, we postulated that the $G\beta\gamma$ subunits may activate Hck and so the physical interaction of $G\beta$ and p-Hck (Hck Tyr⁴¹¹) was examined (B). Lyso-PCs caused the FRET+ interaction of $G\beta$ with p-Hck which was not present in the control PMNs (B). These data represent three separate

experiments on the PMNs from three disparate donors; 25–30 PMNs were analysed in each treatment group.

Figure 8. Intracellular neutralization of clathrin inhibits lyso-PC induced G2A internalization (A) PMNs were loaded with antibodies against α -adaptin (α Ab), a clathrin regulatory protein, clathrin heavy chain (CHC Ab) or isotypic controls (IgG) employing the BioPorter™ endosomal delivery system. The PMNs were either stimulated with albumin (control) or 4.5 μ M lyso-PCs for 60 s at 37 °C. The PMNs were fixed, smeared on to glass slides and incubated with the A-20 antibody against G2A. The PMN images were masked such that the cytosol and nuclear regions were subtracted, leaving only the pixels in the plasma membrane. Each cell was then ‘sliced’ identically using a random plane (white line) that divided the cell to focus on one area of the PMN membrane. G2A immunoreactivity is white. Lyso-PC priming of PMNs induced a decrease in membrane-associated pixels (panel b) as compared with the albumin-treated controls loaded with the isotypic antibody controls (panel a). Intracellular neutralization of α -adaptin-1 (α Ab, panel c) or the clathrin heavy chain (CHC Ab, panel d) inhibited internalization of G2A. These digital micrographs represent 25 PMNs per treatment group and were completed using PMNs from two separate donors and two disparate antibodies against different epitopes of clathrin heavy chain and α -adaptin. Quantification of these data is shown in (B). The histogram illustrates the percentage (voxels) of G2A immunoreactivity in the plasma membrane and demonstrates that, compared with albumin-treated PMNs, lyso-PCs induced a decrease in membrane G2A immunoreactivity, e.g. internalization, which can be inhibited by intracellular neutralization of the clathrin heavy chain or α -adaptin-1 regulatory protein.

Figure 9. Lyso-PCs cause a FRET+ interaction between β -arrestin-1 and clathrin

Isolated PMNs were treated with albumin or 4.5 μ M lyso-PCs for 60 s at 37 °C. The PMNs were then fixed, smeared on to glass slides, porated and incubated with antibodies against β -arrestin-1 or clathrin heavy chain (Clathrin HC). Albumin-treated PMNs (top row) contain immunoreactivity for β -arrestin-1 (green) and clathrin (red) which do not demonstrate any co-localization. Conversely, lyso-PC priming (bottom row) causes a re-distribution of β -arrestin-1 (green) and clathrin (red), such that there is a FRET+ interaction (presented in pseudocolour as A.L.U.F.I.: red, the highest; blue, the lowest) of these two fluorophores with a FRET efficiency of 38 ± 4 %-indication that the immunoreactivity is <6 nm apart. These images are taken from 25–30 PMNs per treatment group and represent two experiments performed on PMNs from different donors.

Figure 10. Lyso-PCs induce the co-localization of GRK6 with β -arrestin-1

Isolated PMNs were incubated with albumin (control) or 4.5 μ M lyso-PCs for 5–60 s at 37 $^{\circ}$ C, then lysed and immunoprecipitated with antibodies against G2A (A-20) or GRK6 overnight at 4 $^{\circ}$ C. The beads were then isolated, digested and the proteins separated by SDS/PAGE (10 % gel) and immunoblotted for GRK6 (A) or β -arrestin-1 (B). As compared with albumin-treated control PMNs, lyso-PCs caused the co-precipitation of GRK6 with the G2A receptor at 30 and 60 s (A). In addition, lyso-PCs also induced co-precipitation of β -arrestin-1 with GRK6 which first appeared at 30 s, was present at 60 s, and was not present in albumin-treated controls (B). These data are representative of three separate experiments using PMNs from three different donors. IP, immunoprecipitation.

Figure 11. Lyso-PCs cause a FRET+ co-localization of G2A with β -arrestin-1

Isolated PMNs were primed with albumin, lyso-PCs or PAF for 3 min at 37 °C, fixed, smeared on to glass slides, porated and incubated with antibodies specific for G2A (A-20) and β -arrestin-1, or PAFr and β -arrestin-1. As compared with albumin-treated controls (A and B), lyso-PCs caused a FRET+ interaction of β -arrestin-1 with G2A (D, white colour), whereas PAF induced a FRET+ interaction of the PAFr with β -arrestin-1 (E, white colour). Importantly, lyso-PCs did not cause a FRET+ interaction between the PAFr and β -arrestin-1 (C), and likewise PAF did not cause a FRET+ interaction between G2A and β -arrestin-1 (F). These data are taken from 25–30 PMNs per treatment group and are representative of two separate experiments performed on PMNs from different donors.