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Phosphatidic Acid and Lysophosphatidic Acid Induce Haptotactic Migration of Human Monocytes*

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The present study was aimed at defining the chemotactic activity of phosphatidic acid, which is rapidly produced by phagocytes in response to chemotactic agonists. Exogenously added phosphatidic acid induced human monocyte directional migration across polycarbonate filters with an efficacy (number of cell migrated) comparable to that of "classical" chemotactic factors. In lipid specificity studies, activity of phosphatidic acid decreased with increasing acyl chain length but was restored by introducing unsaturation in the acyl chain with the most active form being the natural occurring 18:0,20:4-phosphatidic acid. Lysophosphatidic acid was also active in inducing monocyte migration. No other phospholipid and lysophospholipid tested was effective in this response. Monocyte migration was regulated by a gradient of phosphatidic acid and lysophosphatidic acid bound to the polycarbonate filter, in the absence of detectable soluble chemoattractant. Migration was also observed if phospholipids were bound to fibronectin-coated polycarbonate filters. Thus, phosphatidic acid and lysophosphatidic acid, similarly to other physiological chemoattractants (*e.g.* C5a and interleukin-8), induce cell migration by an haptotactic mechanism. Phosphatidic acid caused a rapid increase of filamentous actin and, at higher concentrations, induced a rise of intracellular calcium concentration. Monocyte migration to phosphatidic acid and lysophosphatidic acid, but not to diacylglycerol, was inhibited in a concentration-dependent manner by *Bordetella* pertussis toxin, while cholera toxin was ineffective. In the chemotactic assay, phosphatidic acid and lysophosphatidic acid induced a complete homologous desensitization and only partially cross-desensitized one with each other, or with diacylglycerol and monocyte chemotactic protein-1. Suramine inhibited monocyte chemotaxis with a different efficiency: phosphatidic acid > lysophosphatidic acid ≫ diacylglycerol. On the contrary, monocyte chemotactic protein-1-induced chemotaxis was not affected by the drug. Collectively, these data show that phosphatidic acid induces haptotactic migration of monocytes that is at least in part receptor-mediated. These results support a role for phosphatidic acid and lysophosphatidic acid in the regulation of leukocyte accumulation into tissues.

The recruitment of leukocytes from the blood compartment into tissues is a highly regulated process which involves receptor and counter-receptor interactions and secretion of chemotactic factors (for reviews, see Refs. 1–3). Chemoattractants play a pivotal role in this process by promoting integrin-mediated leukocyte-endothelial cell interaction, leukocyte shape change, and inducing the shedding of L-selectin from leukocyte membrane (2, 4). Since the close interaction among leukocytes, endothelial cells, and chemotactic factors occurs in conditions of lateral shear stress, it is very likely that chemotactic agonists can best accomplish their function if anchored on the surface of the endothelial layer (5). Chemotactic signals, locally produced in response to inflammatory agonists, promote the directional migration of leukocytes. Classical chemotactic factors are formylated peptides, of which fMLP[1] is the prototype, products of complement activation cascade (C5a) and a number of cytokines including the members of the recently discovered family of chemokines (6–9). In addition to these factors, a number of lipids with chemotactic activity have been reported. These include arachidonic acid and products related to the arachidonic acid cascade, such as leukotriene B4, platelet activating factor, and lysophosphatidylcholine and diacylglycerols (6, 10–13).

Phosphatidic acid (PA) is a simple phospholipid which plays a crucial role in lipid biosynthesis (14). Recent studies have focused attention on the possible role of PA as a second messenger (see Refs. 15, 16 for reviews). PA can be produced through the hydrolysis of choline-containing phosphoglycerides by the action of phospholipase D (PLD) in a number of cell types including human phagocytes (15, 16). PA can be converted to 1-radyl-2-acyl-glycerols (DG) by the enzyme PA phosphohydrolase (17–19) or act directly as a second messenger (6, 15, 16, 20). In neutrophils (21–25) and monocytes,[2] chemotactic factors, such as fMLP, C5a, leukotriene B4, interleukin-8, and MCP-1 (monocyte chemotactic protein-1) induce activation of PLD and a number of reports have linked PA formation with the regulation of the oxidative burst (20, 25–30) and granule release (24, 31, 32). More recently, PA accumulation was implicated in neutrophil chemotaxis both *in vitro* and *in vivo* (33). In addition, exogenously added PA and lysoPA were shown to induce a number of biological responses including DNA synthesis (34–36), invasion of hepatoma and carcinoma cells into

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[1] The abbreviations used are: fMLP, formylmethionylleucylphenylalanine; PLD, phospholipase D; PA, phosphatidic acid; DG, 1-radyl-2-acyl-glycerols; MCP-1, monocyte chemotactic protein-1; PBMC, peripheral blood mononuclear cells; PTox, *Bordetella* pertussis toxin; CTox, cholera toxin; 18:0-PA, 1,2-distearoyl-3-*sn*-phosphatidic acid; 14:0-PA, 1,2-dimyristoyl-3-*sn*-phosphatidic acid; 18:0,20:4-PA, 1-stearoyl-2-arachidonyl-3-*sn*-phosphatidic acid; 16:0-PA, 1,2-dipalmitoyl-3-*sn*-phosphatidic acid; 8:0-DG, 1,2-dioctanoyl-rac-glycerol; FCS, fetal calf serum.

[2] M. Locati and S. Sozzani, unpublished observation.

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monolayers of mesothelial cells (37), actin stress fibers assembly (38, 39), and to activate effector enzymes, such as PLD (40, 41), phospholipase A2 (34, 42), and phosphorylation of focal adhesion kinase (43), a tyrosine kinase present at the focal adhesion where stress fibers originate. LysoPA was also reported to induce chemotaxis of *Dictyostelium discoideum amoebae* (44). At least part of these actions appear to be mediated by a putative specific *Bordetella* pertussis toxin-sensitive GTP-binding protein-coupled membrane receptor (45, 46). These findings prompted us to investigate whether PA and lysoPA could also play a role as chemotactic factors for human mononuclear phagocytes.

In this study, we report that PA and lysoPA, bound to polycarbonate filters, are able to induce directional migration of human monocytes. In addition, micromolar concentrations of PA activates actin polymerization and calcium transients. The effect was restricted to PA and lysoPA, since other phospholipids and lysophospholipids were ineffective, and was inhibited by *Bordetella* pertussis toxin and suramine. Since lysoPA is produced in large amounts (1–5 μM in serum) by activated platelets during blood clotting (47, 48), it is possible that these lipids can play an important role in the regulation of phagocyte recruitment in inflammation and wound healing.

