Regulation of Eosinophil Adhesion by Lysophosphatidylcholine via a Non-Store-Operated Ca^{2+} Channel

Xiangdong Zhu, Jonathan Learoyd, Sanober Butt, Lilly Zhu, Peter V. Usatyuk, Viswanathan Natarajan, Nilda M. Munoz, and Alan R. Leff

Section of Pulmonary and Critical Care Medicine, Department of Medicine; and Department of Neurobiology, Pharmacology and Physiology, Pediatrics, Anesthesia and Critical Care, and Committees on Clinical Pharmacology, Cell Physiology and Molecular Medicine, Division of the Biological Sciences, The University of Chicago, Chicago, Illinois

We examined the mechanism by which lysophosphatidylcholine (LPC) regulates β_2 -integrin-mediated adhesion of eosinophils. Eosinophils were isolated from blood of mildly atopic volunteers by negative immunomagnetic selection. β_2 -integrin-dependent adhesion of eosinophils to plated bovine serum albumin (BSA) was measured by residual eosinophil peroxidase activity. LPC caused maximal adhesion of eosinophils to plated BSA at 4 μM . Lysophosphatidylinositol, which has a similar molecular shape, mimicked the effect of LPC on eosinophil adhesion, while neither lysophosphatidylserine nor lysophosphatidylethanolamine had any effect. Phosphatidylethanolamine, a lipid that has a molecular orientation that is the inverse of LPC, blocked eosinophil adhesion caused by LPC. Unlike platelet-activating factor, a G-protein-coupled receptor agonist, LPC did not cause Ca^{2+} -store depletion, but caused increased Ca^{2+} influx upon addition of Ca^{2+} to extracellular medium. This influx was not inhibited by U73122, a phospholipase C inhibitor, demonstrating independence from the G protein-activated phospholipase C pathway. Ca^{2+} influx was inhibited by either preincubation of phosphatidylethanolamine or La^{3+} , a broad spectrum blocker of cation channels. LPC induced up-regulation of the active conformation of CD11b, which was blocked by preincubation with phosphatidylethanolamine. These data suggest that LPC causes a non-store-operated Ca^{2+} influx into eosinophils, which subsequently activates CD11b/CD18 to promote eosinophil adhesion.

Keywords: lysophosphatidylcholine; eosinophils; adhesion; calcium

Eosinophils are the predominant cells recruited to sites of inflammation in asthma and chronic allergic rhinitis (1). An initial step in this process is eosinophil adhesion to receptors on the luminal surface of vascular endothelial cells. The β_2 -integrin subfamily of the CD11b (Mac-1)/CD18 molecule, as well as the CD49 d (VLA-4)/CD29 of the β_1 -integrin subfamily, have been reported to contribute to adhesion of eosinophils to endothelial cells through binding to the endothelial ligands, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (2). Several types of mediator that activate eosinophils and promote eosinophil adhesion to endothelium have been identified, including cytokines (such as IL-5 and granulo-

CLINICAL RELEVANCE

This research focuses on the mechanisms by which lysophosphatidylcholine regulates eosinophil adhesion *in vitro*. This research will add to our knowledge of eosinophil infiltration in allergic inflammation, such as asthma.

cyte macrophage colony-stimulating factor [GM-CSF]) (3), chemokines (eotaxin, RANTES [regulated on activation, normal T cells expressed and secreted]) (4), chemoattractants (formyl-met-leu-phe [fMLP], C5a) (5), and lipid mediators (platelet-activating factor [PAF], leukotriene B_4 [LTB $_4$]) (6).

Prior reports have implicated a group of molecules with a lysophospholipid-like structure in the regulation of leukocyte trafficking (7). The mechanism of lysophospholipid-mediated cell migration has been shown to require the expression of specific G protein-coupled receptors (GPCR), such as the receptors for sphingosine 1-phosphate (S1P) (8) and lysophosphatidic acid (LPA) (9, 10). The immunologic importance of these lysophospholipid receptors is illustrated by the profound immunodeficiency observed in mice lacking expression of the S1P $_1$ receptor in hematopoietic cells (11). S1P $_1$ -deficient T cells fail to egress from the thymus and are therefore completely absent from the periphery. The immunosuppressive drug, FTY720, has been shown to inhibit several S1P receptors, resulting in blocked egress of lymphocytes from the lymph nodes and thymus (7).

Lysophosphatidylcholine (LPC) is produced by the hydrolysis of phosphatidylcholine by various isoforms of phospholipase A_2 or by reactions catalyzed by the enzyme, lecithin-cholesterol acyltransferase (12). LPC was shown to be chemotactic for monocytes and lymphocytes (13), and recently it has been reported that LPC is an apoptotic cell-derived chemotactic factor responsible for the recruitment of phagocytes to remove apoptotic debris (14). Increased expression of adhesion molecules in endothelial cells at sites of inflammation has been found to be a direct effect of LPC (15). LPC also has vasoactive properties, stimulating endothelium-dependent vascular smooth muscle relaxation (16). LPC induces oxidant generation in endothelial cells and smooth muscle cells (17), while conflicting results have been reported on human neutrophils (18, 19). While a prior study showed that LPC elicits oxidant generation in human neutrophils (18), a subsequent investigation has disputed these findings (19). LPC is found in substantial concentrations in the bloodstream ($\sim 200 \mu\text{M}$ [20]). Increased concentrations of LPC have been reported in several diseases, such as endometriosis (21), ovarian cancer (22), asthma, and rhinitis (23). In patients with asthma, the treatment with glucocorticoids resulted in an improvement in the lung function accompanied by a decrease in the plasma

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Correspondence and requests for reprints should be addressed to Dr. Xiangdong Zhu, Section of Pulmonary and Critical Care Medicine, Department of Medicine, MC6076, University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637. E-mail: xzhu@medicine.bsd.uchicago.edu

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LPC level (24). A potential therapeutic use of LPC in sepsis (25) and cerebral ischemia (26) also has been reported. The mechanisms by which LPC protects mice against these two diseases are controversial (18, 19, 25), and prior reports (27, 28) identifying two GPCR, G2A and GPR4, as receptors for LPC now appear to be incorrect. Recent studies have found that LPC induces a change in calcium mobilization via mechanosensitive transient receptor potential channel (TRPC) 6 in cultured human corporal smooth muscle cells (29). LPC can also activate TRPC5 on vascular smooth muscle cells and HEK293 cells overexpressing this channel (30).

