Lysophospholipids of Different Classes Mobilize Neutrophil Secretory Vesicles and Induce Redundant Signaling through G2A

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Lysophosphatidylcholine has been shown to enhance neutrophil functions through a mechanism involving the G protein-coupled receptor G2A. Recent data support an indirect effect of lysophosphatidylcholine on G2A rather than direct ligand binding. These observations prompted the hypothesis that other lysophospholipids (lyso-PLs) may also signal for human neutrophil activation through G2A. To this end, 1-oleoyl-2-hydroxy-sn-glycero-3-[phospho-L-choline], but also C18:1/OH lyso-PLs bearing the phosphoserine and phosphoethanolamine head groups, presented on albumin, were shown to signal for calcium flux in a self- and cross-desensitizing manner, implicating a single receptor. Blocking Abs to G2A inhibited calcium signaling by all three lyso-PLs. Furthermore, inhibition by both pertussis toxin and U-73122 established signaling via the Gαi/phospholipase C pathway for calcium mobilization. Altered plasma membrane localization of G2A has been hypothesized to facilitate signaling. Accordingly, an increase in detectable G2A was demonstrated by 1 min after lyso-PL stimulation and was followed by visible patching of the receptor. Western blotting showed that G2A resides in the plasma membrane/secretory vesicle pathway for calcium mobilization. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

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G2A on the surface of the neutrophils associated with mobilization of the secretory vesicle pool. Together, these data support that neutrophils respond to a variety of lyso-PLs resulting from activation of PL_A2 in a G2A-dependent manner.

Materials and Methods

Reagents

The 1-oleoyl-2-hydroxy-sn-glycerol-3-[phospho-t-serine] (18:1/OH lyso-PS), 1-oleoyl-2-hydroxy-sn-glycerol-3-[phospho-t-choline] (18:1/OH lyso-PC), 1-stearoyl-2-hydroxy-sn-glycerol-3-[phospho-t-choline] (18:0/OH lyso-PC), 1-palmitoyl-2-hydroxy-sn-glycerol-3-[phospho-t-choline] (16:0/OH lyso-PC), 1-oleoyl-2-hydroxy-sn-glycerol-3-[phospho-t-ethanolamine] (18:1/OH lyso-PE), and 1-oleoyl-2-hydroxy-sn-glycerol-3-phosphatidic acid were from Avanti Polar Lipids. Oleic acid and arachidonic acid were purchased from Cayman Chemical. Anti-G2A (N-20), anti-G2A (A-20), and anti-goat IgG-HRP were from Santa Cruz Biotechnology. Anti-CD45 clone BAA-55, fatty acid-free BSA, and fMLP were from Sigma-Aldrich. Platelet-activating factor (PAF) was obtained from Calbiochem. Goat IgG and anti-mouse Cy3 were from Jackson ImmunoResearch Laboratories, and mouse IgG was from BD Pharmingen. Anti-goat Alexa 488, indo-1 AM, FM 1-43, and Hoescht were from Molecular Probes.

Neutrophil isolation and culture

Neutrophils were obtained from normal, healthy donors in accordance with protocol reviewed and approved by the institutional review board. Using endotoxin-free reagents and plasticware, human neutrophils were isolated by the plasma Percoll method, as described previously (19). Of note, neutrophils were isolated and washed in autologous plasma and then removed from autologous plasma for 15–30 min before the start of an experiment. Unless otherwise noted, all incubations were conducted in HEPES buffer (137 mM NaCl, 2.7 mM KCl, 2 mM MgCl2, 5 mM glucose, 10 mM HEPES (pH 7.4)) with designated amounts of fatty acid-free BSA. In pilot studies, isolated neutrophils maintained in these plasma-free conditions for the duration of experimentation were tested for stimulation with autologous plasma because it contains endogenous lyso-PLs in high concentrations (e.g., lyso-PC at 100 μM (20); see below). This reinduction of plasma to neutrophils maintained in HEPES buffer induced neither calcium signaling nor redistribution of G2A, as determined below. In experiments using neutralizing Ab to G2A for the purposes of blocking lyso-PL stimulation, neutrophils (5 × 10^6/ml) in HEPES buffer with 0.05% fatty acid-free BSA were incubated on ice with 10 μg/ml anti-G2A (N-20) or isotype control Abs for 30 min. Following incubation, 2 × 10^6 cells were removed and diluted to 1 ml with HEPES buffer and warmed to 37°C before stimulation with lyso-PLs.