EXPERIMENTAL PROCEDURES

Materials—The following lipids were from Sigma: 1,2-distearoyl-3-*sn*-phosphatidic acid (18:0-PA), 1,2-dimyristoyl-3-*sn*-phosphatidic acid (14:0-PA), 1-stearoyl-2-arachidonyl-3-*sn*-phosphatidic acid (18:0,20:4-PA), 1-oleoyl-2-lysophosphatidic acid (18:1-LPA), phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylglycerol, lysophosphatidylglycerol, phosphatidylinositol, lysophosphatidylinositol, phosphatidylserine, 1,2-dioctanoyl-rac-glycerol (8:0-DG), 1,2-dioleoyl-rac-glycerol (18:1-DG). The following were from Serdary Research Laboratories, Inc. (Port Huron, MI): 1,2-dioleoyl-3-*sn*-phosphatidic acid (18:1,-PA), 1,2-didecanoyl-3-*sn*-phosphatidic acid (10:0-PA), 1,2-dipalmitoyl-3-*sn*-phosphatidic acid (16:0-PA), 1-palmitoyl-2-lysophosphatidic acid (16:0-LPA). All the lipids were dissolved in chloroform; before the experiment, the chloroform was evaporated under a stream of N_2, and the lipids were then dissolved in RPMI 1640 (Biochrom, Berlin, Germany) + 1% fetal bovine serum (FCS; Hyclone, Logan, UT) at room temperature by a stepped microtip equipped sonicator. Propranolol was from Ayrst Laboratories Inc. (New York, NY) and suramine was from Bayer (Leverkussen, Germany). Phosphatidic acid, L-α-dipalmitoyl, [glycerol-^{14}C(U)] (specific activity 144 mCi/mmol) and formyl-L-Met-L-Leu-Phe-OH (specific activity 56.9 Ci/mmol) were from Du Pont de Nemours (Dreiech, Germany). Human recombinant MCP-1 (MCP-1) was from PeproTech Inc. (Rocky Hill, NJ); fMLP was from Sigma.

Mononuclear Cell Preparation—Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy blood donors through the courtesy of Centro Trasfusionale, Ospedale Sacco, Milan, Italy. Blood was washed once with saline at 400 × *g* to remove plasma and platelets and then centrifuged on Ficoll (Biochrom) at 600 × *g* for 30 min at room temperature. PBMC were collected at the interface, washed twice with saline, and resuspended in RPMI 1640 with 1% FCS. In some experiments monocytes were further purified (>85% pure) by centrifugation at 450 × *g* on a 46% iso-osmotic Percoll (Pharmacia, Uppsala, Sweden) gradient, as described previously (49).

Migration Assay—Cell migration was evaluated using a chemotaxis microchamber technique (50) as described previously (49). Twenty-seven ± 1 μl of chemoattractant solution or control medium (RPMI 1640 with 1% FCS) were added to the lower wells of a chemotaxis chamber (Neuroprobe, Pleasanton, CA). A polycarbonate filter (5 μm pore size, Neuroprobe) was layered onto the wells, covered with a silicon gasket and with the top plate. Fifty μl of cell suspension (1.5 × 10^6/ml monocytes in PBMC) were seeded in the upper chamber. The chamber was incubated at 37 °C in air with 5% CO_2 for 90 min. At the end of the incubation, filters were removed, stained with Diff-Quik (Baxter s.p.a., Rome, Italy), and five high power oil-immersion fields were counted. Similar results were obtained if monocyte migration was evaluated in the presence of 0.2% bovine serum albumin (Sigma) instead of FCS or if PA was added in the assay in chloroform (<0.001% final concentration) without previous sonication.

Haptotaxis was assessed using the microchamber technique and polycarbonate filters as described above. Portion of the filter (4 cm^2) were coated on the lower side with the chemoattractant by floating in a solution (1 ml of RPMI, 1% FCS) containing different concentrations of the agonists in 6-well plates (3046 Falcon, Becton Dickinson, Milan, Italy) at 37 °C for 30 min (51, 52). The filters were then extensively washed in assay medium, blotted on filter paper, and mounted in the chemotaxis chamber. The assay was then performed and evaluated exactly as described above. For some experiments, filters were coated with 50 μg/ml fibronectin (Sigma) overnight (53). Then, the filters were washed and coated with phospholipids as described above.

Pertussis (PTox) and Cholera Toxin (CTox) Treatment—Recombinant PTox was a kind gift of Dr. D. Rappuoli (IRIS-Biocine, Siena, Italy); CTox was from Calbiochem. Cells were preincubated with different concentrations of the toxins at 37 °C for 90 min. At the end of the incubation, cells were washed twice with RPMI and tested for their ability to migrate in response to chemotactic agonists (49). Toxin treatment did not alter cell viability (>90%) as evaluated by trypan blue dye exclusion.

Measurement of Intracellular Ca^{2+} Concentration $[Ca^{2+}]_i$—Changes in $[Ca^{2+}]_i$ were monitored using the fluorescent probe Fura-2 as described previously (54) according to the technique reported by Grynkiewicz *et al.* (55). Briefly, Percoll-purified monocytes (10^7/ml) were resuspended in RPMI 1640 and incubated with 1 μM Fura-2 acetoxymethyl ester (Calbiochem) at 37 °C for 15 min. After incubation, monocytes were washed and resuspended in Hanks' buffered salt solution (Biochrom) containing 1.2 mM $CaCl_2$ and kept at room temperature until used. Fura-2 fluorescence was measured in a Perkin-Elmer LS 50B spectrophotometer at 37 °C with cells (5 × 10^6/ml) continuously stirred. Samples were excited at 340 and 380 nm, and emission at 487 nm was continuously recorded.

Membrane Fluidity Determination—Membrane fluidity was assessed in cell suspension according to Shinitzky and Barenholz (56) using 1,6-diphenyl-1,3,5-hexatriene (Janssen, Beerse, Belgium) as probe. Percoll-purified monocytes (10^6/ml) were resuspended in Hanks' buffered salt solution in the presence of 2 μM 1,6-diphenyl-1,3,5-hexatriene. After incubation of the mixture at 37 °C for 30 min, the fluorescence polarization value was determined using a MV-1 microviscosimeter (Elscint, Haifa, Israel) before and after the addition of the lipids. Lipids were added in chloroform (1 μl/ml); this concentration of the solvent was with no effect on membrane fluidity values.