Inhalation of LPC to guinea pigs causes selective eosinophil infiltration in airways (31). However, the mechanism by which LPC causes eosinophil infiltration has not been defined. In this study we investigated the effect of LPC on eosinophil adhesion for β_2 -integrin to ICAM-1 surrogate. On the basis of preliminary data, we hypothesized that LPC causes eosinophil adhesion through activation of mechanosensitive ion channel, not GPCR. Our results demonstrate that LPC induces a non-store-operated Ca^{2+} influx. This effect of LPC on eosinophils, which promotes eosinophil adhesion to β_2 -integrin ligand bovine serum albumin (BSA), appears to be independent of GPCR normally associated with chemokines and chemoattractants.

MATERIALS AND METHODS

Materials

Individual synthetic LPCs (16:0, 18:0, and 18:1 LPC), synthetic 16:0 lysophosphatidylethanolamine (LPE), synthetic 18:1 lysophosphatidylserine (LPS), lysophosphatidylinositol (LPI, 65% 16:0, 11% 18:0, and 7% 18:1) purified from soybean were obtained from Avanti Polar Lipids (Alabaster, AL). The lipids were dissolved in 50% ethanol/ H_2O at stock concentration of 10 mM. PAF (C16), 18:1 dioleoyl-phosphatidylethanolamine (PE), and polyclonal antibody against TRPC6 were purchased from Sigma Chemical Co. (St Louis, MO). U73122 and Fura-2/AM were from Calbiochem (La Jolla, CA). Eosinophil isolation materials were obtained from StemCell Technologies (Vancouver, BC, Canada). Polystyrene 96-well microtiter plates were obtained from Costar (Cambridge, MA). Anti-CD11b mAb (clone, Bear 1) was purchased from Beckman Coulter (Fullerton, CA). The CBRM1/5 mAb against activated CD11b was a gift from Dr. T. A. Springer (Harvard Medical School, Boston, MA). Polyclonal antibody against TRPV1 was purchased from Santa Cruz (Santa Cruz, CA).

Isolation of Human Eosinophils

Eosinophils were isolated by a modification of the negative immunomagnetic selection technique (32). The method is based on Percoll centrifugation (density 1.089 g/ml) to isolate granulocytes, hypotonic lysis of red blood cells, and finally, immunomagnetic depletion of neutrophils by the magnetic cell separation system using anti-CD16-coated MACS particles. Eosinophil purity of $\geq 98\%$ was routinely obtained. Cells were kept on ice until use.

Cell Adhesion Assay

β_2 -integrin-dependent eosinophil adhesion to plated BSA was assessed as residual eosinophil peroxidase (EPO) activity of adherent cells (5). Plated BSA has been established as a full surrogate of ICAM-1 for β_2 -integrin-dependent eosinophil adhesion (5, 33). Briefly, 96-well microplates were coated with 10 $\mu\text{g}/\text{ml}$ BSA overnight at 4°C . The plates were washed three times with Hanks' balanced salt solution (HBSS) and saved at 4°C before use. Eosinophils ($1 \times 10^4/100 \mu\text{l}$ HBSS/0.1% gelatin) were preincubated with different concentrations of LPC for 10 min at 37°C . Cells then were added to each well of BSA-coated microplates and incubated for 30 min. After three washes with HBSS, 100 μl of HBSS/0.1% gelatin was added to the reaction wells, and serial dilutions of original cell suspension were added to the empty wells to generate a standard curve. One hundred microliters of EPO substrate (1 mM H_2O_2 , 1 mM OPD, and 0.1% Triton X-100 in Tris buffer, pH 8.0) was then added to the wells. After 30 min incubation at room

temperature, 50 μl of 4 M H_2SO_4 was added to stop the reaction. Absorbance was measured at 490 nm in a microplate reader (Thermomax; Molecular Devices, Menlo Park, CA). All assays were performed in duplicate. Data storage and analysis were facilitated by the use of computer software interfaced with the reader (Softmax; Molecular Devices).

Measurement of Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} of eosinophils was determined by measurement of fura-2 fluorescence in a cuvette with constant stirring as described (34). In brief, cells were incubated with 2 μM Fura-2/AM for 30 min at 37°C , divided into 10^6 cells/tube, and kept on ice until use. Just before use, cells were warmed to 37°C and rapidly centrifuged to remove any extracellular fura-2. Cells then were transferred to a cuvette containing 2 ml HBSS without calcium and with 0.3 mM EGTA added. Fura-2 fluorescence was measured by excitation at 340 and 380 nm, with emissions recorded at 505 nm. After assessing R_{max} using 10 μM ionomycin and R_{min} using 5 mM EGTA, the intracellular Ca^{2+} was finally calculated as described by Grynkiewicz and coworkers (35).

Analysis of Surface Integrin Expression by Flow Cytometry

Eosinophils were treated with various concentrations of LPC for 15 min. Thereafter, eosinophils were centrifuged at $400 \times g$ for 10 min, and the pellets were resuspended in PBS/0.5% BSA. Aliquots of 5×10^5 eosinophils were incubated with 10 μl of fluorescein isothiocyanate-conjugated mAb against CD11b (Clone Bear 1), activated CD11b (Clone CBRM1/5), or isotype-matched control antibody for 30 min at 4°C . The cells were washed twice, resuspended in 1% paraformaldehyde and kept at 4°C until analyzed. Flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, CA). Fluorescence intensity was determined on at least 5,000 cells from each sample. The results were expressed as mean fluorescence intensity (MFI).

Determination of Toxicity of LPC on Human Eosinophils

To determine the cytotoxic effect of LPC on eosinophils, propidium iodide staining was assessed in eosinophils treated with LPC. Aliquots of 0.5×10^6 eosinophils were incubated for 30 min at 37°C with 0–20 μM LPC in a total volume of 250 μl . Propidium iodide at 5 $\mu\text{g}/\text{ml}$ was added to the medium of drug-treated cells, and the cell suspension was immediately analyzed by flow cytometry. Red fluorescence intensity was determined on at least 10,000 cells from each sample, and the percentage of stained cells was analyzed using the Cellquest software.