Calcium flux

Cells (10 × 10^5/ml in HEPES buffer with 0.5% fatty acid-free BSA) were loaded with 2 μM indo-1 AM for 30 min at 37°C. Cells were centrifuged and resuspended in HEPES buffer with 0.5% fatty acid-free BSA at 10 × 10^6/ml. For calcium flux, 2 × 10^6 cells were then added once warmed on ice with 0.05% fatty acid-free BSA in HEPES buffer, resuspended in 1 ml, and warmed to 37°C. Just before the assay, propidium iodide (to monitor permeabilization) was added at a final concentration of 1 μg/ml. Calcium flux was monitored by changes in FLS/P4L fluorescence following stimulation. All results shown are representative of at least three independent experiments.

Surface G2A and CD45 staining

For G2A and CD45 surface staining, neutrophils (2 × 10^6/ml) were stimulated for the indicated times at 37°C. Following a quick spin (20 s at 14,000 rpm), cells were resuspended in 200 μl of ice-cold HEPES buffer with 0.05% fatty acid-free BSA, and then 1 × 10^6 cells (100 μl) were incubated with 10 μg/ml anti-G2A (N-20) and anti-CD45 (1:100) or isotype controls for 1 h on ice. Cells were washed once with ice-cold HEPES buffer and incubated with anti-goat Alexa 488 (1:100) and anti-mouse Cy3 (1:100) for 30 min on ice. The excess secondary Ab was removed by washing once with ice-cold HEPES buffer. Cells were either analyzed directly by flow cytometry or prepared for fluorescence microscopy. All results shown are representative of at least three independent experiments.

For fluorescence microscopy, cells were fixed after staining with 3% paraformaldehyde, washed twice in PBS for 30 min on ice and washed once with ice-cold HEPES buffer. Excess liquid was removed from the cell pellet by aspiration, and cells were resuspended in 8 μl of Gel Mount (Biomed) containing 5 μg/ml Hoescht, mounted onto slides, and viewed with a Zeiss fluorescence microscope using a ×100 oil (N.A. 1.4) Axiovert 200 M objective. Images were analyzed using Slidebook software. All results shown are representative of at least three independent experiments.

Subcellular fractionation and Western blot analysis

Neutrophil granules were isolated by Percoll density gradients from control neutrophils using the following markers: myeloperoxidase (primary granules), lactoferrin (secondary granules), gelatinase (tertiary granules), and HLA (secretory vesicles and plasma membranes), as previously described (21). For Western blots, cell equivalents from the subcellular fractionation were loaded onto a 10% SDS-PAGE gel, blocked with 5% BSA/5% milk in TBST for at least 1 h. Membranes were incubated overnight at 4°C with goat anti-G2A (A-20) diluted 1/1,000 in 1% BSA/1% milk in TBST, washed twice with 2 × 50-mm, and incubated with HRP-conjugated anti-goat IgG diluted 1/10,000 in 0.1% BSA/0.1% milk in TBST for 45 min. After two washes with TBST, proteins were visualized by ECL.

FM 1-43 staining

Neutrophils in HEPES buffer (2.0 × 10^6 cells in 400 μl) with 0.05% BSA and 1 mM CaCl2 were stimulated with lipids for 1 min at 37°C in the presence of FM 1-43 (2 μM final concentration) added along with the stimulus in 100 μl. Stimulation was stopped with an equal volume of ice-cold HEPES buffer, followed by centrifugation. Cells were resuspended in 500 μl of ice-cold HEPES buffer and analyzed by flow cytometry.