Measurement of F-actin—F-actin levels were determined as described previously (57). Briefly, Percoll-purified monocytes (5 × 10^6/ml) in Hanks' buffered salt solution + 0.05% bovine serum albumin were prewarmed at 37 °C for 5 min and then stimulated with the chemotactic agonists. The reaction was stopped by withdrawal of aliquots of cells at different times. Cells were fixed with 3.7% (final concentration) formaldehyde for 10 min. Following permeabilization with 50 ng/ml lysophosphatidylcholine (Sigma), cells were stained with 0.165 μM *N*-(7-nitrobenz-2-oxa-1,3-diazo(-4-yl)(NBD)-phallacidin (Molecular Probes Inc., Eugene, OR). Cells were then washed, and fluorescence was evaluated by a FACStar^{Plus} (Beckon Dickinson). The results are expressed as the relative F-actin content calculated as the ratio of the fluorescence intensity of stimulated cells over that of unstimulated cells at the same time point.

Statistical Analysis—Chemotaxis experiments were performed in triplicate. Results are presented as mean (±S.D.) of a representative experiment or as mean (±S.E.) of several experiments. Statistical significance was assessed by Student's *t* test.

RESULTS

Induction and Structural Requirements for Monocyte Migration by PA and LysoPA—A first series of experiments was performed to evaluate the chemotactic properties of PA. Fig. 1 shows that 18:0,20:4-PA induced a statistically significant ($p <$ 0.01 by paired Student's *t* test) migration of human monocytes at the concentration of 100 μM and reached maximal values at 1 mM. These concentrations are in the range of the active concentrations described for other related chemotactic lipids, such as DG (13) and lysophosphatidylcholine (11, 12) in *in vitro* chemotactic assays. As reported in Table I, at the optimal concentration of 1 mM, the number of monocytes migrating to 18:0,20:4-PA was 67 ± 6% (n = 12) of the migration observed in the presence of an optimal concentration of the "classical" chemoattractant fMLP (10^{-8} M) and ~80% of the migration observed with a similar concentration of the most active molecu-

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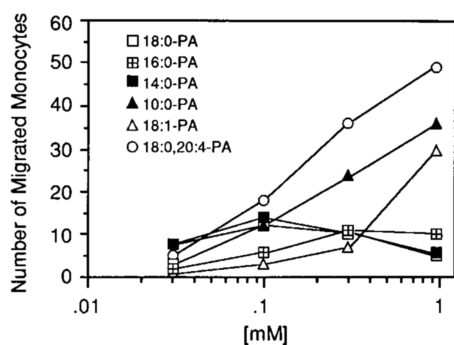

FIG. 1. **Ability of various PAs to induce human monocyte migration.** Human monocytes (1.5×10^6/ml in PBMC) were tested for their ability to migrate across a polycarbonate filter in response to different forms of PAs. PAs were prepared by sonication in RPMI 1% FCS as detailed under "Experimental Procedures" and added to the lower compartments of the chemotactic chamber. At the end of the incubation (90 min), the number of monocytes in five high power microscope-immersion fields was evaluated. Results are the average numbers of two to five different experiments, each one performed in triplicate, at the net of basal migration (against medium; 24 ± 3, $n = 25$). For each experimental point, the variation between the experiments was less than 15%. In the same assay conditions, net monocyte migration in response to an optimal (10^{-8} M) concentration of fMLP was 65 ± 5 ($n = 25$). PAs used were: didecanoyl PA (*10:0-PA*), dimyristoyl PA (*14:0-PA*), dipalmitoyl PA (*16:0-PA*), distearoyl PA (*18:0-PA*), dioleoyl PA (*18:1-PA*), and 1-stearoyl-2-arachidonyl PA (*18:0,20:4-PA*).

TABLE I
Chemotactic activity of PA and of protein and lipid chemoattractants

Human monocytes (1.5×10^6/ml in PBMC) were tested for their ability to migrate across a polycarbonate filter in response to an optimal concentration of the chemotactic factors. At the end of the incubation (1.5 h), the number of monocytes present in five high power oil-immersion fields was counted.

Chemoattractant	Conc.	% input[a]	Chemotactic index	Relative efficacy
	molarity			
fMLP (13)[b]	10^{-8}	16.7 ± 6	3.9 ± 1	100
rMCP-1 (9)	6×10^{-9}	14.2 ± 5	3.5 ± 1	90
8:0-DG (8)	10^{-3}	13.4 ± 5	3.2 ± 1	85
AA[c] (2)	10^{-6}	12.5	3.1	82
18:0,20:4-PA (12)	10^{-3}	9.7 ± 4	2.6 ± 1	67
LTB_4 (3)	3×10^{-7}	9.1 ± 5	2.2 ± 0	56
PAF (5)	10^{-7}	8.1 ± 2	1.5 ± 0	38

[a] % of migrated monocyte with respect to the input (7.5×10^4 monocytes/well).
[b] No. of experiments.
[c] AA, arachidonic acid.

lar species (8:0-DG) of DG (13). On the contrary, 18:0,20:4-PA appeared to be equally or more active than two classical lipid chemotactic agonists, leukotrine B4 and platelet-activating factor (6, 10). At the optimal concentration (1 mM), 18:0,20:4-PA was not toxic (>90% viable cells at the end of the chemotactic assay, by trypan blue dye exclusion) and did not alter monocyte membrane fluidity (3.287 ± 0.004 and 3.227 ± 0.004 poise for control and PA, respectively).

The ability of PA with various chemical structures to induce monocyte migration was evaluated (Fig. 1). All the different forms were active, though with a different relative potency and dose-response curves. Activation by saturated PAs decreased with increasing acyl chain length with 10:0-PA>14:0-PA = 18:0-PA>16:0-PA. Activation was restored by introducing acyl chain unsaturation in long chain phosphatidic acids (*e.g.* 18:1-PA *versus* 18:0-PA), with the most active being the naturally occurring 18:0,20:4-PA.