RT-PCR Analysis of mRNA Expression of Mechanosensitive TRP Channel

To determine the expression of TRP channels in human eosinophils by RT-PCR, mRNA enriched total RNA was isolated by a Qiagen RNeasy mini kit according to the manufacturer's instructions (Valencia, CA). Approximately 80 ng of RNA was isolated from 8×10^6 eosinophils. The entire amount of RNA obtained from each isolation was then used for cDNA synthesis. First-strand cDNA was synthesized using Bio-Rad's iScript cDNA synthesis kit (Hercules, CA). For PCR, the reaction solution (50 μl) contained 10 μl of cDNA solution, 1 μM primer (each for reverse and forward primers), 2 mM MgCl_2 , 0.2 mM dNTP, and 1.25 units of Taq polymerase in $1 \times$ PCR buffer supplied by Fermentas, Inc. (Hanover, MD). The cycling conditions were: 95°C for 10 min, followed by 35 cycles, each consisting of denaturation at 95°C for 1.5 min, annealing at 63°C for 2 min, and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were analyzed on 2% agarose gels and visualized by staining with ethidium bromide. Primer pairs were the same as those used by Heiner and colleagues (36). Primers for cyclophilin were used as a housekeeping gene control.

Western Blot Analysis of TRPC6 and TRPV1

Eosinophils (10^6) were lysed in 40 μl of lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 2 mM Na_2VO_4 , 50 mM NaF, 2.5 mM DFP, 1% SDS, and protease inhibitor cocktail) and then mixed with loading buffer and boiled for 5 min. Samples then were subjected to SDS-PAGE, using 10% acrylamide gels under reducing condition (15 mA/gel). Electrophoretic transfer of proteins from the gels to nitrocellulose membrane was

achieved using a semi-dry system (400 mA, 60 min). The membrane was blocked for 60 min with 1% BSA, which then was incubated with 1:1,000 anti-TRPC6 or TRPV1 antibody diluted in Tris-buffered saline plus 0.05% Tween 20 (TBS-T) overnight. The membranes then were washed three times for 20 min with TBS-T. Anti-rabbit or mouse IgG conjugated with horseradish peroxidase was diluted 1:3,000 in TBS-T and incubated with nitrocellulose membrane for 60 min. The membrane was again washed three times with TBS-T and assayed by an enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

Statistics

Data are expressed as mean $\pm$ SEM. The significance of difference in adhesion between experimental groups was determined by ANOVA. When ANOVA indicated a significant difference between groups, a *t* test was done to determine which intergroup differences were significant. Statistical significance was claimed whenever $P < 0.05$.

RESULTS

Effect of LPC on Eosinophil Adhesion

We assessed the effect of LPC (16:0) on eosinophil adhesion to plated BSA. We have previously established that eosinophil adhesion to plated BSA is β_2 -integrin CD11b/CD18 dependent (5) and that BSA is a full and complete surrogate ligand for β_2 -integrin. LPC-induced eosinophil adhesion was concentration dependent. LPC increased eosinophil adhesion to plated BSA from $11.3 \pm 1.9\%$ for nonstimulated control to a maximum adhesion of $22.2 \pm 2.1\%$ at $4 \mu\text{M}$ (Figure 1A, $P < 0.01$, $n = 5$). Eosinophil adhesion then decreased with increasing concentrations of $> 4 \mu\text{M}$ LPC. Concentrations $\geq 6 \mu\text{M}$ were toxic to the cells and caused progressive de-adhesion. At a concentration of $6 \mu\text{M}$ LPC, $81.2 \pm 2.2\%$ cells stained positive with propidium iodide as compared with control of $2.0 \pm 1.5\%$ ($P < 0.01$, Figure 1B, $n = 4$).

Because other LPC species (e.g., 18:0 and 18:1) normally are present along with 16:0 LPC in plasma and tissues, we also examined the effects of these LPC species on eosinophil adhesion. Both 18:0 and 18:1 LPC caused adhesion of eosinophils that was similar to that of 16:0 LPC, but was somewhat less potent and less efficacious for 18:1 LPC (Figure 1C, $n = 5$).

Effect of Shape of Lysophospholipids on Eosinophil Adhesion

We next examined the effect of the charge and shape of lysophospholipids on eosinophil adhesion. Four lysophospholipids with different polar heads (ethanolamine, serine, choline, and inositol) and comparable acyl chains were used in these experiments (Figure 2A). LPI and LPS are negatively charged, whereas LPC and LPE are neutral. LPC has a large polar head with a diameter of ~ 5.2 nm and a thin hydrophobic acyl chain tail with a cross-section estimated to be 1.8–2.0 nm, and can be pictured as a cone with its base at the polar interface (37). LPC and LPI have larger head groups than LPE and LPS. As shown in Figure 2B, LPE and LPS have no effect on eosinophil adhesion. By contrast, LPI, a similar cone-shaped lipid with a large inositol head group and a single fatty acid side chain, mimicked the effect of LPC on eosinophil adhesion (toxic effect was also observed at $\geq 6 \mu\text{M}$; data not shown). These results indicate that the shape but not the charge of the lysophospholipids determines their effect on eosinophil adhesion.

LPC Mobilizes Ca^{2+} Independently of G Protein Signaling in Eosinophils

We next tested whether LPC increases intracellular Ca^{2+} in eosinophils. LPC was applied to isolated human eosinophils in the absence of extracellular Ca^{2+} to study separately Ca^{2+} store depletion and influx. LPC was applied at $t = 100$ s in Ca^{2+} -free buffer in the presence of 0.3 mM EGTA, followed by addition

Figure 1. Effect of LPC on eosinophil adhesion. (A) Concentration-dependent effect of LPC on eosinophil adhesion. Eosinophils were treated with various concentrations of 16:0 LPC for 10 min and adhered to BSA-coated plate for 30 min. Eosinophil adhesion was measured as residual EPO activity as described in MATERIALS AND METHODS ($n = 5$). (B) Effect of 16:0 LPC on eosinophil toxicity. Eosinophils were treated with various concentrations of 16:0 LPC for 40 min and propidium iodide-stained cells were measured by flow cytometry. Data are expressed as mean $\pm$ SEM from four independent experiments. (C) Effect of LPC species on eosinophil adhesion. Eosinophils were treated with various concentrations of three different species of LPC for 10 min and adhesion was measured as above ($n = 5$).

of 2 mM Ca^{2+} at $t = 200$ s. Unlike PAF, LPC at $4 \mu\text{M}$ did not cause Ca^{2+} store depletion but significantly increased Ca^{2+} influx in eosinophils. LPC induced sustained Ca^{2+} influx for at least 15 min. However, there was no propidium iodide staining even at 40 min (*see below*), indicating that calcium influx caused by $4 \mu\text{M}$ LPC did not result from a cytotoxic effect. A representative Ca^{2+} trace with $4 \mu\text{M}$ LPC is shown in Figure 3A. Typical Ca^{2+} store depletion transients caused by GPCR agonists, such as PAF (Figure 3B), were not detected on addition of LPC. However,

Figure 2. (A) Shape and charge of lysophospholipids. (B) Effect of lysophospholipids on eosinophil adhesion. Eosinophils were treated with various concentrations of lysophospholipids for 10 min and adhered to plated BSA for 30 min. Eosinophil adhesion was then measured as residual EPO activity. Data are expressed as mean $\pm$ SEM from three independent experiments.