Results

Calcium mobilization on lyso-PC depends on mode of presentation and acyl chain substituent

Calcium mobilization in response to lyso-PC has been demonstrated in human neutrophils and is required for priming of the NADPH oxidase, enhanced adherence, and degranulation (5). Given the potential for detergent-like properties of lyso-PLs, and the demonstrated variability of their biological effects in other publications, the concentration, method of presentation (solvents or carrier proteins), and/or the physical state of the lipid (monomers, micelles, or liposomes) are most likely of critical importance (2, 5, 17). Using simultaneous staining with propidium iodide to detect permeabilization of the plasma membrane (17), we assessed calcium flux in response to C16:0/OH, C18:0/OH, and C18:1/OH lyso-PC species in human neutrophils. All of these had previously been shown to exhibit biological activity in both human and murine neutrophils (5, 7). Initially, lyso-PC species (10 μM) were added in methanol. Although methanol (0.4%) alone was neither stimulating nor permeabilizing (data not shown), calcium mobilization following addition of each of these lyso-PC species in methanol was in all instances associated with membrane permeability, as demonstrated by propidium iodide incorporation (Fig. 1A; C18:1/OH shown). As such, loss of integrity of the plasma membrane was the likely mechanism of the demonstrated prolonged calcium flux, and is reminiscent of published receptor-independent responses of neutrophils to lysophosphatidic acid (lyso-PA) and sphingosine 1-P, added either in water or methanol, respectively (14, 22).

Calcium flux in response to lyso-PC species presented on albumin was next examined. C18:1/OH lyso-PC (10 μM presented on 0.05% albumin) stimulated transient calcium flux peaking at ~60 s, without evidence of membrane permeabilization (Fig. 1B). Permeabilization was not evident even after multiple additions (see below and Fig. 2A). By contrast, C18:0/OH and C16:0/OH lyso-PC (10 μM) both stimulated calcium flux with some membrane permeabilization, although less and more variable than that seen with presentation in methanol (Fig. 1C; C16:0/OH lyso-PC shown). As shown, membrane permeabilization became even more evident with a second addition of these saturated lyso-PC species. For this reason, C18:1/OH lyso-PC was used in subsequent studies.
Lyso-PLs, including lyso-PC, are competitively bound by albumin, which is thought to prevent inappropriate stimulation during circulation in the blood (see Discussion). As such, increasing albumin concentration has been shown to neutralize lyso-PL-induced membrane perturbation and other biological activities (23–28). The effect of suspending neutrophils in increasing albumin concentration was tested on their response to C18:1/OH lyso-PC. As shown in Fig. 1D, whereas lyso-PC signaling was demonstrated in neutrophils suspended in albumin up to 1%, as predicted, signaling was reproducibly ablated when cells were suspended in 4% albumin (data shown) or plasma. These results support the hypothesis that lyso-PL stimulation is highly regulated within the milieu in which they are produced (see Discussion), and suggest that plasma membrane insertion of lyso-PC without evidence of obvious lysis may be mechanistically required for calcium flux (see below).

C18:1/OH lyso-PC signals for receptor-mediated calcium flux via G2A

Having ruled out the likelihood of plasma membrane permeabilization as the cause for calcium flux, evidence for a receptor-mediated process was sought. A second administration of lyso-PC C18:1/OH (10 μM in 0.05% albumin) applied after return of calcium levels to near baseline (between 4 and 5 min) resulted in markedly blunted calcium responses suggestive of canonical receptor desensitization (Fig. 2A). This was not evident for the heterologous stimulus, PAF, confirming findings of Stilliman et al. (5). Several GPCR for lyso-PC have been proposed as follows: G2A, GPR4, and the PAFR (5, 7, 29–31). Of these, neutrophils are known to express G2A and the PAFR, but not GPR4 (2, 7). Signaling by C18:1/OH lyso-PC through the PAFR, as shown above.

FIGURE 1. Calcium flux in neutrophils stimulated with lyso-PC is dependent on fatty acyl substituent and method of delivery. A, Calcium flux (top tracing) stimulated with 18:1/OH lyso-PC (10 μM) delivered in 0.4% MeOH was mediated by permeabilization of the membrane (detected by simultaneous propidium iodide (PI) staining, bottom tracing). B, Delivery of 18:1/OH lyso-PC (10 μM) in 0.05% albumin resulted in calcium flux without permeabilization. C, Stimulation with 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-L-cho- line] (10 μM) induced calcium flux (top panel) with modest permeabilization (bottom tracing). However, calcium flux in response to a second addition was clearly the result of membrane permeabilization. D, Stimulation with 18:1/OH Lyso-PC (10 μM) delivered in 1% BSA (top tracing) induced transient calcium flux (in the absence of permeabilization; data not shown), whereas no flux occurred when neutrophils were suspended in 4% BSA (bottom tracing). All tracings are representative of three to five experiments.