To clarify whether monocyte migration in response to 18:0,20:4-PA was dependent on the presence of a chemotactic gradient between the two compartments of the chamber, checkerboard experiments were performed using polycarbonate filters. As reported in Table II, maximal migration occurred in the presence of a positive concentration gradient (higher concentration in the lower well). In the presence of a negative gradient (higher concentration in the upper well) or in the absence of gradient (equal concentration in the upper and lower wells) no enhanced migration occurred. Although the type of assay used does not formally exclude a chemokinetic component, these results strongly suggest that monocyte migration in response to 18:0,20:4-PA is chemotaxis (directional migration) rather than chemokinesis (activated random migration).

TABLE II
Checkerboard analysis of PA stimulation of monocyte migration

Different concentrations of 1-stearoyl-2-arachidonyl PA (PA) were placed in the upper and/or lower compartments of the chemotaxis chamber. Results represent the number of migrated monocytes (±S.D.) in five oil fields with three replicates.

Below	Above			
	Medium	PA 0.1	PA 0.3	PA 1
		mM		
Medium	51 ± 1	41 ± 3	34 ± 4[b]	31 ± 2[b]
PA 0.1	61 ± 2[a]	52 ± 2	47 ± 3	38 ± 2
PA 0.3	75 ± 4[b]	67 ± 2[a]	51 ± 5	42 ± 5
PA 1	96 ± 4[a]	80 ± 3[a]	59 ± 3	49 ± 1

[a] $p < 0.01$ *versus* migration to control medium (above and below the filter) by Student's *t* test.
[b] $p < 0.05$ *versus* migration to control medium (above and below the filter) by Student's *t* test.

In order to evaluate if other lipids could mimic PA action on human monocytes, a number of different phospholipids and their corresponding lyso-derivatives were tested in the chemotaxis assay. LysoPAs (18:1-lysoPA and 16:0-lysoPA) stimulated monocyte migration. From a quantitative point of view, lysoPAs were similar (16:0-LysoPA) or weaker (18:1-LysoPA) than the corresponding molecular species of PA (Fig. 2*A*). On the contrary, all the other phospholipids and lysophospholipids tested were not active (Fig. 2*B*).

Monocyte Migration Induced by Filter-bound PA and LysoPA—In a modified Boyden chemotaxis chamber, cells migrate across a polycarbonate filter in response to a chemotactic gradient between the lower well (which contains the chemoattractant) and the upper well where effector cells are seeded (58). By the use of labeled PA it was observed that less then 1.5% of PA present in the lower chamber diffused to the upper chamber at the end of the 1.5-h chemotaxis assay. In the same experimental conditions, ~20% of labeled fMLP, a prototypic chemotactic agonist used for comparison, diffused to the upper chamber (data not shown). In addition, a detectable amount (2.5%, $n = 2$) of labeled PA was found to be associated with the polycarbonate filter even after extensive washing. This result suggested that PA bound to the filter could act as an haptotactic factor. Thus, filters were floated in solutions containing different concentrations of PA for 30 min, washed, and then tested in the migration assay. Fig. 3*A* shows that monocyte migration in response to filter-bound PA in the absence of PA in the lower well (haptotaxis) was similar in potency and efficacy to that observed when PA was also seeded in the lower well (haptotaxis + chemotaxis; Fig. 3*A*) or when PA was directly added to the lower well (Fig. 1). In an effort to elucidate whether migration of monocytes to filter-bound PA was also dependent on the presence of a concentration gradient between the lower and upper compartments of the chamber, filters were coated on one side with different concentrations of PA and assembled in the chemotactic chamber upside down (*i.e.* with the coated surface in contact with the cells). As reported in Fig. 3*A*, in these conditions no monocyte migration was observed either in the absence or in the presence of the respective PA

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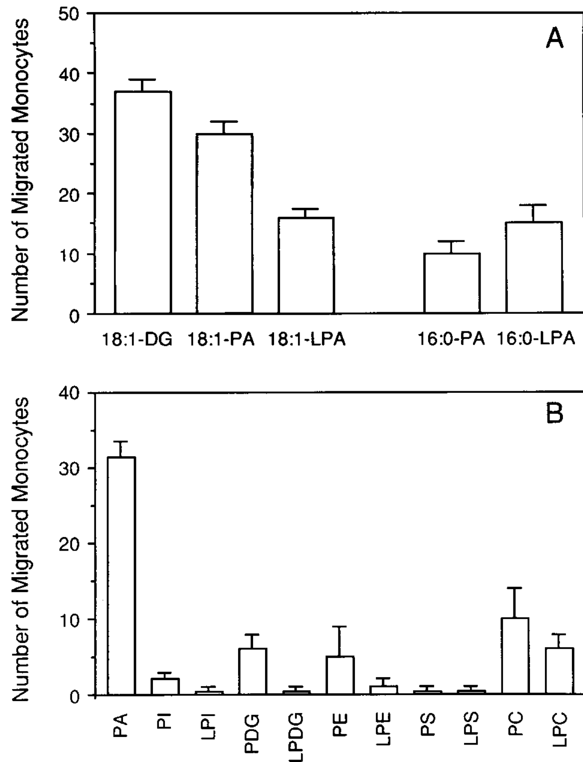

FIG. 2. **Ability of various phospholipids to induce human monocyte migration.** Human monocytes (1.5×10^6/ml in PBMC) were tested for their ability to migrate across a polycarbonate filter in response to a fixed concentration (300 μM) of different phospholipids. Phospholipids were prepared by sonication in RPMI 1% FCS as detailed under "Experimental Procedures" and added to the lower compartments of the chemotactic chamber. At the end of the incubation (90 min), the number of monocytes in five high power microscope-immersion fields was evaluated. Results are the average numbers of three different experiments, each one performed in triplicate, at the net of basal migration (against medium; 21 ± 4, $n = 22$). In the same assay conditions, net monocyte migration in response to an optimal (10^{-8} M) concentration of fMLP was 78 ± 6 ($n = 22$). Phospholipids used were: *panel A*, 1,2-dioleylglycerol (*18:1-DG*), dioleoyl PA (*18:0*), oleoyl lysoPA (*18:1-LPA*), dipalmitoyl PA (*16:0-PA*), and palmitoyl lysoPA (*16:0-LPA*); *panel B*, 1-stearoyl-2-arachidonyl PA (*18:0,20:4-PA*), phosphatidylinositol (*PI*), lysophosphatidylinositol (*LPI*), phosphatidylglycerol (*PDG*), lysophosphatidylglycerol (*LPDG*), phosophatidylethanolamine (*PE*), lysophsophatidylethanolamine (*LPE*), phosphatidylserine (*PS*), lysophosphatidylserine (*LPS*), phosphatidylcholine (*PC*), lysophosphatidylcholine (*LPC*).