Ca^{2+} influx was detected immediately upon addition of external calcium in LPC-treated cells at 200 s. In the absence of LPC, Ca^{2+} entry in eosinophils was minimal (Control, Figure 3A). By contrast, PAF applied at $t = 100$ s caused transient release of Ca^{2+} from intracellular stores followed by Ca^{2+} influx upon addition of external calcium at $t = 200$ s (Figure 3B). At 600 s LPC caused further Ca^{2+} influx, indicating that calcium influx caused by LPC is not mediated by store-operated calcium channels.

To elucidate the signaling cascade for LPC-induced Ca^{2+} entry, we next examined the involvement of G protein-coupled phospholipase C (PLC) signaling pathway. U73122, a specific inhibitor of PLC (38), was added at $t = 30$ s. U73122 had no effect on Ca^{2+} influx caused by LPC (Figure 3C). However, pretreatment with U73122 subsequently abolished Ca^{2+} -store depletion transient caused by PAF (Figure 3D). After addition of external Ca^{2+} , Ca^{2+} entry was detected in PAF-stimulated cells; LPC caused further entry from ~ 250 nM to ~ 800 nM (Figure 3D). These data indicate that G protein-coupled PLC is not the pathway that is used for Ca^{2+} entry caused by LPC in eosinophils.

To investigate further the mechanism of Ca^{2+} influx induced by LPC, we next examined the effect of lanthanum and SKF 96365, a store-operated Ca^{2+} channel blocker (45) in human eosinophils. Pretreatment eosinophils with $100 \mu\text{M}$ La^{3+} (Figure 3E), a nonselective TRP channel blocker (39), attenuated the rate of Ca^{2+} influx caused by LPC. By contrast, SKF96365, had no effect on calcium influx caused by LPC (Figure 3F). 2-APB, another store-operated calcium channel blocker, did not block Ca^{2+} entry either (data not shown).

We next examined the effect of the shape of LPC on calcium influx in eosinophils. Previous study has shown that externally applied LPC to lipid bilayers increases large mechanosensitive channel (MscL) activity (40). PE (which has a small ethanolamine head group and two acyl chains as a large tail group, Figure 3G, *inset*), a lipid with shape oppositely oriented to that of LPC, neutralizes the effect of LPC on MscL activity (40). In our study, preincubation of eosinophils with $10 \mu\text{M}$ PE blocked almost completely Ca^{2+} entry caused by LPC (Figure 3G), indicating that the effect of LPC on eosinophils also is caused by the same mechanism of outer membrane insertion of LPC.

Finally, we investigated whether BSA neutralized LPC-induced Ca^{2+} entry. LPC in plasma is present mainly in albumin- and lipoprotein-bound form (41). Pretreatment of eosinophils with $3 \mu\text{M}$ BSA abolished effect of LPC on Ca^{2+} entry (Figure 3F). These data suggest that free LPC, but not the protein bound LPC, has the ability to cause Ca^{2+} entry.

Blockade of LPC Induced Eosinophil Adhesion by PE, Soluble BSA, or EGTA

Since PE blocked Ca^{2+} entry caused by LPC, we then examined the effect of PE on eosinophil adhesion caused by LPC. 18:1 dioleoyl-phosphatidylethanolamine blocked eosinophil adhesion caused by LPC (Figure 4A). Adhesion of nonstimulated eosinophils to plated BSA was $10.4 \pm 2.1\%$. Adhesion caused by $4 \mu\text{M}$ LPC was blocked by PE from 23.2 ± 4.0 to $11.6 \pm 1.1\%$ at $3 \mu\text{M}$, and was further blocked to 9.5 ± 1.5 at $10 \mu\text{M}$ ($P < 0.01$ versus control).

Previous investigations have shown that albumin binds to lysophospholipids, thereby sequestering these lipids away from the cell surface (41, 42). We therefore examined the effect of increasing concentrations of soluble albumin on eosinophil adhesion caused by LPC. At concentrations of BSA approaching $1\text{--}3 \mu\text{M}$, eosinophil adhesion caused by LPC was completely blocked (Figure 4B).

To examine whether the presence of extracellular Ca^{2+} was required for LPC-induced eosinophil adhesion, eosinophil adhesion in response to LPC was measured in the calcium-free HBSS + 0.1% gelatin in the presence of 0.3 mM EGTA (Figure 4C). In the regular HBSS buffer, which contains 1.3 mM Ca^{2+} , LPC at $4 \mu\text{M}$ induced eosinophil adhesion from buffer control of $8.2 \pm 2.0\%$ to $25.1 \pm 2.0\%$ ($P < 0.01$). In the absence of extracellular Ca^{2+} , no adhesion was induced by LPC (Figure 5C, $P = \text{NS}$ versus control without calcium).

Effect of LPC on Surface Mac-1 (CD11b/CD18) Expression and Active Conformational Change

We next examined the role of two previously postulated mechanisms of integrin-mediated adhesion: (1) up-regulation of surface integrin molecules, and (2) the affinity/conformational changes of the β_2 -integrin (43). We first determined the effect of LPC in regulating Mac-1 (CD11b/CD18) expression by flow cytometry. Stimulation of eosinophils with LPC did not increase significantly the expression of the CD11b α -chain of Mac-1 on the eosinophil surface (baseline control of 18.0 ± 1.3 versus 19.5 ± 2.1 MFI for treatment [$P = \text{NS}$]) (Figures 5A and 5E). Treatment of eosinophils with $10 \mu\text{M}$ PE had no effect on CD11b surface expression either (Figure 5E, 17.8 ± 1.9 versus nonstimulated control, $P = \text{NS}$). By contrast, CD11b expression was up-regulated by PAF, from 18.0 ± 1.3 to 31.6 ± 2.0 ($P < 0.01$). Pretreatment of PE did not prevent the surface up-regulation of CD11b caused by PAF (Figures 5C and 5E).