FIGURE 2. Calcium flux induced by 18:1/OH lyso-PC is self-desensitizing, does not signal via the PAFR, and is dependent on G2A. A, 18:1/OH lyso-PC (10 μM) induced calcium flux, but demonstrated self-desensitization to a second administration, suggesting a receptor-mediated process independent of the PAFR (top). No permeabilization was evident (bottom). B, Pretreatment of neutrophils with neutralizing Abs to G2A (black tracing) abrogated calcium flux in response to 18:1/OH lyso-PC, but not fMLP, whereas isotype control Abs (gray dashed tracing) had no effect. All tracings are representative of five experiments.
(Fig. 2A), was unlikely in the current experiments because there was no evidence of desensitization to PAF. Given previous reports of neutrophil expression of the G protein-linked receptor, G2A, and lyso-PC signaling through this receptor (2, 7), signaling in the presence of blocking Abs to G2A was tested. As shown, and as in the investigation of Yan et al. (7), blocking Abs to G2A (but not isotype control Abs) inhibited calcium flux in response to C18:1/OH lyso-PC (Fig. 2B). Inhibition of calcium flux by anti-G2A occurred in a stimulus-specific manner, in that neither fMLP (data shown) nor PAF responses were inhibited.

C18:1/OH lyso-PS and lyso-PE also signal for G2A-dependent calcium flux

Although lyso-PC was originally reported to be a direct ligand of G2A (10), more recent investigations have failed to corroborate this assertion (11, 12). These observations prompted us to test whether signaling by other classes of lyso-PLs, previously reported to stimulate biological responses, was likewise mediated through G2A. Both lysophosphatidylserine (lyso-PS) and lysophosphatidylethanolamine (lyso-PE) have been shown to induce variable enhancement of the neutrophil oxidative burst in response to a number of stimulatory agents (1, 8), in some, but not other investigations (7). As with lyso-PC, C18:1/OH lyso-PS (10 μM) presented on albumin was also found to induce transient calcium flux in a self-desensitizing manner without evidence of permeabilization (data not shown), suggesting a receptor-mediated event (Fig. 3A). Furthermore, cross-desensitization was demonstrated between lyso-PS and lyso-PC regardless of which was added initially or subsequently (Fig. 3B). Notably, C18:1/OH lyso-PE also induced similar, though blunted calcium flux without permeabilization, and with identical patterns of self-desensitization and cross-desensitization with lyso-PC, confirming that it, too, signals redundantly with the other lyso-PLs in neutrophils (Fig. 3C). As predicted, given the finding that blocking Ab to G2A inhibited responses to lyso-PC, calcium flux induced by lyso-PS and lyso-PE was also inhibited by blocking Ab to G2A in an identical manner (Fig. 3D; lyso-PS is shown).

As a control, oleic acid (C18:1, 10 μM in 0.05% albumin) was administered and found not to induce calcium flux (data not shown). Although delivery of oleic acid to the cells was not measured in these experiments, calcium flux under identical conditions induced by arachidonic acid, known to activate neutrophils via an undefined pathway (32) (and not sensitive to anti-G2A blockade; data not shown), suggests that delivery of the fatty acid was achieved. These data strongly suggest that the glycerol backbone of the lyso-PLs is essential for subsequent G2A signaling. Additionally, the downstream conversion of lyso-PC, lyso-PS, and lyso-PE to lyso-PA by phospholipase D was considered. However, stimulation by C18:1/OH lyso-PA did not result in calcium flux (data not shown), and thus, the demonstrated redundancy in signaling is unlikely to be due to enzymatic loss of the head group.

Lyso-PL-induced G2A-dependent signaling is associated with membrane perturbation

Together, these data suggest that both the glycerol backbone and a relatively bulky head group are required for induction of G2A signaling. Reasoning that insertion of such cone-shaped lipids, known to differentially expand the membrane leaflet (18, 33–35), might accompany and even support G2A signaling (see below), we sought evidence using FM 1-43, a stain that preferentially inserts into loosely packed membranes to test for lyso-PL-induced plasma membrane perturbation (36, 37). In support of this hypothesis, probing the plasma membrane of neutrophils at 1 min after stimulation with lyso-PLs showed that lyso-PC, lyso-PS, and lyso-PE enhanced FM 1-43 staining, whereas lyso-PA and oleic acid had minimal effects (Fig. 4A). As such, induction of membrane perturbation as identified by FM 1-43 staining appears to