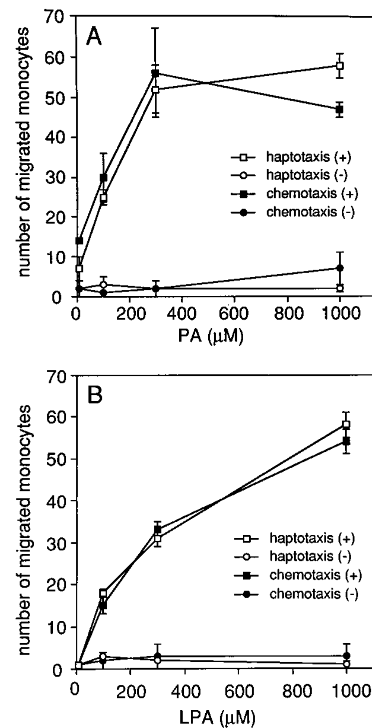

FIG. 3. **Ability of PA and lysoPA to induce monocyte migration by haptotaxis.** Human monocytes (1.5×10^6/ml in PBMC) were tested for their ability to migrate in response to filter-bound 18:0,20:4-PA (*PA*) and 18:1-lysoPA (*LPA*) (*haptotaxis*) or filter-bound + soluble PA and LPA (*chemotaxis*). Filters were coated on one side with different concentrations of PA and LPA for 30 min, washed, and assembled in the chemotactic chamber with the coated side facing the lower compartment (positive gradient, +) or with the coated surface facing the upper compartment (negative gradient, −). At the end of the incubation (90 min), the number of monocytes in five high power microscope-immersion fields was evaluated. Average number of triplicate determinations at the net of basal values (24 ± 2) of one experiment representative of three is shown.

concentration in the lower well. Similar results were obtained when lysoPA was used (Fig. 3*B*). Table III also shows that in the same experimental conditions, fMLP was only minimally able to promote monocyte haptotaxis, in agreement with a previews report (52). On the contrary, MCP-1, which shears structural homology with IL-8, showed a clear haptotactic component in the induction of monocyte migration. Interestingly, DG that was previously shown to induce neutrophil migration in a Boyden microchamber chemotactic assay (13) appears also to act by an haptotactic mechanism rather than by chemotaxis. At the optimal concentration of 0.3 mM PA, the amount of PA bound to filter and able to induce directional migration of 10% of input cells (see Table I) was 6 fmol/mm^2 ($n = 2$). PA and lysoPA could induce haptotactic migration also when filters were first coated with fibronectin, a natural component of extracellular matrix (Table III).

Cross-desensitization between PA and LysoPA—The ability of 18:0,20:4-PA and 18:1-lysoPA to cross-desensitize human monocytes in terms of chemotaxis was investigated. In these experiments, monocytes were first exposed to an optimal chemotactic concentration of PA or lysoPA, washed, and then tested for their ability to migrate in response to each other; MCP-1 was used as reference chemoattractant. Results reported in Fig. 4 show that each of the agonists induced at least 85% of homologous desensitization and caused a partial (~30%) heterologous desensitization. However, PA was more efficient to desensitize against PA than against lysoPA ($p < 0.05$) and DG ($p < 0.01$), and lysoPA was more efficient to desensitize against itself than for PA ($p < 0.05$) or DG ($p < 0.01$).

Effect of PTox and CTox on PA-induced Monocyte Migration—The action of most chemotactic agonists is sensitive to PTox but not to CTox treatment (6–9, 25, 49). Therefore, the action of these two toxins on monocyte migration in response to 18:0,20:4-PA, 18:1-lysoPA, and 8:0-DG was investigated. Fig. 5*A* shows that PTox was able to inhibit, in a concentration-dependent manner, monocyte chemotaxis to PA. On the contrary, monocyte migration to DG was not affected by PTox treatment. CTox, up to 1 μg/ml, did not affect monocyte activation by PA or DG. At the highest concentration tested (1 μg/ml), PTox treatment inhibited monocyte migration of 43 ± 4% ($n = 5$), 51 ± 6% ($n = 3$), and 17 ± 2% ($n = 5$) for PA, lysoPA, and DG, respectively (Fig. 5*B*). In the same assay conditions, chemotaxis to MCP-1 was inhibited more than 90%, as previously reported (49).

Activation of Actin Polymerization by PA—Formation of filamentous actin is a prerequisite for cell motility (6, 59). Thus, it was of interest to determine the action of PA on actin polymerization. Incubation of Percoll-purified human monocytes with 18:0,20:4-PA rapidly increased the amount of F-actin with a

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TABLE III
Haptotactic activity of PA and lysoPA: comparison with other chemotactic agonists

Human monocytes (1.5×10^6/ml in PBMC) were tested for their ability to migrate across polycarbonate filters in response to an optimal concentration of the chemotactic factors. The filters were pretreated with the respective chemotactic factor, and migration was evaluated either in the absence or in the presence of the chemoattractant in the lower well. At the end of the incubation (1.5 h) the number of monocytes present in five high power oil-immersion fields was counted.

Agonist	Chemoattractant in the lower well	Fibronectin coating	No. cell migrated[a]	Ratio[b]
18:0,20:4-PA	−	−	69 ± 6	
	+	−	70 ± 3	1.0
16:0-LPA	−	−	58 ± 3	
	+	−	54 ± 4	1.0
8:0-DG	−	−	77 ± 4	
	+	−	54 ± 4	1.4
fMLP	−	−	16 ± 2	
	+	−	87 ± 6	0.2
rMCP-1	−	−	28 ± 4	
	+	−	68 ± 1	0.4
18:0,20:4-PA[c]	−	+	76 ± 5	
	+	+	80 ± 7	0.9
16:0-LPA[c]	−	+	59 ± 2	
	+	+	74 ± 4	0.8

[a] Results, at the net of basal migration (45 ± 2), of one experiment, performed in triplicate, representative of two to four similar experiments. Agonists were used for filter pretreatment and as chemoattractants, in the lower well, at their optimal concentration (1 mM for PA, lysoPA, and DG, 10^{-8} M fMLP, and 6×10^{-9} M MCP-1).

[b] Haptotactic response/chemotactic (+haptotactic) response ratio.

[c] Migration was assessed using fibronectin-coated filters as described under "Experimental Procedures." Results, at the net of basal migration (37 ± 2), of one experiment, performed in triplicates, representative of two.