We next determined whether LPC up-regulates the change to active conformation for Mac-1 (CD11b/CD18). The activated conformation of Mac-1, as identified by the mAb CBRM1/5 (2), was induced after treatment with LPC from 19.3 ± 1.3 to

Figure 3. Effect of LPC on Ca^{2+} influx into eosinophils. Eosinophils were labeled with fura-2, and resuspended in Ca^{2+} -free buffer with 0.3 mM EGTA added. Ca^{2+} store-depletion and Ca^{2+} influx were measured as described in MATERIALS AND METHODS. Calcium-free buffer control (A, B), 1 μM U73122 (C, D), 100 μM La^{3+} (E), 30 μM SKF96365 (F), 10 μM PE (G), or 3 μM BSA (H) was added at $t = 30$ s, followed by addition of calcium-free buffer control (A), LPC (A, C, E–H) or PAF (B, D) at $t = 100$ s as indicated, and thereafter 2 mM Ca^{2+} was added at $t = 200$ s. LPC was added at $t = 600$ s (B) and at $t = 500$ s (D) to confirm that further Ca^{2+} influx can be induced by LPC in PAF-pre-treated cells. Fura-2 fluorescence was measured by excitation at 340 and 380 nm with emissions recorded at 505 nm. Intracellular Ca^{2+} was calculated as described ($n = 4$).

32.5 ± 1.2 MFI ($P < 0.01$, Figure 5B). PE alone had no effect on the expression of active CD11b (Figure 5F); however, it blocked the active conformation of Mac-1 caused by LPC, from 32.5 ± 1.2 to 20.3 ± 1.4 MFI (Figures 5B and 5F, $P < 0.01$). By contrast, PAF-mediated up-regulation of active conformational change of Mac-1 (Figures 5D and 5F) was not attenuated by PE.

TRP Expression in Eosinophils

As our data suggested the involvement of non-store-operated Ca^{2+} channels, we next investigated the potential of TRP channel involvement in this phenomenon. Recent studies have suggested

that LPC may incorporate into the outer membrane of the lipid bilayer, thereby changing the membrane curvature and activating the mechanosensitive ion channel within the lipid membrane (30, 40). We examined the profile of TRP channel expression in human eosinophils. Specific primer pairs were used to screen for the presence of TRP mRNA species in the highly purified human eosinophils. The expression profile for members of the TRP family is illustrated in a representative gel of RT-PCR products shown in Figure 6A. PCR products for TRPC6 and TRPV1 were found in all eosinophil samples. To confirm TRPC6 and TRPV1 protein expression, we examined eosinophil lysates by Western blot analysis. A specific antibody for TRPC6 revealed

Figure 4. Inhibitory effect of PE, soluble BSA, or lack of extracellular Ca^{2+} on eosinophil adhesion caused by LPC. Eosinophils were pretreated with various concentrations of PE (A) or BSA (B) for 10 min at 37°C , before incubation with $4\ \mu\text{M}$ LPC for another 10 min. (C) Eosinophils were resuspended in Ca^{2+} -free HBSS containing 0.1% gelatin and 0.3 mM EGTA or HBSS containing 0.1% gelatin and 1.3 mM Ca^{2+} and incubated with $4\ \mu\text{M}$ LPC for 10 min. Eosinophil adhesion was measured at 30 min after adhering to plated BSA. Each point represents the mean $\pm$ SEM of four separate experiments.

a strong band of $\sim 105\ \text{kD}$ (Figure 6B). Antibody for TRPV1 revealed a band of $\sim 110\ \text{kD}$ in NIH3T3 cells (Figure 6C, lanes 2 and 3); however, only very weak expression of TRPV1 was ever found in human eosinophils (Figure 6B, lane 2, and Figure 6C, lane 1). To determine if this very weak band was either nonspecific or nonfunctional, we administered $100\ \mu\text{M}$ capsaicin,

a known activator of TRPV1, to aliquots of 10^6 eosinophils for up to 10 min. No calcium influx was demonstrated.

DISCUSSION

The major findings in this study are as follows. (1) Extracellular LPC elicits increased β_2 -integrin-mediated eosinophil adhesion to immobilized BSA. This effect is mimicked by the same cone-shaped lipid, LPI, and cancelled by PE, a lipid with shape opposite to that of LPC. (2) In contrast to G protein receptor agonists, LPC increases Ca^{2+} entry without having any detectable effect on Ca^{2+} store depletion. (3) This Ca^{2+} influx is blocked by preincubation with either PE or La^{3+} , a general blocker of mechanosensitive channels. (4) LPC causes active conformational change of CD11b that is prevented by preincubation with PE. These findings provide the evidence that LPC causes a non-store-operated Ca^{2+} influx, which causes activation of β_2 -integrin and subsequent eosinophil adhesion.

The mechanism by which LPC alters cell function has remained elusive. Our studies indicate that rather than GPCR activation as suggested previously (18, 19, 44), Ca^{2+} influx through a non-store-operated calcium channel is important for the induction of eosinophil adhesion by LPC. We show that eosinophil adhesion caused by LPC is regulated by Ca^{2+} influx. The inhibition of Ca^{2+} influx by PE (Figure 3G), BSA (Figure 3H), or in the absence of extracellular Ca^{2+} (Figure 3) corresponded to the decreased eosinophil adhesion (Figure 4) caused by these agents. Other evidence suggests that Ca^{2+} influx caused by LPC is independent of GPCR. (1) The marked Ca^{2+} entry (Figure 3A) in the absence of store depletion in response to LPC suggests a mechanism other than G protein receptor-dependent store depletion and subsequent store-operated calcium ion entry, such as in the case of fMLP or PAF. (2) LPC caused further Ca^{2+} entry in the presence of PAF, which caused store-operated calcium entry, suggesting that LPC-induced Ca^{2+} influx comes through routes other than store-operated calcium channels (Figure 3B). (3) Pharmacologic inhibition of PLC by U73122 had no effect on calcium entry caused by LPC. This excludes the role of Gi or Gq-PLC pathway in LPC-induced eosinophil adhesion. Gs-mediated cAMP production has been linked to decreased eosinophil adhesion, excluding the possible involvement of Gs in LPC-induced eosinophil adhesion (45). Involvement of $\text{G}_{\alpha 13}$ is also unlikely, as $\text{G}_{\alpha 13}$ caused-Rho activation promotes eosinophil detachment (46). (4) The calcium entry caused by LPC was blocked by La^{3+} , suggests a role of a mechanosensitive cation channel. (5) LPC induced eosinophil adhesion in a narrow concentration ranging from $1\text{--}4\ \mu\text{M}$, compared with receptor-mediated agonist, which activates eosinophils over a range of $2\text{--}3\ \log$ concentrations (5, 47, 48). The narrow dose-response curve of LPC on eosinophil adhesion also argues against the likelihood of its action on a cell surface receptor. (6) Prior reports on the putative receptor for LPC, including G2A and GPR4, have been retracted from publication (27, 28). Altogether, our data suggest that Ca^{2+} entry and subsequent eosinophil adhesion caused by LPC is mediated by an integral membrane cation channel rather than by a GPCR.