FIGURE 3. Calcium flux is also stimulated by 18:1/OH lyso-PS and 18:1/OH lyso-PE, which cross-desensitize to 18:1/OH lyso-PC. A, Stimulation with 18:1/OH lyso-PS (10 μM) induced transient calcium flux in the absence of permeabilization (data not shown) with self-desensitization to a second addition in a manner independent of PAF stimulation. B, Lyso-PC and lyso-PE were cross-desensitizing; calcium flux stimulated with 18:1/OH lyso-PE prevented a second calcium flux induced by 18:1/OH lyso-PS (top). Similarly, calcium flux stimulated with 18:1/OH lyso-PS cross-desensitized for subsequent calcium flux induced by 18:1/OH lyso-PC (bottom). C, 18:1/OH lyso-PE (10 μM) induced calcium flux and was self-desensitizing (top) and cross-desensitizing with 18:1/OH lyso-PC (bottom). D, Pretreatment with neutralizing Abs to G2A (black tracing) abrogated calcium flux induced by 18:1/OH lyso-PS (10 μM), but not fMLP. Isotype Ab control (gray dashed tracing) had no effect. All tracings are representative of three to five experiments.
be associated with the ability of lyso-PLs to induce G2A signaling. Of note, pretreatment of the cells with blocking Abs to G2A had no effect on enhancement of FM 1-43 staining, demonstrating that membrane perturbation is not downstream of G2A signaling (Fig. 4B).

C18:1/OH lyso-PLs signals via G2A to Goi and PLC

The downstream mechanism of calcium flux induced by lyso-PC, lyso-PS, and lyso-PE was investigated. Given the prominence of GPCR→Goi→PLC signaling in neutrophils, this pathway was studied first. As precedent, lyso-PC stimulation has been associated with G2A signaling through Goi to PLC in various cell types (3–5, 24), although not others, where G2A signaling through other Go subunits has been demonstrated (e.g., Go13 and Gαq) (38–41). Similarly, lyso-PS induced calcium flux in leukemic cells in a manner inhibited by pertussis toxin (whereas murine fibroblasts were insensitive) and by the PLC inhibitor, U-73122 (42, 43). Pretreatment of neutrophils with pertussis toxin inhibited calcium flux to all three lyso-PLs, confirming signaling through Goi (Fig. 5A; lyso-PS is shown). Finally, all three lyso-PLs activated PLC downstream of Goi as demonstrated by suppression of calcium flux by U-73122, the PLC inhibitor (Fig. 5B; lyso-PS is shown). As expected, calcium mobilization in response to fMLP was also blocked by PLC inhibition.

Lyso-PLs enhance surface localization and redistribution of G2A by mobilizing secretory vesicles

Earlier work by Wang et al. (11) demonstrated that overexpressed G2A was spontaneously internalized to an early endosomal compartment in fibroblast cells maintained in the absence of serum. Conversely, treatment with lyso-PC over several hours enhanced or stabilized plasma membrane localization of the overexpressed G2A receptor and was required for lyso-PC-induced migratory responses (11). Thus, we asked whether lyso-PLs would alter plasma membrane levels of G2A as analyzed in the flow cytometer. As shown, the presence of G2A was detectable on the surface of unstimulated neutrophils, and enhanced surface staining was demonstrated as early as 1 min following lyso-PL stimulation (Fig. 6), a time coinciding with calcium responses and FM 1-43 staining. Given the rapidity of the enhanced G2A staining following treatment with lyso-PL, mobilization from a latent pool of secretory vesicles or granules was hypothesized. Western blotting of granular and membrane fractions from neutrophils showed that G2A is found in the plasma membrane/secretory vesicle fraction, but not in primary, secondary, or tertiary granule fractions (Fig. 6B). Predictably, CD45, a marker of secretory vesicles (44), was concomitantly mobilized with lyso-PL stimulation (Fig. 6A). Additionally, phenylarsine oxide, a nonspecific inhibitor of many neutrophil
functions, inhibited the enhanced staining of both CD45 and G2A following stimulation (data not shown). These data support the hypothesis that lyso-PL stimulation results in secretion of a highly labile secretory vesicular pool resulting in enhanced plasma membrane G2A. As a negative control, oleic acid (C18:1, 10 μM) was administered and found not to induce G2A up-regulation (data not shown). Of note, pretreatment of the cells with anti-G2A inhibited lyso-PL-induced secretory vesicle mobilization determined by CD45 staining, demonstrating that G2A signaling is upstream of secretory vesicle mobilization (Fig. 7).