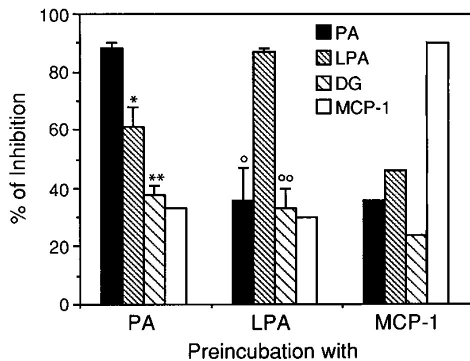

FIG. 4. **Homologous and heterologous desensitization of monocyte migration by PA, lysoPA, and MCP-1.** Monocytes (1.5×10^6/ml in PBMC) were preincubated with 1 mM 1-stearoyl-2-arachidonyl-PA (*PA*), 1 mM oleoyl lysoPA (*LPA*) or 6×10^{-9} M MCP-1 at 37 °C for 30 min. The cells were then washed and assayed for their migration toward homologous or heterologous stimuli. Results are expressed as percent of inhibition with respect to relative control group (cell preincubated with medium and tested against the three single agonists). The mean numbers (±S.E.) of four separate experiments performed in triplicate are reported. * $p < 0.05$, ** $p < 0.01$ with respect to PA. °$p < 0.05$ and °°$p < 0.01$ with respect to LPA, by Student's *t* test.

significant increase observed at 15 s and maximal effect reached at 30 s after stimulation (data not shown). As shown in Fig. 6, the response was concentration dependent starting at 0.1 μM and reaching a plateau at 100 μM, with an EC_{50} of 9.5 ± 2 μM ($n = 6$). In parallel experiments the effect of MCP-1 was tested for comparison. MCP-1 (6×10^{-9} M) gave a rapid rise in fluorescence with a peak observed at the shortest time point evaluated (5 s), and the magnitude of its effect was about 1.5-fold higher than that of PA (data not shown).

Effect of PA on $[Ca^{2+}]_i$—A common action of most chemotactic factors is the induction of a rise of $[Ca^{2+}]_i$. Thus, the effect

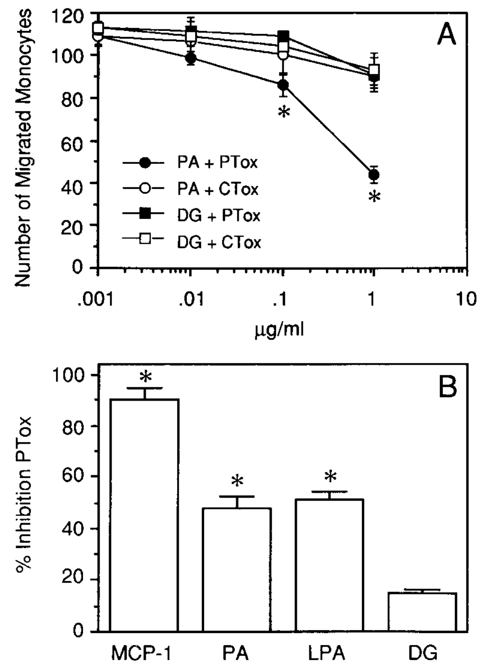

FIG. 5. **Effect of PTox and CTox pretreatment on monocyte migration.** Monocytes (1.5×10^6/ml in PBMC) were incubated at 37 °C with different concentrations of the toxins for 90 min. At the end of the incubation, cells were washed twice, resuspended in RPMI 1% FCS, and tested in the migration assay. Phospholipids tested were 1-stearoyl-2-arachidonyl PA (*PA*), 1,2-dioctanoylglycerol (*DG*), and oleoyl lysoPA (*LPA*) at the concentration of 1 mM, or 6×10^{-9} M MCP-1. *Panel A*, results are average numbers ± S.D. of triplicate determinations of one of two experiments at the net of basal migration (45 ± 5). *Panel B*, monocytes were incubated with 1 μM PTox and then tested in the chemotaxis assay. The mean values ± S.D. of 5 (*PA*, *DG*, and *MCP-1*) and 3 (*LPA*) different experiments performed in triplicate are reported. Results are expressed as percent of inhibition. Migration values of cells incubated in the absence of the toxins in response to the different agonists were assumed as 100%. *$p < 0.01$ against respective control group (no PTox).

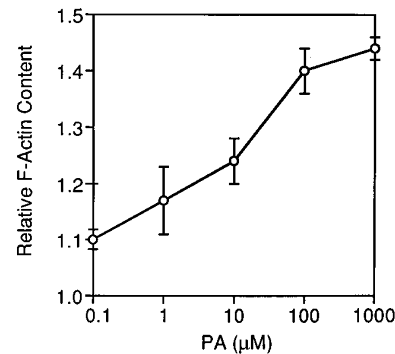

FIG. 6. **Effect of PA and lysoPA on F-actin content.** Percoll-purified human monocytes (10^6/ml) were incubated with 1-stearoyl-2-arachidonyl PA (*PA*) for 30 s. Staining of the cells with NBD-phallacidin and determination of F-actin content was performed as described under "Experimental Procedures." Data are the mean values ± S.D. of three to six independent experiments. The results are expressed as the relative F-actin content calculated as the ratio of the fluorescence intensity of stimulated over that of unstimulated cells. All the points showed are statistically significant ($p < 0.05$ by Student's *t* test, with respect to control (untreated cells).

of PA on intracellular calcium levels in human monocytes was examined. The addition of 0.3–1 mM 18:0,20:4-PA to Fura-2-loaded monocytes induced a rapid increase in $[Ca^{2+}]_i$ (Fig. 7, *A* and *B*). At both concentrations the effect was biphasic with a very rapid increase of $[Ca^{2+}]_i$, followed by a slower phase which peaked about 10 s after stimulation and declined very slowly. The concentration of 0.1 mM, active in inducing monocyte mi-