Perozo and coworkers have hypothesized a mechanism by which LPC causes activation of mechanosensitive channels (40). They propose that asymmetry in the lateral pressure between the two leaflets of the bilayer initiates the sequence of mechanical transduction steps that leads to the open state of mechanosensitive channels. According to the authors' hypothesis, the cone-shaped LPC causes the release of intrabilayer lateral pressure due to its insertion into the outer monolayer of the plasma membrane. By sensing this pressure change, mechanosensitive channels undergo conformational changes and subsequently

Figure 5. Effect of LPC on CD11b expression. Eosinophils (0.5×10^6) were preincubated with buffer or PE for 10 min, followed by stimulation with 4 μ M LPC (A, B) or 1 μ M PAF (C, D) for 10 min. Expression of surface CD11b (A, C) and activation conformation change of CD11b (C, D) was measured by flow cytometry as described in MATERIALS AND METHODS. Representative overlay histograms from four experiments are shown (A–D). E (CD11b) and F (activated CD11b) are the MFI from these four experiments.

open. Application of Perozo's hypothesis leads us to the conclusion that eosinophil calcium entry channels are activated by the same mechanism. Our data support this hypothesis. We find that LPI, a lipid with shape similar to LPC, mimics the effect of LPC on eosinophil adhesion (Figure 2). PE, a lipid with a shape opposite to that of LPC, blocked the effect of LPC on Ca^{2+} entry (Figure 3), eosinophil adhesion (Figure 4), and CD11b

active conformational change (Figure 5). As LPC activates mechanosensitive channels on both prokaryotic and eukaryotic cells, we believe that LPC may represent an evolutionarily conserved crude modulator of integral membrane proteins.

We find that preincubation of eosinophils with soluble BSA substantially attenuated the bioactivity of LPC. Soluble albumin has been shown to contain one to three high-affinity binding

Figure 6. Transient receptor potential channel expression in eosinophils. (A) RT-PCR analysis of TRP mRNA expression in eosinophils. mRNA-enriched total RNA were extracted from human eosinophils and reverse-transcribed to cDNA. PCRs were then performed using specific primer for each TRP channel and the products were separated on 2% agarose gels. Cyclophilin was used as a housekeeping gene control ($n = 3$). (B) Western blot analysis of TRP protein expressions. Eosinophils were lysed and cell lysates were separated by electrophoresis on 10% SDS-PAGE. The membrane was cut into strips, and TRPC6 and TRPV1

expression on each strip was detected by specific antibodies ($n = 2$). (C) Western blot analysis of TRPV1 expression in various cell lysates. Equal amount of proteins (20 μ g) were loaded on 10% SDS-PAGE gel and separated by electrophoresis. After transfer, the membrane was probed with anti-TRPV1 antibody. Lane 1, eosinophils; lane 2, heat-treated NIH 3T3 cells; lane 3, untreated NIH 3T3 cells ($n = 3$).

sites for LPC (49). LPC in plasma is present mainly in albumin- and lipoprotein-bound form (41). These forms may play an important role in delivering fatty acids and choline to tissues such as the brain (41), but may be in a form that is unable to stimulate eosinophil functions. It has been shown that some of the effects of LPC are decreased in the presence of albumin (50). Thus, the functionally available concentration of LPC *in vivo*, and the stimulatory action of eosinophil function, may be controlled by the low concentrations of free LPC. Our results shown in Figure 4B appear to support this notion. The presence of 3 μ M BSA also reversed the ability of LPC to cause calcium influx (Figure 2H). The physiologically relevant concentrations of LPC *in vivo* will be better understood when estimates of unbound LPC concentrations in specific tissues can be reliably made. These results suggest that effective proinflammatory activity of LPC can occur in tissues either in which there is elevated LPC concentration that exceeds the binding capacity of albumin or under conditions of decreased albumin levels. We do not think immobilized BSA on 96-well plates interfered with the effect of LPC on eosinophil adhesion, as free LPC was preincubated with eosinophils first.

Compared with Ca^{2+} release from intracellular stores, Ca^{2+} entry still is not well elucidated. Members of the TRP family of ion channels represent the first potential candidates for calcium entry channels in nonexcitable cells. In this study, we found that TRPC6 and TRPV1 both are expressed as mRNA in eosinophils. These two mRNAs are also found in human neutrophils (36). We further confirmed the protein expression of TRPC6 by Western blot. By contrast, we found weak or no TRPV1 protein expression in eosinophils. Capsaicin, a TRPV1 agonist, also caused no increase in intracellular calcium in eosinophils. Although the role of TRPC6 could not be defined specifically, as selective blockade is not yet technically possible, a role for TRPV1 as a regulator of Ca^{2+} influx in these studies was excluded.

We conclude that the naturally occurring lysophospholipid LPC causes a non-store-operated Ca^{2+} influx, which subsequently activates CD11b/CD18 to cause eosinophil adhesion. This effect of LPC on eosinophil adhesion appears to be independent of GPCR. This newly identified LPC signaling mechanism could provide insight into the molecular mechanisms of the multiple actions of LPC in cells involved in various inflammatory diseases. However, the role of LPC blockade as a potential therapeutic approach for attenuating eosinophilic inflammation or inflammation in other inflammatory cells remains to be elucidated.