Interestingly, surface G2A mobilized by lyso-PL stimulation appeared to peak at 3 min of stimulation and began to decline slightly thereafter, but remained well above control levels for at least 10 min (Fig. 6A). Neutrophils stimulated with lyso-PLs and stained for G2A were also examined by fluorescence microscopy to determine whether secretory vesicle mobilization resulted in obvious changes in the distribution of the surface G2A. Although no obvious changes in receptor distribution were identified at early time points coinciding with calcium flux and secretory vesicle mobilization (1 min), obvious patching of the G2A into prominent discrete aggregates occurred at later time points coinciding with the period of desensitization to subsequent lyso-PL stimulation (4–5 min) (Fig. 8; lyso-PC stimulation shown). Although receptor internalization has been reported as accompanying desensitization of many GPCRs following stimulation (45–47), the maintenance of elevated surface G2A through the period of functional desensitization suggested that G2A desensitization in neutrophils is more clearly associated with patching of the receptor than to its internalization. As such, we reasoned that any stimulus mobilizing secretory vesicles and patching G2A would lead to a nonresponsive state to subsequent lyso-PL stimulation. To this end, stimulation with 10 nM PAF (or fMLP not shown) rapidly induced calcium flux (peaking ~10 s after addition; Fig. 2A) and readily mobilized secretory vesicles, as demonstrated by marked enhancement of surface staining of both CD45 and G2A (Fig. 9A, and compare scale to Fig. 6A). However, in contrast to lyso-PL stimulation, obvious patching of G2A was rapidly induced, detectable by the first minute following PAF stimulation (Fig. 9B), and was sustained for at least 10 min. As predicted, lyso-PL stimulation delivered following dissipation of PAF-induced calcium flux (at 3 min) and during PAF-induced G2A patching, resulted in minimal calcium flux (Fig. 10). Notably, pretreatment with anti-G2A had no demonstrable effect on PAF-induced calcium mobilization mediated by the PAFR (Fig. 10), demonstrating that rapid G2A mobilization and aggregation in the absence of lyso-PL stimulation do not lead to detectable G2A-dependent calcium flux. Although we hypothesize that this is because of rapid desensitization associated with G2A receptor patching, such G2A-dependent calcium flux may also be simply undetectable in the more robust GPCR stimulation provided by PAF (or fMLP; data not shown).

Taken together, this investigation has shown that G2A resides on the neutrophil plasma membrane and is latent in neutrophil secretory vesicles, and upon lyso-PL stimulation is rapidly mobilized and signals for calcium flux via Goi and PLC (Fig. 11). Use of blocking Abs to G2A demonstrates that whereas signaling via G2A is not required for lyso-PL-induced...
membrane perturbation, it is required for calcium signaling and accompanying secretory vesicle mobilization. As such, membrane perturbation occurs either as a parallel process, or is in fact, upstream of G2A signaling (see Discussion).

**Discussion**

A novel finding of this investigation is that in addition to calcium signaling by lyso-PC, lyso-PLs bearing phosphoserine and phosphoethanolamine head groups also signal for calcium flux mediated by the GPCR G2A (Fig. 3). We hypothesize that redundancy in signaling by lyso-PLs bearing different head groups is explained by their similar perturbing effects on the plasma membrane that may lead to G2A dimerization/oligomerization, a probable requirement for GPCR signaling. Of note, biological activities, both in vitro and in vivo, have been described previously for lyso-PS and lyso-PE. Lyso-PS has been shown to stimulate calcium flux in ovarian and breast cancer cell lines (48), leukemia cells (42), mast cells (49), and fibroblasts (43), but in other instances has had no biological effect when compared with lyso-PC (7, 42). Although a lyso-PS-specific GPR34 has been recently identified in mast cells (50), its limited tissue distribution makes it unlikely to have mediated many effects previously reported for lyso-PS. Fewer reports of biological activity are found for lyso-PE (15, 16), and in many instances, it appears biologically inert when compared with other lyso-PLs (7, 11, 43). In the current experiments, lyso-PE (even at concentrations of up to 50 μM) gave clearly blunted responses in comparison with lyso-PC and lyso-PS for both calcium flux and G2A mobilization. Lyso-PE is considerably less polar and hydrated than lyso-PC and less membrane perturbing (51). Furthermore, lyso-PA was unable to induce calcium flux, suggesting that the presence of a bulky head group is a requirement for G2A signaling (see below). Additionally, little is known of lyso-PL degradation or reacylation, which may be head group specific (52–55) and may also alter biological activity in diverse in vitro and in vivo systems.