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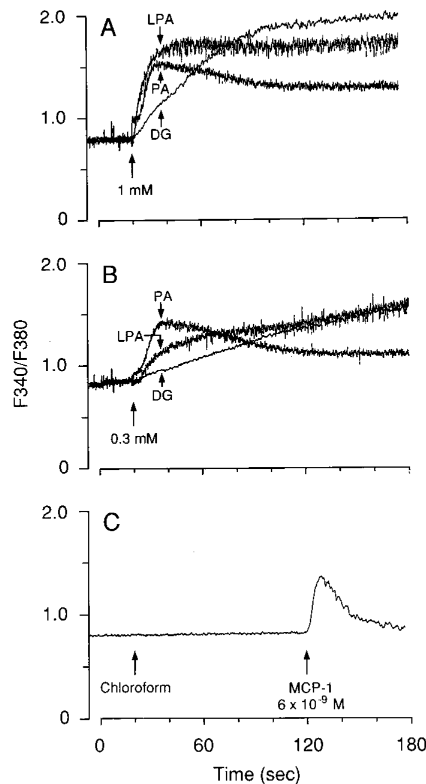

FIG. 7. **Effect of PA, lysoPA, and DG on $[Ca^{2+}]_i$.** Percoll-purified monocytes (10^7/ml) were incubated with Fura-2 acetoxymethyl ester (1 μM) at 37 °C for 15 min, washed, and then exposed in cuvette (5×10^6/ml) to different concentrations of the agonists (1-stearoyl-2-arachidonyl PA (*PA*), 1,2-dioctanoylglycerol (*DG*), lyso oleoyl-PA (*LPA*) or MCP-1). One experiment representative of at least four is shown. Results are expressed as ratio of fluorescence at two excitation wavelengths (340 and 380 nm) and emission at 487 nm.

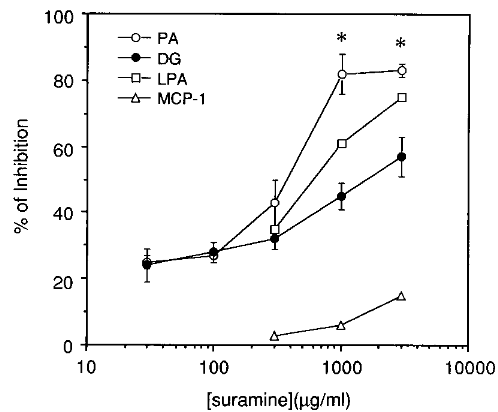

FIG. 8. **Effect of Suramine on PA-, lysoPA-, DG-, and MCP-1-induced monocyte migration.** Human monocytes (1.5×10^6/ml in PBMC) were tested for their ability to migrate across a polycarbonate filter in response to a fixed concentration (1 mM) of 1-stearoyl-2-arachidonyl PA (*PA*), 1,2-dioctanoylglycerol (*DG*), lyso oleoyl-PA (*LPA*), or 6×10^{-9} M MCP-1. Phospholipids were prepared as detailed under "Experimental Procedures" and added to lower compartments of the chemotactic chamber. At the end of the incubation (90 min), the number of monocytes in five high power microscope-immersion fields was evaluated. Suramine was dissolved in RPMI 1% FCS and added to the cell suspension just before use. Results are the average numbers ±S.E. of four different experiments (PA and DG) or the average number of two different experiments (LPA and MCP-1) each one performed in triplicate. Numbers represent percent of inhibition of chemotactic activity. Cell migration in the absence of suramine at the net (100%) of basal values (45 ± 87) was 62 ± 5, 62 ± 2 ($n = 4$), 47, and 65 ($n = 2$) for PA, DG, LPA, and MCP-1, respectively. $^*p < 0.05$ by Student's t test of PA *versus* DG.

gration (Figs. 1 and 3) gave borderline inconsistent results (data not shown). Thus, the concentrations curve for PA, and lysoPA, in haptotaxis and calcium assays are slightly dissociated with the first response being more sensitive than the latter (EC_{50} of 115 ± 8 and 250 ± 32 mM for migration and calcium rise, respectively; $n = 3$; $p < 0.05$). Fig. 7, *A* and *B*, also shows that 18:1-lysoPA and 8:0-DG were active. However, the calcium curves originated by the three lipids showed different kinetics. The rise in $[Ca^{2+}]_i$ after lysoPA stimulation was monophasic and more sustained in the time. On the contrary, DG stimulation resulted in a much slower rise of $[Ca^{2+}]_i$. Similar results were obtained with 10:0-PA, 16:0-PA, and 16:0-lysoPA (data not shown). The effect of the three lipids was different from that of a classical chemotactic agonists, such as MCP-1 (Fig. 7*C*) or fMLP (data not shown and 49, 54, 60), which is characterized by a rapid, but transient, increase of $[Ca^{2+}]_i$.

Sensitivity of PA-, LysoPA-, and MCP-1-induced Monocyte Migration to Suramine—Suramine is a polyanionic compound that was recently described to inhibit PA- and lysoPA-induced DNA synthesis and phosphoinositide hydrolysis in Rat-1 fibroblasts (61) and lysoPA binding to its receptor (45). In Fig. 8 it is shown that the chemotactic response to 18:0,20:4-PA, 18:1-lysoPA, and 8:0-DG was decreased in a concentration-dependent manner by the drug. PA-induced chemotaxis was more sensitive to the effect of suramine than that of DG with respective IC_{50} values of 0.35 ± 0.09 and 1.52 ± 0.22 mg/ml ($n = 4$; $p < 0.05$). LysoPA showed an intermediate sensitivity to the action of the drug with an IC_{50} of 0.6 mg/ml ($n = 2$). In the same experimental conditions, suramine (up to 3 mg/ml) failed to inhibit monocyte chemotaxis to MCP-1 (Fig. 8).

DISCUSSION

Exogenously added PA and lysoPA were shown to produce numerous effects, including calcium mobilization (62–64), activation of MAP kinases (65, 66), induction of actin polymerization (38, 39), and activation of focal adhesion kinase (43) in several cell types. These events are known to be implicated in the induction of leukocyte chemotaxis (6, 59).

The present study shows that PA was able to activate directional migration of human monocytes in a polycarbonate filter assay with an efficacy (peak percentage migration) comparable to that of other chemotactic factors, such as fMLP, MCP-1, leukotriene B4, and DG (Table I). Monocyte migration was PTox sensitive (Fig. 5) and was dependent on a positive gradient of the chemotactic agonist, as evaluated by checkerboard-type analysis (Table II and Fig. 3). Interestingly, PA and LPA act as membrane-bound haptotactic agonists. Other physiological chemotactic agonists were found to induce cell migration by haptotaxis, such as casein (67), C5a (68), and interleukin-8 (51, 52). In addition, membrane-bound molecules, such as platelet-activating factor (69), MIP-1β, and RANTES (regulated upon activation, normal T expressed) (70, 71) where shown to promote cell adhesion and migration. Chemotactic factors play a crucial role in the regulation of leukocyte-endothelial cells interaction by the induction of leukocyte shape change and modulating adhesion molecules (2, 4, 5). In normal conditions of lateral shear stress, it is unlikely that a soluble gradient of chemotactic factors can accomplish this role. Thus, migration to an immobilized gradient of chemotactic agonists present on the surface of endothelial cells or extracellular matrix components represents a physiological condition (5).