Conflict of Interest Statement: X.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.V.U. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. V.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.R.L. has received \$3,000 in the past 3 years for serving on a GlaxoSmithKline Advisory Board, \$13,000 per year for a Merck Advisory Board, \$24,000 per year for Broncus/Asthmatx Advisory Board, and \$3,500 for a Pfizer Advisory Board.

References

1. Rothenberg ME, Hogan SP. The eosinophil. *Annu Rev Immunol* 2006; 24:147–174.
2. Weber C, Katayama J, Springer TA. Differential regulation of beta 1 and beta 2 integrin avidity by chemoattractants in eosinophils. *Proc Natl Acad Sci USA* 1996;93:10939–10944.
3. Fattah D, Page KR, Bezbaruah S, Priest RC, Horgan CM, Solari R. A rapid activation assay for human eosinophils based on adhesion to immobilized ICAM-1, VCAM-1 and IgG. *Cytokine* 1996;8:248–259.
4. Liu J, Munoz NM, Meliton AY, Zhu X, Lambertino AT, Xu C, Myo S, Myou S, Boetticher E, Johnson M, *et al.* Beta2-integrin adhesion caused by eotaxin but not IL-5 is blocked by PDE-4 inhibition and beta2-adrenoceptor activation in human eosinophils. *Pulm Pharmacol Ther* 2004;17:73–79.
5. Zhu X, Subbaraman R, Sano H, Jacobs B, Sano A, Boetticher E, Munoz NM, Leff AR. A surrogate method for assessment of beta(2)-integrin-dependent adhesion of human eosinophils to ICAM-1. *J Immunol Methods* 2000;240:157–164.
6. Lynch OT, Gienbycz MA, Barnes PJ, Lindsay MA. Pharmacological comparison of LTB(4)-induced NADPH oxidase activation in adherent and non-adherent guinea-pig eosinophils. *Br J Pharmacol* 2001; 134:797–806.
7. Halin C, Scimone ML, Bonasio R, Gauguier JM, Mempel TR, Quackenbush E, Proia RL, Mandal S, von Andrian UH. The S1P-analog FTY720 differentially modulates T-cell homing via HEV: T-cell-expressed S1P1 amplifies integrin activation in peripheral lymph nodes but not in Peyer patches. *Blood* 2005;106:1314–1322.
8. Fieger CB, Huang MC, Van Brocklyn JR, Goetzl EJ. Type 1 sphingosine 1-phosphate G protein-coupled receptor signaling of lymphocyte functions requires sulfation of its extracellular amino-terminal tyrosines. *FASEB J* 2005;19:1926–1928.
9. Zheng Y, Kong Y, Goetzl EJ. Lysophosphatidic acid receptor-selective effects on Jurkat T cell migration through a Matrigel model basement membrane. *J Immunol* 2001;166:2317–2322.
10. Masiello LM, Fotos JS, Galileo DS, Karin NJ. Lysophosphatidic acid induces chemotaxis in MC3T3–E1 osteoblastic cells. *Bone* 2006; 39:72–82.
11. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 2004;427:355–360.
12. Prokazova NV, Zvezdina ND, Korotaeva AA. Effect of lysophosphatidylcholine on transmembrane signal transduction. *Biochemistry (Mosc)* 1998;63:31–37.
13. Jing Q, Xin SM, Zhang WB, Wang P, Qin YW, Pei G. Lysophosphatidylcholine activates p38 and p42/44 mitogen-activated protein kinases in monocytic THP-1 cells, but only p38 activation is involved in its stimulated chemotaxis. *Circ Res* 2000;87:52–59.
14. Lauber K, Bohn E, Krober SM, Xiao YJ, Blumenthal SG, Lindemann RK, Marini P, Wiedig C, Zobywalski A, Baksh S, *et al.* Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* 2003;113:717–730.
15. Kume N, Cybulsky MI, Gimbrone MA Jr. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. *J Clin Invest* 1992;90:1138–1144.
16. Eizawa H, Yui Y, Inoue R, Kosuga K, Hattori R, Aoyama T, Sasayama S. Lysophosphatidylcholine inhibits endothelium-dependent hyperpolarization and N omega-nitro-L-arginine/indomethacin-resistant endothelium-dependent relaxation in the porcine coronary artery. *Circulation* 1995;92:3520–3526.
17. Takeshita S, Inoue N, Gao D, Rikitake Y, Kawashima S, Tawa R, Sakurai H, Yokoyama M. Lysophosphatidylcholine enhances superoxide anions production via endothelial NADH/NADPH oxidase. *J Atheroscler Thromb* 2000;7:238–246.
18. Silliman CC, Elzi DJ, Ambruso DR, Musters RJ, Hamiel C, Harbeck RJ, Paterson AJ, Bjornsen AJ, Wyman TH, Kelher M, *et al.* Lysophosphatidylcholines prime the NADPH oxidase and stimulate multiple neutrophil functions through changes in cytosolic calcium. *J Leukoc Biol* 2003;73:511–524.
19. Lin P, Welch EJ, Gao XP, Malik AB, Ye RD. Lysophosphatidylcholine modulates neutrophil oxidant production through elevation of cyclic AMP. *J Immunol* 2005;174:2981–2989.
20. Xu Y. Sphingosylphosphorylcholine and lysophosphatidylcholine: G protein-coupled receptors and receptor-mediated signal transduction. *Biochim Biophys Acta* 2002;1582:81–88.
21. Murphy AA, Santanam N, Morales AJ, Parthasarathy S. Lysophosphatidylcholine, a chemotactic factor for monocytes/T-lymphocytes is elevated in endometriosis. *J Clin Endocrinol Metab* 1998;83:2110–2113.
22. Okita M, Gaudette DC, Mills GB, Holub BJ. Elevated levels and altered fatty acid composition of plasma lysophosphatidylcholine(lysoPC) in ovarian cancer patients. *Int J Cancer* 1997;71:31–34.
23. Mehta D, Gupta S, Gaur SN, Gangal SV, Agrawal KP. Increased leukocyte phospholipase A2 activity and plasma lysophosphatidylcholine levels in asthma and rhinitis and their relationship to airway sensitivity to histamine. *Am Rev Respir Dis* 1990;142:157–161.