Previous investigation in fibroblasts has demonstrated that overexpressed G2A resided in an early endosomal pool and was mobilized or stabilized on the plasma membrane over several hours following lyso-PC stimulation; whether this redistribution of G2A was the result of decreased internalization or increased recycling or exocytosis to the surface, and whether the expressed and tagged protein behaved identically to native G2A were not determined (11). In these current studies in neutrophils, both G2A mobilization and G2A-dependent calcium flux occurred rapidly, within 1–3 min of lyso-PL stimulation (Figs. 1, 2, and 6). Without the means to selectively inhibit the mobilization of secretory vesicles, it is not possible to determine the requirement of mobilization for G2A-dependent calcium signaling. It does appear, however, that G2A signaling is entirely analogous to that of other GPCRs (e.g., PAFR or fMLP receptor): signaling begins with receptors localized on the plasma membrane, which stimulates rapid recruitment of other receptors to the plasma membrane via secretory vesicle mobilization.
Evidence suggests that for many GPCR, dimerization (or oligomerization) is required for signaling (56, 57), and we hypothesize that G2A dimerization is sensitive to changes in the lipid environment upon insertion of lyso-PL (Fig. 8). Along these lines, lyso-PC and lyso-PE have been shown to stabilize the pentameric form of the membrane protein, phospholamban, and prevent its dissociation to monomers (58). It is hypothesized that membrane insertion of these perturbing, cone-shaped lipids (18, 33–35, 51, 59) supports G2A dimerization/oligomerization (56, 57).

Significantly, within minutes of stimulation, lyso-PC, lyso-PS, and lyso-PE each self- and cross-desensitized for subsequent stimulation of G2A-dependent calcium flux (Fig. 3). Such observations are typical of GPCRs. However, unlike many studies of GPCR desensitization (45–47), G2A desensitization was not accompanied by obvious loss of the receptor from the cell surface; rather, desensitization to lyso-PL stimulation was temporally associated with visible G2A patching (Fig. 8), perhaps the result of progressive receptor self-association (Fig. 11). Furthermore, rapid mobilization and patching of G2A induced by other stimuli (e.g., PAF) also inhibited subsequent lyso-PL stimulation, suggesting that receptor patching may be a step in heterologous desensitization as well (Figs. 9 and 10). Of note, this later finding is unidirectional, in that initial lyso-PL stimulation does not inhibit subsequent stimulation through the PAFR (Fig. 2A) or the fMLPR, and indeed primes for these responses, by enhancing their stimulation of neutrophil superoxide production (5, 8) (data not shown). These observations of self-, cross-, and heterologous desensitization corroborate functional studies made with other GPCRs in which involvement of GPCR kinases, arrestins, and protein kinase C has been variously demonstrated. Sensitive molecular approaches will be needed to fully understand the events of G2A signaling, both its activation and inactivation (45–47, 60, 61), and are exceedingly difficult to accomplish in short-lived, terminally differentiated neutrophils.

Importantly, the data suggest that lyso-PL stimulation of neutrophils will only occur where local concentrations of lyso-PLs are high relative to concentrations of neutralizing proteins such as albumin. Appropriately, plasma lyso-PLs are not stimulating: although present at concentrations of 100 μM or more (all lyso-PL species combined) (20), the lyso-PLs are protein-bound, mostly to inflammatory cells. Though present at concentrations of 100 μM or more, lyso-PLs will only occur where local concentrations of lyso-PLs are stimulatory, the fact remains that lyso-PC and lyso-PS administered in vivo have been shown to have profound effects (7, 65–67). Furthermore, recent investigations linking loss of G2A to both atherosclerosis (20) and autoimmunity (68), and demonstrating the therapeutic use of lyso-PC in sepsis (7), underscore that a better understanding of lyso-PL stimulation is essential.

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