Studies in other cell types have shown that exogenous PA can act by itself, via a specific cell-surface receptor (35, 36, 72), while others have attributed this effect to lysoPA contaminating the commercial preparation of PA (64). In addition, there is evidence that in some cell types PA can be converted to lysoPA by the action of a PA-hydrolyzing phospholipase A2 (47, 48, 73),

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raising the possibility that lysoPA is the effector molecule responsible for the biological action of PA. In our study, among the different phospholipids and lysophospholipids tested, lysoPA was indeed the only phospholipid that could substitute for PA in inducing monocyte migration. In this study, lysoPA showed, at best, an identical concentration curve as PA (Figs. 2 and 3), and in cross-desensitization experiments, PA and lysoPA induced only a partial (~30%) heterologous desensitization (Fig. 4). Taken together, these data suggest that contamination of the PA preparation with lysoPA cannot account for the present observation.

There is evidence that some cell types (17, 18), including neutrophils (19, 74, 75), possess a membrane-bound PA phosphohydrolase which is able to convert PA to DG. Thus, it is possible that the effect of PA on chemotaxis is not direct but mediated by PA-derived DG. Several findings argue against this hypothesis and support a direct role of PA in the induction of monocyte activation: (i) in chemotaxis assays, PA induced a nearly complete (88 ± 5%) homologous desensitization and only a partial (38 ± 3) cross-desensitization with DG (Fig. 4); (ii) PA-induced monocyte chemotaxis was much more sensitive to the effect of suramine than DG (IC_{50} = 0.35 and 1.52 mg/ml, respectively; $p < 0.05$; Fig. 8); (iii) PA and DG induced an increase of $[Ca^{2+}]_i$ with different kinetics. The effect of PA was rapid and biphasic, while that of DG was monophasic and slower (Fig. 7); (iv) propranolol (up to 60 μM), a PA phosphohydrolase inhibitor (17, 74), did not alter monocyte chemotaxis to PA at concentrations able to inhibit the conversion of intracellularly formed PA to DG (19, 20, 74), (data not shown). However, it is possible that the membrane-bound and the intracellular forms of PA phosphohydrolase have a different sensitivity to the effect of inhibitors (74, 75); (v) finally, and most convincing, PA-induced monocyte chemotaxis was inhibited in a concentration-dependent manner by PTox, while in the same assay conditions the action of DG was not affected (Fig. 5).

Actin polymerization is known to be an important step in several biological processes, including cell motility (59). Recently, it was reported that PA induces F-actin formation in fibroblasts (39) and that PA is one of the second messengers of lysoPA-induced actin polymerization (41). Also in monocytes, PA induced a rapid activation of actin polymerization in monocytes (Fig. 6). The kinetics of the effect was slightly slower than that of MCP-1, a classical chemotactic factor, but was more sensitive than haptotaxis to the action of PA (EC_{50} = 9.5 and 115 μM for F-actin and haptotaxis, respectively).

At higher concentrations (0.3–1 mM), PA and lysoPA induced a rapid (within seconds) increase of $[Ca^{2+}]_i$, in monocytes (Fig. 7). These concentrations are more than one log higher than those able to induce the same biological response in other cell types, including Rat-1 fibroblasts, platelets, PC12 cells, and *Xenopus laevis* oocytes (35, 36). In these cells, lysoPA induces calcium transients at concentrations in the nanomolar range (64). Nanomolar concentrations are compatible with the reported affinity of the putative lysoPA receptor (45, 46) that also binds PA although with 100-fold lower affinity (46). However, concentrations in the micromolar range are required to induce DNA synthesis (45, 61, 76), platelet activation (77), integrin activation (78), and to activate effector enzymes, such as phosphatidylinositol-specific phospholipase C (45, 64, 77, 79), phospholipase D (40), and phospholipase A2 (42, 45). Thus, it is possible that more than one receptor is responsible for PA and lysoPA activity or, alternatively, that PA can act directly as a second messenger (29, 42, 80–84). Phospholipids can cross the lipid bilayer membrane (85, 86), and there is evidence that exogenous PA is incorporated into the outer leaflet of lipid bilayer and internalized into cells (85, 87). The data reported here suggest that monocyte activation by PA is mediated, at least in part, by the activation of a PTox-sensitive GTP-binding protein-coupled membrane receptor. Monocyte migration in response to PA is inhibited by PTox treatment, and PA can induce complete homologous desensitization. However, inhibition by PTox was only partial (~50%) and in preliminary experiments, PA-induced actin polymerization was not sensitive to the action of the toxin.[3] Thus, it is possible that in monocytes, exogenously added PA also may bypass the activation of a surface receptor. PA is produced by phagocytes in response to chemotactic stimuli (21–25). It has been calculated that PA concentration can reach micromolar levels in receptor-stimulated neutrophils (21, 88) and millimolar levels in stimulated rat hepatocytes *in vivo* (80). Platelet activation during coagulation of whole blood results in serum concentrations of 1–5 μM lysoPA (48). The results reported here raise the intriguing possibility that PA leaking out of some cell types and interacting with specific receptors may amplify the action of chemotactic agents. A recent report has shown that lysoPA enhances fibronectin binding and assembly to cultured fibroblastic cells (89) implicating a role for this lipid in extracellular matrix deposition. PA and lysoPA were also reported to induce the release of transforming growth factor β in mouse skin (90). Thus, PA and lysoPA could act at multiple levels in the regulation of leukocyte infiltration, acting directly as an attractant signal, regulating extracellular matrix component deposition, and inducing the release of chemotactic factors, such as transforming growth factor β.

In conclusion, we report that immobilized PA and lysoPA are able to induce haptotactic migration of human monocytes *in vitro* and that this action resembles, on several aspects, that of classical chemotactic factors. Since PA is produced in monocytes after chemotactic receptor stimulation and released by activated platelet *in vivo* it is possible that this simple phospholipid may play an important role in regulating monocyte infiltration into tissues.

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