24. Chhabra SK, Khanduja A, Jain D. Decreased sodium-potassium and calcium adenosine triphosphatase activity in asthma: modulation by inhaled and oral corticosteroids. *Indian J Chest Dis Allied Sci* 1999; 41:15–26.
25. Yan JJ, Jung JS, Lee JE, Lee J, Huh SO, Kim HS, Jung KC, Cho JY, Nam JS, Suh HW, *et al.* Therapeutic effects of lysophosphatidylcholine in experimental sepsis. *Nat Med* 2004;10:161–167.
26. Blondeau N, Lauritzen I, Widmann C, Lazdunski M, Heurteaux C. A potent protective role of lysophospholipids against global cerebral ischemia and glutamate excitotoxicity in neuronal cultures. *J Cereb Blood Flow Metab* 2002;22:821–834.
27. Zhu K, Baudhuin LM, Hong G, Williams FS, Cristina KL, Kabarowski JH, Witte ON, Xu Y. Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4. *J Biol Chem* 2001;276:41325–41335.
28. Kabarowski JH, Zhu K, Le LQ, Witte ON, Xu Y. Lysophosphatidylcholine as a ligand for the immunoregulatory receptor G2A. *Science* 2001;293:702–705.
29. So I, Chae MR, Kim SJ, Lee SW. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces the change of calcium mobilization via TRPC ion channels in cultured human corporal smooth muscle cells. *Int J Impot Res* 2005;17:475–483.
30. Flemming PK, Dedman AM, Xu SZ, Li J, Zeng F, Naylor J, Benham CD, Bateson AN, Muraki K, Beech DJ. Sensing of lysophospholipids by TRPC5 calcium channel. *J Biol Chem* 2006;281:4977–4982.
31. Nishiyama O, Kume H, Kondo M, Ito Y, Ito M, Yamaki K. Role of lysophosphatidylcholine in eosinophil infiltration and resistance in airways. *Clin Exp Pharmacol Physiol* 2004;31:179–184.
32. Hansel TT, De Vries IJ, Iff T, Rihs S, Wandzilak M, Betz S, Blaser K, Walker C. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J Immunol Methods* 1991;145:105–110.
33. Sano M, Leff AR, Myou S, Boetticher E, Meliton AY, Learoyd J, Lambertino AT, Munoz NM, Zhu X. Regulation of interleukin-5-induced beta2-integrin adhesion of human eosinophils by phosphoinositide 3-kinase. *Am J Respir Cell Mol Biol* 2005;33:65–70.
34. Sano A, Zhu X, Sano H, Munoz NM, Boetticher E, Leff AR. Regulation of eosinophil function by phosphatidylinositol-specific PLC and cytosolic PLA(2). *Am J Physiol Lung Cell Mol Physiol* 2001;281:L844–L851.
35. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; 260:3440–3450.
36. Heiner I, Eisefeld J, Halaszovich CR, Wehage E, Jungling E, Zitt C, Luckhoff A. Expression profile of the transient receptor potential (TRP) family in neutrophil granulocytes: evidence for currents through long TRP channel 2 induced by ADP-ribose and NAD. *Biochem J* 2003;371:1045–1053.
37. Maingret F, Patel AJ, Lesage F, Lazdunski M, Honore E. Lysophospholipids open the two-pore domain mechano-gated K(+) channels TREK-1 and TRAAK. *J Biol Chem* 2000;275:10128–10133.
38. Smith RJ, Justen JM, McNab AR, Rosenbloom CL, Steele AN, Detmers PA, Anderson DC, Manning AM. U-73122: a potent inhibitor of human polymorphonuclear neutrophil adhesion on biological surfaces and adhesion-related effector functions. *J Pharmacol Exp Ther* 1996; 278:320–329.
39. Gillo B, Chorna I, Cohen H, Cook B, Manistersky I, Chorev M, Arnon A, Pollock JA, Selinger Z, Minke B. Coexpression of Drosophila TRP and TRP-like proteins in *Xenopus* oocytes reconstitutes capacitative Ca²⁺ entry. *Proc Natl Acad Sci USA* 1996;93:14146–14151.
40. Perozo E, Kloda A, Cortes DM, Martinac B. Physical principles underlying the transduction of bilayer deformation forces during mechanosensitive channel gating. *Nat Struct Biol* 2002;9:696–703.
41. Croset M, Brossard N, Polette A, Lagarde M. Characterization of plasma unsaturated lysophosphatidylcholines in human and rat. *Biochem J* 2000;345:61–67.
42. Mochizuki M, Zigler JS Jr, Russell P, Gery I. Serum proteins neutralize the toxic effect of lysophosphatidyl choline. *Curr Eye Res* 1982;2:621–624.
43. Myou S, Zhu X, Boetticher E, Myo S, Meliton A, Lambertino A, Munoz NM, Leff AR. Blockade of focal clustering and active conformation in beta 2-integrin-mediated adhesion of eosinophils to intercellular adhesion molecule-1 caused by transduction of HIV TAT-dominant negative Ras. *J Immunol* 2002;169:2670–2676.
44. Yuan Y, Schoenwaelder SM, Salem HH, Jackson SP. The bioactive phospholipid, lysophosphatidylcholine, induces cellular effects via G-protein-dependent activation of adenylyl cyclase. *J Biol Chem* 1996; 271:27090–27098.
45. Bryant RE, Sutcliffe MC. The effect of 3',5'-adenosine monophosphate on granulocyte adhesion. *J Clin Invest* 1974;54:1241–1244.
46. Alblas J, Ulfman L, Hordijk P, Koenderman L. Activation of RhoA and ROCK are essential for detachment of migrating leukocytes. *Mol Biol Cell* 2001;12:2137–2145.
47. Zhu X, Jacobs B, Boetticher E, Myou S, Meliton A, Sano H, Lambertino AT, Munoz NM, Leff AR. IL-5-induced integrin adhesion of human eosinophils caused by ERK1/2-mediated activation of cPLA2. *J Leukoc Biol* 2002;72:1046–1053.
48. Zhu X, Munoz NM, Kim KP, Sano H, Cho W, Leff AR. Cytosolic phospholipase A2 activation is essential for beta 1 and beta 2 integrin-dependent adhesion of human eosinophils. *J Immunol* 1999;163:3423–3429.
49. Portman OW, Illingworth DR. Lysolecithin binding to human and squirrel monkey plasma and tissue components. *Biochim Biophys Acta* 1973;326:34–42.
50. Yuan Y, Jackson SP, Newnham HH, Mitchell CA, Salem HH. An essential role for lysophosphatidylcholine in the inhibition of platelet aggregation by secretory phospholipase A2. *Blood* 1995;86:4166–4174.